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Petition for the Determination of Nonregulated Status for Glyphosate Tolerant Creeping Bentgrass Event ASR368

The undersigned submits this petition under 7 CFR § 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR Part 340

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Monsanto Petition Number: TR054-15U1

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CERTIFICATION

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioner that are unfavorable to the petition.

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EXECUTIVE SUMMARY

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. APHIS regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

The Scotts Company ("Scotts") and Monsanto Company ("Monsanto") are submitting this request to APHIS for a determination of nonregulated status for the biotechnology-derived glyphosate tolerant creeping bentgrass ASR368. Although this petition seeks non-regulated status for ASR368, Scotts and Monsanto have no intention to and will not commercialize or further propagate such plants in the future. Further, Scotts and Monsanto will not grant a license to or otherwise allow other entities to obtain, use, or propagate such plants. Volunteers from prior regulated field productions of ASR368 have been successfully managed for ten years, and appropriate management measures will continue to address any possibility of future volunteers.

Product Description

Glyphosate tolerant creeping bentgrass ASR368 was developed by the insertion of a 5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) gene from *Agrobacterium* sp. strain CP4 (*cp4 epsps*) into the creeping bentgrass genome. When creeping bentgrass plants containing the inserted gene are treated with glyphosate herbicide, the plants are unaffected since the continued action of the expressed tolerant CP4 EPSPS enzyme provides the plant's need for aromatic amino acids. The CP4 EPSPS protein as expressed in ASR368 is identical to that expressed in Roundup Ready® corn (96-317-01p) and other glyphosate tolerant crops (e.g., cotton, soybean, corn, sugar beet, canola, alfalfa) that have been previously reviewed and granted nonregulated status by USDA-APHIS.

Data and Information Presented Confirms the Lack of Plant Pest Potential and the Food and Feed Safety of ASR368 Compared to Conventional Creeping Bentgrass

The data and information demonstrates that ASR368 is agronomically, phenotypically, and compositionally comparable to conventional cultivated creeping bentgrass, with the exception of the introduced trait. Moreover, the data and information presented demonstrate ASR368 is not expected to pose an increased plant pest or weediness risk compared to conventional creeping bentgrasses. The food, feed, and environmental safety of ASR368 was confirmed based on multiple, well-established lines of evidence:

® Roundup Ready is a registered trademark of Monsanto Technology LLC.

- Creeping bentgrass (*Agrostis stolonifera* L.) is a familiar grass, used widely as a turfgrass and occassionally for forage that has a history of safe consumption as animal feed, and serves as an appropriate basis of comparison for ASR368.

- A detailed molecular characterization of the inserted DNA demonstrates a single, intact copy of the T-DNA insert in a single locus within the creeping bentgrass genome.

- The CP4 EPSPS protein in ASR368 is identical to the CP4 EPSPS protein produced in several other commercially-available glyphosate tolerant crops that have been previously reviewed and deregulated by USDA (*e.g*., soybean, maize, cotton, sugarbeet, canola, and alfalfa). The safety of CP4 EPSPS proteins present in biotechnology-derived crops has been thoroughly assessed, and is the subject of numerous publications. The mode-of-action of CP4 EPSPS protein and how it confers glyphosate tolerance has been extensively studied and is well documented in peer reviewed publications.

- A compositional assessment supports the conclusion that ASR368 forage is compositionally equivalent to forage of conventional creeping bentgrass.

- An extensive evaluation of ASR368 phenotypic and agronomic characteristics and environmental interactions demonstrates ASR368 has no increased plant pest risk or weediness compared to conventional creeping bentgrasses. Additional research on wind-scattered ASR368 seeds in the central Oregon environment conducted within the last 12 years by researchers at EPA and Oregon State University has not identified any biological characteristics of ASR368 that indicate it poses plant pest or weediness risks or environmental impacts any different than that of conventional creeping bentgrass. These additional studies also validate the phenotypic and agronomic evaluations of ASR368.

- A survey of weed scientists belonging to the Weed Science Society of America reported that, "creeping bentgrass is currently widespread throughout the U.S., however, it is relatively non-aggressive, their presence is rarely considered a problem that warrants management and thus they are generally not managed as weeds."

- APHIS-PPQ analysis of glyphosate tolerant creeping bentgrass noted no material difference in terms of weediness between conventional creeping bentgrass and glyphosate tolerant creeping bentgrass.

- Interspecific and intergeneric hybridization with ASR368 confers no novel growth characteristics that would make hybrid plants any more weedy than the respective parent species.

- An assessment of potential impact to non-target organisms (NTOs), including organisms beneficial to agriculture, indicates that ASR368 is not expected to

affect other organisms as compared to conventional creeping bentgrass under typical management practices.

Conventional Creeping Bentgrass Cultivars are Appropriate Comparators to ASR368

ASR368 was chosen from among more than four hundred transformation events because of its commercially acceptable agronomic and phenotypic characteristics and tolerance to glyphosate herbicides. Using a forward breeding strategy, clones of the ASR368 R0 generation were crossed with a number of Elite Parent Plants to develop the R1 and F1 progeny populations. As a result of this unique breeding strategy, each individual plant of an ASR368 seedling population is genotypically and phenotypically distinct yet representative of *A. stolonifera*.

A number of different comparators were employed for ASR368 in the experiments. Due to the forward breeding process and the potential for somaclonal variation among plants regenerated from tissue culture, near isogenic or tissue culture lines were not used as comparators for ASR368. The following were employed as comparators: (1) commercial cultivars that represent the range of *A. stolonifera* agronomic and phenotypic characteristics, (2) Elite Parent Plants (EPPs), which were selected from commercially available *A. stolonifera* cultivars developed before 1994 and were crossed with ASR368 R0 generation plants to produce the R1, F1 and F2 progeny populations and/or, (3) null segregant or "Glyphosate Susceptible" (GS) plants from which non-transgenic populations were developed. The commercial cultivars, EPPs and null segregants were considered the most appropriate comparators for assessing the plant pest risk of ASR368 than either near-isogenic or tissue culture lines.

Comparators included the following: B99061R (a non-transgenic tissue culture line), EPPs, commercial cultivars Backspin, Penncross, Penn A-4, Crenshaw, and others. This collection of conventional genotypes is generally representative of the genotypic variability of *A. stolonifera* and are appropriate comparators to assess whether glyphosate tolerant creeping bentgrass ASR368 has been altered in a biologically meaningful manner.

Molecular Characterization Verified the Integrity and Stability of the Inserted DNA in ASR368

Glyphosate tolerant creeping bentgrass ASR368 was produced by particle acceleration technology using a linear DNA segment from plasmid PV-ASGT08 containing two *cp4 epsps* gene expression cassettes. The first *cp4 epsps* gene expression cassette contained the *cp4 epsps* coding sequence under the regulation of the rice actin promoter, a rice actin intron, a chloroplast transit peptide (CTP2) sequence and a nopaline synthase (NOS) 3' polyadenylation sequence. The second *cp4 epsps* gene expression cassette contained the *cp4 epsps* coding sequence under the regulation of the cauliflower mosaic virus (CaMV) enhanced 35S plant promoter (e35S), a maize heat-shock protein 70 (*Zm*HSP70) intron, CTP2 and the NOS 3' polyadenylation sequence. The *ctp2 cp4 epsps* coding region used to produce ASR368 is the same as that employed in several glyphosate tolerant crops,

such as Roundup Ready soybean, which have been previously reviewed and granted nonregulated status by the USDA.

Molecular characterization determined that ASR368 contains one insertion of the integrated DNA located on a 10 kb *Hin*d III restriction fragment. This insert contains one copy of the segment used in transformation. The individual genetic components in each of the two *cp4 epsps* gene expression cassettes in the integrated DNA are intact. The genome of event ASR368 does not contain any detectable backbone sequences from the plasmid vector. Sequences of the 5' and 3' ends of the insert were determined by genome walking and confirmed by PCR amplification and nucleotide sequencing. In addition, the *ctp2-cp4 epsps* coding regions were confirmed to be identical to those in plasmid PV-ASGT08. These data establish that only the expected full-length CTP2-CP4 EPSPS protein should be encoded by the insert in event ASR368. In addition, the genetic stability of the inserted DNA was demonstrated by Southern blot analysis on genomic DNA of the R0, F1 and F2 generations of event ASR368. Furthermore, segregation analysis for nineteen creeping bentgrass populations derived from the reciprocal crosses made between ASR368 F1 progeny and conventional elite parental plants corroborate that a single T-DNA insert in ASR368 is integrated in the plant genome and is inherited as a single locus following a Mendelian one-locus model in a stable manner.

Data Confirm CP4 EPSPS Protein Safety

A multistep approach was used to characterize the CP4 EPSPS protein expressed in ASR368 resulting from the genetic modification. This detailed characterization confirms the CP4 EPSPS protein is safe for human (although creeping bentgrasss is not consumed by humans) and animal consumption. The assessment involved: 1) identity and function of the CP4 EPSPS protein produced in ASR368; 2) demonstration of the equivalence of the plant-produced and *E. coli*-produced CP4 EPSPS proteins; 3) the level of the CP4 EPSPS protein in ASR368 plant tissues; 4) assessment of the potential allergenicity of the CP4 EPSPS protein produced in ASR368; and 5) the food/feed and environmental safety assessment of the CP4 EPSPS protein produced in ASR368. The CP4 EPSPS protein from ASR368 was determined and shown to be equivalent to *E. coli*-produced CP4 EPSPS, used previously for human and animal safety studies, and commercial Roundup Ready soybean (event 40-3-2). An assessment of the allergenic potential of the protein supports the conclusion that the CP4 EPSPS protein does not pose an allergenic risk to humans or animals. The donor organisms for the CP4 EPSPS coding sequence, *Agrobacterium* sp. strain CP4, is ubiquitous in the environment and not commonly known for human or animal pathogenicity or allergenicity. The CP4 EPSPS protein lacks structural similarity to allergens, toxins or other proteins known to have adverse effects on mammals. The CP4 EPSPS protein is rapidly digested in simulated digestive fluid and demonstrate no oral toxicity in mice at the level tested. Based on the above information, the consumption of the CP4 EPSPS protein from ASR368 or its progeny is considered safe for humans and animals. Furthermore, since Scotts and Monsanto will not commercialize ASR368, exposure to humans, who do not consume creeping bentgrass, and animals is expected to be negligible. Given the assessed protein safety data, the identical nature of the CP4 EPSPS protein in ASR368 to CP4 EPSPS contained in other products that have been deregulated by USDA-APHIS, CP4 EPSPS contained in

ASR368 is also considered as safe for humans, animals, and the environment as conventional creeping bentgrass.

ASR368 is Compositionally Equivalent to Conventional Creeping Bentgrass

Compositional analyses were conducted on leaf forage samples from ASR368, the non-transformed parent, B99061R and three conventional varieties produced from four replicated field sites across the U.S. during 2000-2001. Single samples of four additional conventional, commercial varieties were also included to establish commercial ranges and 99% tolerance intervals to provide additional information on the range of natural variability for each component. Comparative analyses of proximates (protein, fat, ash and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber, minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium and zinc) and carbohydrates by calculation were performed. In all, 17 different components were analyzed to assess the composition of ASR368.

In a combined-site analysis in which the data were pooled among the sites, there were no statistically significant differences observed between ASR368 and the control B99061R for any of the analytical components. In an individual-site analysis of the data, four statistically significant differences were observed between ASR368 and B99061R among three different analytical components. Statistically significant differences were detected for the content of moisture (1 site), phosphorus (1 site), and NDF (2 sites). Of the four comparisons observed to be statistically different between ASR368 and B99061R, all values of ASR368 were within the range and 99% tolerance interval of the conventional, commercial varieties. The statistically significant differences were only observed at one or two sites, not in the combination of all the field sites, and were not considered to be biologically meaningful from a food and feed safety or nutritional perspective. Therefore, it is concluded that ASR368 is compositionally equivalent to and as safe and nutritious as the forage produced from other conventional creeping bentgrass varieties.

ASR368 Does Not Change Creeping Bentgrass Plant Pest Potential or Environmental Interactions

Plant pest potential of a biotechnology-derived crop is assessed from the basis of familiarity and is recognized as an important underlying concept in risk assessment. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant hybrid or variety whose biological properties and plant pest potential are well known. Familiarity considers the biology of the plant, the introduced trait, the receiving environment, and the interactions among these factors. This provides a basis for comparative risk assessment between a biotechnology-derived plant and the conventional control. Thus, the phenotypic, agronomic, and environmental interaction assessment of ASR368 included genetically similar conventional controls as comparators. This evaluation used a weight of evidence approach and considered statistical differences between ASR368 and conventional controls. Comparison to a range of conventional, commercial references grown concurrently established the range of natural variability for creeping bentgrass and provided a context from which to further evaluate any statistical differences. Characteristics assessed included: seed germination,

physiology and plant establishment, vegetative establishment, relative growth, flowering and pollen characteristics, fecundity, botanical characteristics, and disease and pest susceptibility. The phenotypic and agronomic assessments demonstrate that ASR368 is comparable to conventional creeping bentgrass. Thus, ASR368 is not expected to have increased weediness or plant pest potential as compared to conventional creeping bentgrass.

Bare soil and competitive turf seed establishment studies were conducted in 2001 and 2002 at four locations encompassing irrigated and non-irrigated conditions, variation in competition and seasonal establishment. These studies demonstrated that: (1) the establishment and persistence of ASR368 tended to fall within the range of the commercial cultivars, (2) the establishment and persistence of ASR368 was generally low for all genotypes when seeded in bare soil (< 30%) and (3) the establishment and persistence of seedlings of all genotypes was unsuccessful when seeded into an existing competitive situation. Given the results from these experiments, which further confirm reports in the scientific literature, seed of ASR368 would not be expected to germinate, establish or persist in unmanaged competitive and non-competitive ecosystems differently from conventional creeping bentgrasses.

Vegetative establishment studies were conducted in 2001 and 2002 at six different locations, including irrigated and non-irrigated conditions. These studies demonstrated that: (1) the vegetative establishment and persistence of ASR368 tended to fall within the range of conventional genotypes, (2) ASR368 plants are not different from conventional creeping bentgrass cultivars in their ability to produce new tillers from viable stolon nodes, and (3) the establishment of all creeping bentgrass genotypes, including ASR368, was much reduced under non-irrigated versus irrigated conditions.

The growth of ASR368 was compared to conventional bentgrasses in bare soil and competitive turf at eleven locations representing the northern (cool), southern (warm) and transition climate zones in 2000, 2001, 2002 and 2003. These studies demonstrated that: (1) ASR368 displayed no increase in vegetative growth, aggressiveness, invasiveness or relative fitness compared to conventional creeping bentgrass cultivars when established in bare soil with no competition or with competition from other turfgrasses in cool, warm and transition climates, (2) ASR368 displayed no competitive advantage in comparison to conventional creeping bentgrass cultivars in direct sun vs. shade or reduced light, and (3) the relative growth of ASR368 was within the normal range for other conventional creeping bentgrass cultivars. Given the results of these experiments, which further confirm reports in the scientific literature, ASR368 and its progeny would not be expected to grow in a different manner in either managed or unmanaged ecosystems as compared to conventional creeping bentgrass.

Flowering characteristics of ASR368 and conventional creeping bentgrass genotypes were evaluated in the greenhouse and at four different field locations in 2001 and 2002. These studies demonstrated that: (1) ASR368 genotypes were within the range of B99061R and the conventional cultivars for date of first inflorescence, anthesis initiation, anthesis completion and the duration of anthesis; and (2) results were consistent with the findings of other researchers who observed that the flowering characteristics of ASR368

and a number of other *Agrostis* species were not different in Washington in 2001 and 2002. Given the results from these experiments, which further confirm reports in the scientific literature, ASR368 and its progeny would not be expected to flower differently as compared to conventional creeping bentgrass.

The size and longevity of pollen collected from plants of ASR368 and conventional creeping bentgrass genotypes, grown in the greenhouse and field, were evaluated in 2001 and 2002. These studies demonstrated that: (1) the diameter of pollen from ASR368 and conventional creeping bentgrass cultivars was not significantly different; (2) the longevity of pollen from ASR368 and conventional creeping bentgrass cultivars was not significantly different; and (3) the size and longevity of pollen from ASR368 or its progeny are within the normal ranges of these characteristics as compared to conventional creeping bentgrass cultivars. Given the results from the ASR368 pollen experiments, which are consistent with reports in the scientific literature regarding the longevity of grass pollen, the pollen size and longevity of ASR368 would not be expected to be different than that of conventional creeping bentgrass.

Fecundity characteristics of ASR368 were evaluated in the greenhouse and at four different field locations in 2001 and 2002. These studies demonstrated that: (1) ASR368 open-and self- pollinated seed set is not significantly different from other conventional creeping bentgrass cultivars; (2) ASR368 self-pollinated seed set is low, as expected, due to self-incompatibility systems known to exist in the *Agrostis* genus; and (3) seed production is variable among creeping bentgrass cultivars and that the ASR368 genotypes are within the range of the representative conventional creeping bentgrass cultivars. Given the results of the 2001 and 2002 fecundity studies, which encompassed three generations of ASR368, it is not expected that ASR368 and its progeny would differ in their ability to produce seed as compared to conventional creeping bentgrass.

Physiological characteristics of the seed of ASR368 were compared to conventional genotypes in a number of germination tests performed in the laboratory. These studies demonstrated that: (1) ASR368 seed does not germinate differently than non-transgenic ASR368 GS seed under standard, suboptimal and supra-optimal conditions; (2) the survival and germination of ASR368 seed were not different than those of ASR368 GS seed under the stressful conditions of the Accelerated Aging Test, which is also a measure of seed longevity, and (3) the germination energy and seedling vigor of ASR368 GT seed were not significantly different across germination conditions from those of the ASR368 GS. Given the results of the seed physiology studies, it is not expected that seed of ASR368 would differ in its ability to germinate under stressful conditions or have greater longevity than seed of conventional creeping bentgrass.

ASR368 botanical characteristic evaluations were conducted in the greenhouse and at four different field locations in 2001 and 2002. These studies demonstrated that: (1) the visual inspection and evaluation of a number of botanical characteristics indicate no gross aberration or deviation between the ASR368 genotypes and conventional, commercial creeping bentgrass cultivars and (2) insertion and expression of the *cp4 epsps* gene did not alter the morphology, floral or vegetative features of ASR368 in any significant way. Given the results from these experiments, which further confirm reports in the scientific

literature, the botanical characteristics of ASR368 would not be expected to be different from conventional creeping bentgrass.

Observations of plant growth and disease and insect pest susceptibility of ASR368 were documented for 65 field releases performed between 1999 and 2002. These observations demonstrate that: (1) there are no discernible differences in plant growth between ASR368 and conventional creeping bentgrass plants; (2) there are no discernible differences in disease severity between ASR368 and conventional creeping bentgrass plants and (3) there are no discernible differences in insect infestation between ASR368 and conventional creeping bentgrass.

Furthermore, a strong empirical record of evaluating feral glyphosate tolerant creeping bentgrass plants in Jefferson County and Malhuer County, Oregon and Canyon County, Idaho has been established. Over eleven years of evaluations demonstrate that, as expected, ASR368 creeping bentgrass plants are primarily limited to disturbed, mesic sites near seed production fields and behave similar to conventional creeping bentgrass. Additionally, even when ASR368 established in Kentucky bluegrass seed production fields, ASR368 seed was not present in the final Kentucky bluegrass seed product.

In summary, the phenotypic and agronomic assessments were evaluated to characterize ASR368, and to assess whether the trait introduced in ASR368 alter the plant pest or weediness potential compared to conventional creeping bentgrass. The evaluation, using a weight of evidence approach, considered the reproducibility, magnitude, and direction of detected differences between ASR368 and conventional controls, and comparison to the range of the conventional reference cultivars. Results from the phenotypic and agronomic assessments and evaluations of feral ASR368 plants in the environment demonstrate that ASR368 does not possess increased weediness characteristics, increased susceptibility or tolerance to specific abiotic stressors, diseases or insect pests, or characteristics that would confer a plant pest risk as compared to conventional creeping bentgrass and is unlikely to increase the weediness of any other cultivated plant or native wild species with which *A. stolonifera* can interbreed.

ASR368 Will Not Negatively Affect NTOs, Including Those Beneficial to Agriculture

Evaluation of the impacts of ASR368 on non-target organisms (NTO) is a component of the plant pest risk assessment. Since ASR368 does not possess pesticidal activity, all organisms that interact with ASR368 are considered to be NTOs. The safety of the EPSPS family of proteins, and specifically CP4 EPSPS, as produced in a number of glyphosate tolerant crops that have been granted nonregulated status by USDA-APHIS and have a history of safe use, has been well characterized and demonstrated. Furthermore, observations from multi-year U.S. field trials of ASR368 and progeny support conclusions of no adverse impacts of glyphosate tolerant creeping bentgrass on NTOs and no changes to plant-disease interactions as compared to conventional creeping bentgrass. Taken together, these data support the conclusion that the potential for ASR368 to adversely impact NTOs, including threatened and endangered species, is no different relative to that of conventional creeping bentgrass.

Conclusion

Based on the data and information presented in this petition, it is concluded that ASR368 is not expected to be a plant pest. Therefore, Scotts and Monsanto request a determination from USDA-APHIS that ASR368 and any naturally occurring progeny derived from crosses between ASR368 and sexually compatible species be granted nonregulated status under 7 CFR § 340.

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ABBREVIATIONS AND DEFINITIONS[1]

~ ....Approximately
§.............................Section
3'............................The distal, or growing end, of an mRNA transcript; the end nearest to or containing the polyA sites
5'............................The proximal, or start end, of an mRNA transcript; the end nearest to the promoter
A............................Adenine
A. stolonifera.........*Agrostis stolonifera*
AOSA....................Association of Official Seed Analysts
AOSCA.................Association of Official Seed Certification Agencies
bp...........................Nucleotide base pairs
B99061R ....non-transgenic tissue culture line also referred to as B99061R/99028
C............................Cytosine
ca. ....Approximately
CaMV....................Cauliflower mosaic virus
CP4 EPSPS ....EPSPS protein from *Agrobacterium* sp. strain CP4
cp4 epsps...............Gene encoding the CP4 EPSPS protein (enzyme)
CTAB....................Cetyltrimethylammonium bromide
CTP ....Chloroplast Transit Peptide
ctp2........................DNA sequence coding for CTP variant 2
DNA......................Deoxyribonucleic acid
dATP ....Deoxyadenosine triphosphate
dCTP ....Deoxycytidine triphosphate
E9 3' ....3' polyadenylation region of the pea *rbcS* E9 gene
E. coli....................*Escherichia coli*
EDTA....................Ethylenediaminetetraacetic acid
ELISA ....Enzyme-linked immunosorbent assay
EPSP......................5-Enolpyruvylshikimate-3-phosphate
EPSPS ....5-Enolpyruvylshikimate-3-phosphate synthase
G............................Guanine
HC1 ....Hydrochloric acid
NPTII ....Neomycin phosphotransferase II
Kb..........................Nucleotide kilobase pairs
kD..........................kiloDalton
LB..........................Left Border
M...........................Molar
mL ....milliliter
mM........................millimolar
mRNA ....Messenger RNA

---

[1] Standard abbreviations, e.g., units of measure, will be used according to the format described in 'Instructions to Authors' in the Journal of Biological Chemistry.

ABBREVIATIONS AND DEFINITIONS (cont)

MW .... Molecular weight
NaC1 .... Sodium chloride
NaOH .... Sodium hydroxide
Na_2HPO_4 .... Sodium phosphate dibasic
NOS .... Nopaline synthase
OECD .... Organization for Economic Co-operation and Development
on .... Origin of replication
on-V .... Bacterial origin of replication from RK2 plasmid
on-322 .... Bacterial origin of replication from *E.coli* plasmid pBR322
OSCS .... Oregon State University Seed Certification Service
OSU .... Oregon State University
PCR .... Polymerase chain reaction
PEP .... phosphoenolpyruvate
PV-ASGTO8L .... DNA plasmid vector used to transform event ASR368
RB .... Right Border
rpm .... Revolutions per minute
GG .... Genotype that is homozygous for glyphosate tolerance
Gg .... Genotype that is hemizygous for glyphosate tolerance
gg .... Genotype that is homozygous for glyphosate sensitivity
RT .... Room temperature
S3P Shikimate-3-phosphate
SDS .... Sodium dodecyl sulfate
sp. .... Species
spp .... Sub-species
SSC .... Saline-sodium citrate buffer. 20X SSC is 3 M sodium chloride, 0.3 M
.... sodium citrate
T .... Thymine
T-DNA .... Transferred DNA
TE buffer .... Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
TMB .... (3,3',5,5' Tetramethylbenzidene) peroxidase substrate
Tris .... Tris (hydroxymethyl)aminomethane
U.S.C .... United States Code

I. RATIONALE FOR THE DEVELOPMENT OF ASR368

I.A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR § 340.6

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. sections 7701-7772), to prevent the introduction and dissemination of plant pests into the United States. The APHIS regulations, at 7 C.F.R. Part 340.6, provide that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition must be granted, thereby allowing unrestricted introduction of the article.

The Scotts Company ("Scotts") and Monsanto Company ("Monsanto") are submitting this request to APHIS for a determination of nonregulated status for the biotechnology-derived glyphosate tolerant creeping bentgrass ASR368 and any progeny derived from naturally-occurring crosses between those ASR368 plants and compatible species. Scotts and Monsanto have no intention to and will not commercialize or further propagate such plants in the future. Further, Scotts and Monsanto will not grant a license to or otherwise allow other entities to obtain, use or propagate such plants.

I.B. Rationale for the Development of Glyphosate Tolerant Creeping Bentgrass Event ASR368

Scotts and Monsanto developed glyphosate tolerant creeping bentgrass event ASR368 (event ASR368) that is tolerant to the herbicide glyphosate, the active ingredient in numerous industrial, turf and ornamental herbicides. The EPSPS enzyme from *Agrobacterium* sp. strain CP4 is functionally similar to plant EPSPS enzymes but has a greatly reduced affinity for glyphosate (Padgette et al., 1996). In conventional plants, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids thereby preventing plant production of these essential compounds (Steinrucken and Amrhein, 1980; Padgette et al., 1996). In glyphosate tolerant creeping bentgrass, metabolic requirements for the plant's growth and development are met by the continued action of the glyphosate tolerant CP4 EPSPS enzyme in the presence of glyphosate.

Creeping bentgrass with glyphosate tolerance would have enabled the use of glyphosate herbicides for effective control of weeds occurring in the production of grass seed and to maintain superior quality turf on golf courses. Glyphosate is highly effective against the majority of annual and perennial weeds common to grass seed and turf production. Scotts and Monsanto, however, will not commercialize or license to other entities glyphosate tolerant creeping bentgrass.

I.C. Submissions to Other Regulatory Agencies

Under the Coordinated Framework for Regulation of Biotechnology (CFR) (USDA-APHIS, 1986), the responsibility for regulatory oversight of biotechnology-derived crops falls primarily on three U.S. agencies: U.S. Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and in the case of plant incorporated protectants, the Environmental Protection Agency (EPA). Deregulation of ASR368 by USDA constitutes only one component of the overall regulatory oversight and review of this product. As a practical matter, ASR368 cannot be released and marketed until FDA and USDA have completed their reviews and assessments under their respective jurisdictions. Additionally, EPA must complete its review and assessments prior to approving the use and allowable residues of glyphosate on ASR368.

I.C.1. Submission to FDA

Event ASR368 falls within the scope of the 1992 FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (U.S. FDA, 1992). A food/feed safety and nutritional assessment summary document was submitted to the FDA in September 2002 and completed its consultation with the FDA, identified under BNF No. 000079, on September 23, 2003 (http://www.fda.gov/food/foodscienceresearch/geplants/submissions/ucm155781.htm).

I.C.2. Submission to EPA

As Scotts and Monsanto will not commercialize ASR368, there is no need to obtain a label amendment to allow the use of glyphosate on ASR386. Therefore, neither Monsanto nor Scotts are seeking, or will seek in the future, a label amendment from EPA with respect to ASR368.

I.C.3. Submissions to Foreign Government Agencies

Scotts and Monsanto does not intend to make any submissions for approval of ASR368 to foreign governments.

II. THE BIOLOGY OF CREEPING BENTGRASS (*AGROSTIS STOLONIFERA* L.)

The following section provides an in-depth review of the scientific literature regarding the origin, use, biology, taxonomy, genetics, related species and potential weediness of creeping bentgrass. Although a wind-pollinated and an obligate outcrossing species, this review provides substantial evidence to support the following statements regarding *A. stolonifera*:

- seed has been successfully produced in the U.S. for more than 110 years and for the past 85 years in essentially a single defined geographic area without becoming either an uncontrolled weed of or impacting more than 80 other turfgrass species;

- the formation of hybrids with related species is possible but declines precipitously with increasing distance from a source plant;

- hybrids are typically intermediate of their parents and predominantly sterile; and

- while the species has some weedy characteristics (Baker, 1974), it is not considered a major weed in U.S. agriculture (Holm et al., 1979; Uva et al., 1997; USDA 2015; USDA-APHIS 2015a).

Furthermore, when considered in conjunction with the data and information provided in Section VII of this document, it is apparent that ASR368 is no more likely to become a weed than conventional creeping bentgrass.

II.A. Origin and cultural history of *A. stolonifera*

II.A.1. Geographic origin

Complete agreement in the literature as to the geographical origin of *A. stolonifera* does not exist. However, the species is thought to have originated in cooler climes of both Eurasia and North America (Hitchcock, 1950; Hubbard, 1984) but is now naturalized in similar environments in other regions of the world (Hubbard, 1984; Hitchcock, 1950). Native and naturalized habitats of *A. stolonifera* tend to be moist and humid, and include coastal areas (Hitchcock, 1950; Hubbard, 1984), highland meadows (Hubbard, 1984), flood plains (Bradshaw, 1958a), and early succession forested areas (Collet et al., 1996). It apparently has been a common component of Eurasian pasturelands for centuries (Bradshaw, 1958a; King, 1962; Duich, 1985).

II.A.1.1. Sporting uses of *A. stolonifera*

A. stolonifera has been planted as a playing surface on golf course putting greens, tees and fairways in cool season turfgrass growing areas of North America for over 110 years (Duich,

1985; Hurley and Murphy, 1996). There is practically no current home lawn or institutional market for *A. stolonifera* because of the high level of management required to maintain its aesthetic character (Hanson et al., 1969; Turgeon, 2002).

The tolerance of *A. stolonifera* to extremely close mowing and its stoloniferous, spreading habit make it especially well adapted to the frequent, close mowing and recuperative requirements of golf course putting greens and modern fairways (Reese, 2000). *A. stolonifera* excels and is most competitive at mowing heights between 0.125 and 0.75 inch that are common to golf course greens and fairways, and is not reported to produce mature flowers, shed pollen nor produce viable seed that are disruptive to play at these mowing heights (Lush, 1988a; Quemada, 1999). Its vegetative growth response to consistent water, nitrogen-based fertilizers and sunlight allows it to recover from the damage due to traffic and golf club action (Beard, 2002). Surrounding areas (golf course roughs) are typically planted to turfgrasses of different genera with less intensive management inputs (Beard, 2002).

II.A.1.2. Cultural history

Although potentially native to this continent (Hitchcock, 1950), a North American origin of the *A. stolonifera* varieties used for golf course green turf has been questioned (Duich, 1985; Meyer and Funk, 1989; Hurley and Murphy, 1996). The earliest *A. stolonifera* golf putting greens were established in the U.S. by greens keepers who imported "South German" bentgrass seed in the late 19th century from Europe (Duich, 1985).

South German bentgrass was an inconsistent mixture of *Agrostis* species, primarily colonial bentgrass (*Agrostis tenuis* Sibth now accepted as *A. capillaris* L.) variably mixed with velvet bentgrass (*Agrostis canina* L.), redtop (*Agrostis alba* L., now accepted as *A. gigantea* Roth.) and typically only a trace of *A. stolonifera*. That trace was sufficient, however, to produce greens of predominantly *A. stolonifera* or mixtures with *A. canina* over a period of 20 or 30 years (Huff and Landschoot, 2000). The selection and use of vegetatively propagated varieties of *A. stolonifera* from these mature greens began as early as 1917 (Holt and Payne, 1951) and was prompted in part as a means to mitigate the incidence of diseases common to other vegetative varieties (Hurley and Murphy, 1996). Popular vegetative varieties included 'Congressional', 'Toronto', 'Washington' and 'Cohansey', names that reflect the geographic or golf course source of the original selection (Meyer and Funk, 1989).

Interestingly, the variable mixture of South German bentgrass seed was harvested from naturalized *Agrostis* pastures in Europe where some frequency of *Agrostis* hybridization should have been expected. However, records or reports of *Agrostis* hybrid survival at the expense of typical *Agrostis* species started from seed on golf courses in the U.S. could not be found.

The breeding of and conversion to seeded *A. stolonifera* varieties began by the mid-1900s (Hurley and Murphy, 1996). The first commercially available Certified seeded variety, 'Seaside', became available in the 1920s. Hitchcock (1950) refers to Seaside bentgrass as a seeded variety of native origin, while Hurley and Murphy (1996) refer to it as a naturalized species, developed with no formal breeding. Regardless of its origin, Seaside and other early

seeded *A. stolonifera* varieties were not improved through plant breeding but harvested from nearly pure, naturally occurring stands (North and Odland, 1935; Schoth, 1930).

An entire edition of *The Bulletin*, a publication of the United States Golf Association Green Section, was devoted to early bentgrass seed production in North America. Articles recount some of the pioneers and techniques used by the bentgrass seed industry in the Pacific Northwest, Rhode Island and Canada (USGA, 1930). Odland (1930) reports that bentgrass is mentioned in U.S. records from at least 1747, although no reference is cited. In 1924, Lyman Carrier, USDA, traveled to the Coos Bay, Oregon region with H.A. Schoth, USDA, to look for creeping bentgrass stands identified by Schoth and Roland McKee of the Bureau of Plant Industry on a previous visit in 1923 (Schoth, 1930). They found nearly pure stands in dairy pastures along coastal streams. Don Brewer, former Oregon Seed Certification Director, recounted the early years of creeping bentgrass seed production in Oregon (Brewer, 1992; Oregon State University, personal communication, 2002). Brewer wrote that Carrier resigned from the USDA, then immediately moved to Oregon and formed a company called 'Cocoos' that would purchase bentgrass hay from the growers of the pure creeping bentgrass stands and then thresh, condition and market the seed as Cocoos bentgrass. Carrier was competing with imported German bentgrass that was being sold for $5.00 per pound.

Apparently, Professor George Hyslop at Oregon State University learned of Carrier's operation and felt growers were losing a valuable opportunity. Hyslop then apparently encouraged growers to harvest and process the seed themselves and offered OSU to "certify" their seed as Seaside variety creeping bentgrass (Brewer, 1992; OSU personal communication, 2002). Hyslop (1930) also recounts the difficulty in distinguishing redtop and creeping bentgrass seed at the time. The creeping bentgrass, Seaside, was first harvested in 1926 and the first Certified Seaside creeping bentgrass was harvested in 1927 (Hyslop, 1930).

The first seeded variety improved through breeding and selection was Penncross, introduced by the Pennsylvania State University in 1954 (Duich, 1985). The breeding of seeded varieties has progressed considerably, with more than 25 new varieties released in the last 30 years (Cattani et al., 1996; Hurley and Murphy, 1996). Some of the selection and breeding of current cultivars was the result of crossbreeding of early named vegetative selections along with other high quality clones from turf areas or conventional cultivars such as Penncross and selecting and reselecting among the progeny resulting from these crosses.

II.A.1.3. Establishment

Establishment strategies for *A. stolonifera* on golf courses focus on high populations and the most rapid germination and grow-in possible, so that play may commence at the earliest possible date. *A. stolonifera* seeds are among the smallest grass seeds (6-7 million/pound) (Musser and Perkins, 1969) and therefore limited in carbohydrate reserves. Successful establishment of commercial *A. stolonifera* by seed requires a highly prepared seedbed and substantial inputs of water and nutrients to encourage germination and rooting (Burton, 1992).

Fall seeding or stolon sprigging is preferred over spring for seed production because floral induction of buds is required to promote reproductive floral initiation the following spring (Turgeon, 2002). The economic outcome of this physiological process is that in some geographic areas fall seedings produce a crop in approximately twelve months instead of eighteen for spring seedings. Fall establishment also decreases annual weed pressure and provides a longer development period before the following summer golf season (White, 2000; Schumann et al., 1998).

A. stolonifera prefers a slightly acid soil pH between 5.5 and 6.5. Soil acidity should be adjusted prior to planting according to a soil test (Beard, 2002). Likewise, soil nutrient levels are adjusted both prior to planting based on a soil test and then frequently after establishment based on either a soil or foliar test (Beard, 2002). Mature fairway plantings require between 80 and 160 pounds of nitrogen per acre per season and greens are generally fertilized at 1.5 times the fairway recommendation (Beard, 2002). Newly established stands are fertilized more intensively and frequently than mature stands to hasten root growth, plant development and to establish a tight closed plant stand (Beard, 2002).

Seeding rate recommendations for new turfgrass establishment of *A. stolonifera* range from as low as 20 to 40 seeds per square inch or 0.5 to 1.0 pound per 1000 square feet (Madison, 1966) to as high 3.0 pounds per 1000 square feet (White, 2000; Beard, 2002) for some putting greens. The lower range of rates is most commonly recommended and will provide an adequate stand but some practitioners believe the higher rates will provide a denser stand in less time. Evidence suggests, however, that increased spatial competition in these plantings can lead to higher disease rates (Rossi et al., 1999) and increased consumption of water and nutrients and that the presumed temporal benefit is seldom achieved (White, 2000). Lush (1990) proposed the use of the power rule to ascertain the potential wear tolerance of turfgrasses. Cattani (2000), using this formula, showed that wear resistance potential equilibrated by 12 weeks after seeding different rates of creeping bentgrass seed, even though there were distinct visual differences in the turf.

II.B. Biological characteristics of *A. stolonifera*

II.B.1. Taxonomy

Agrostis stolonifera is a flat or involute-leaved, stoloniferous, perennial species with stolons up to 200 cm in length and smooth stems from 5 – 100 cm. The panicle inflorescence is characteristic of the genus *Agrostis*. The inflorescence ranges from 1 to 30 cm in length with branches spreading only at anthesis. They often bear green to purplish spikelets of 2 to 3 mm in length and consist of a single perfect floret (Pohl, 1953; Tutin, 1980), which opens in the morning (Cattani pers. comm., 2002; Davies, 1953). *A. stolonifera* is wind-pollinated and an essentially obligate outcrossing species (Bradshaw, 1958a).

Agrostis stolonifera has been ascribed to the family *Poaceae;* subfamily, *Pooideae;* tribe *Agrostideae* (Hitchcock, 1971). A recently accepted classification is as follows:

Family: Poaceae

Subfamily: Pooideae

Tribe: Poeae

Subtribe: Agrostidinae

Genus: Agrostis (Soreng et al., 2001)

Creeping bentgrass used for turf is currently accepted as *A. stolonifera* L. (Soreng et al., 2001). The species has also been referred to as *A. palustris* (Huds.) (Beard, 1973; Christians, 1998; Meyer and Funk, 1989) or *A. stolonifera* var. *stolonifera* and A. *stolonifera* var. *palustris* (Philipson, 1937). Breeders and taxonomists continue using both *A. stolonifera* L. and *A. palustris* Huds. synonymously or in combination as the species name for creeping bentgrass. We use *A. stolonifera* L. in this paper because it is the oldest and currently accepted usage.

The common name, "creeping bentgrass", has been erratically and erroneously applied to several *Agrostis* species including *A. alba* or more commonly "redtop" (Beal, 1896; Budd and Best, 1964). Gould and Shaw (1968) misapplied *A. stolonifera* to redtop (*A. alba* syn. *A. gigantea*). Common name usages have also contributed to many of the taxonomic difficulties associated with *A. stolonifera* L. (Bradshaw, 1958b; Cattani, pers. comm., 2002).

The misapplication of both common and scientific names to the various *Agrostis spp.* is a common problem with this genus and difficult to discern when comparing different taxonomic references (Stuckey and Banfield, 1946). This is due to frequent taxonomic classification based on adaptation and phenotypic characters, which reflect observed differences by particular taxonomists (Philipson, 1937). For example, Hitchcock (1950) describes *A. palustris* as similar to *A. stolonifera* except in a few phenotypic characters including length of stolons and its propagation for turf use. However, stolon length appears to exhibit considerable variability. Cattani et al. (1996) reported significant internode length differences between commercial *A. stolonifera* varieties. Cattani (1999) also found differences in stolon and internode length between two *A. stolonifera* varieties that were reportedly closely related. In addition, Burg et al., (1979) reported the use of lemma and palea characteristics in species determination of *Agrostis spp.* with similar seed size but Davies (1953) uses palea length to divide the commonly occurring U.K. *Agrostis* sp. into two types.

Varied attempts to definitively characterize *Agrostis spp.* or any other species suggest that the use of phenotypic differences, while convenient, is not always clear and should not be relied upon as the sole method to distinguish between closely related species (Philipson, (1937; Barkworth and Dewey, 1985). Finally, *A. stolonifera* has been observed to hybridize with other *Agrostis* species, particularly *A. tenuis* Sibth. (Bradshaw, 1958a; Davies, 1953). Morphology of the hybrids is variably intermediate between the two parent species (Bradshaw, 1958a) and may account for some of the confusion.

Hitchcock (1950, 1971) considers *A. palustris* (*A. stolonifera)* as a potential native species to northern North America. Meyer and Funk (1989) and Hanson et al. (1969) consider creeping bentgrass to be introduced to North America from Eurasia. The USDA, NRCS-Plants Database lists *A. stolonifera* as a native species (USDA, NRCS, 2001), perhaps based on Hitchcock's hypothesis (Hitchcock, 1950).

II.C. Genetics

The cytological work, although currently limited, may eventually prove more useful in determining species status. A cytological characterization of the bentgrass species was conducted by Keith Jones (1956a,b,c). His elegant work, although conducted in the 1950's, is still considered the standard reference on bentgrass genetics. He reports on the cytological characteristics of velvet bentgrass (*A. canina* var. *canina* and *A. canina* var. *montana*), colonial bentgrass (*A. tenuis,* currently recognized as *A. capillaris*), creeping bentgrass (*A. stolonifera* syn. *A. palustris*) and redtop (*A. gigantea*). Jones also characterized the cytology of *Agrostis* hybrids including, *A. canina* var. *canina* (currently recognized as A. *canina*) × *A. canina* var. *montana* (currently recognized as *A. vinealis*), *A. canina* var. *montana* × *A. tenuis*, *A. canina* var *montana* × *A. stolonifera*, *A. gigantea* × *A. tenuis* and *A. gigantea* × *A. stolonifera*. Chromosome counts were conducted on all species and hybrids (Table II.2). The basic chromosome number of all *Agrostis* species is ×=7. Creeping bentgrass was found to be a strict allotetraploid with 2n=4x=28 with 14 bivalents forming at meiosis (Jones, 1956b; Church 1936). Jones (1956a,b,c) and Stebbins (1971) reported that the genus *Agrostis* might also have accessory or B chromosomes.

Warnke et al. (1998) confirmed the allotetraploid genomic constitution of 2n=28 for creeping bentgrass. The following genomic constitution of each species (or subspecies) was proposed by Jones (1956a,b,c) showing the currently recognized species designations:

Table II-1. Genomic constitution of several common bentgrass species (Jones 1956a,b,c)

Species	Chromosome Number	Genome Constitution
Velvet bentgrass (*A. canina*)	14	A_1A_1
Brown velvet bentgrass (*A.vinealis*)	28	$A_1A_1A_1A_1$
Colonial bentgrass (*A. capillaris*)	28	$A_1A_1A_2A_2$
Creeping bentgrass (*A.palustris, A. stolonifera*)	28	$A_2A_2A_3A_3$
Redtop (*A. gigantea*)	42	$A_1A_1A_2A_2A_3A_3$

Jones' idea for the cytological work arose due to significant taxonomic and cytological confusion concerning all of the species in the genus *Agrostis*. Work conducted prior to Jones mentioned numerous conflicting reports about the cytology and taxonomy of *Agrostis* species and putative interspecific hybrids. Differences in chromosome numbers within a species, differences in adaptation and occurrence of interspecific hybrids and differences in

the possibilities and success of interspecific crosses beyond the F_1 generation are reported in the literature dating back to the late 1890s.

Jones (1956a,b,c) and Bradshaw (1958a) concluded that, while interspecific F1 hybrids were possible, genomic differences between species, incomplete homology between genomes, low seed set of F_1 hybrid plants and potential for functionally sterile aneuploid gametes and/or aneuploid plants would act as an effective barrier to gene exchange between species.

In possible support of the theories of Jones and Bradshaw, Bjorkman (1954) identified 600 tetraploids (2n=28), 160 pentaploids (2n=35) and 135 hexaploids (2n=42) from his 897-plant *A. stolonifera* collection. Only two aneuploid plants were recovered from his broad geographic collection of vegetative material. The hexaploid and pentaploid types produced almost exclusively aneuploid progeny (17/18 and 31/32 respectively) upon self-pollination. When he crossed hexaploid and tetraploid cytotypes artificially, he recovered 26 progeny and 25 of them were aneuploid. When he crossed pentaploid and tetraploid plants, he recovered 7 progeny, 6 of which were aneuploid. Jones (1956b) found it interesting to note that Bjorkman's field collections had been almost exclusively selected for euploid chromosome numbers in light of the high proportion of pentaploids in the original population and the proportion of aneuploid progeny. However, the artificial nature of Bjorkman's forced crosses and seedling establishment under noncompetitive greenhouse conditions does not mimic field conditions.

If Bjorkman's plants were interspecific hybrids, then the lack of aneuploids among the original collected parent plants with such a large proportion of 2n=35 types suggests that F_2 hybrids and/or F_1 backcrosses to the parent species are uncommon or are unlikely to persist in nature. Sterility of creeping bentgrass F_1 interspecific hybrids due to meiotic irregularity (demonstrated in *Agrostis* by Jones, 1956a,b,c and Bradshaw, 1958b) or poor fitness and survival of typically weak aneuploid F_2 and backcross plants are possible explanations for Bjorkman's results.

Bradshaw (1958a) studied the natural hybridization between *A. stolonifera* and *A. tenuis* (believed to be *A. capillaris* since it was the tetraploid form). Bradshaw (1958b) believed that the study area also contained a few putative F_2 and backcross hybrid plants based on plant morphological characteristics. There were varying degrees of fertility in the F_1, F_2 and backcross plants, with putative backcross plants having a higher degree of fertility. Bradshaw (1958a,b) also noted that his hybrids tended to be morphologically intermediate to the parent species, which makes absolute taxonomic diagnosis without cytology very difficult.

However, Kik et al. (1992) reported a similar range of ploidy levels within *A. stolonifera*. They later demonstrated both ploidy level and somatic chromosome number variation in individual plants of *A. stolonifera* (Kik et al., 1993). Therefore, another possible explanation for the lack of aneuploids in Bjorkman's population is chimeric development of tissues with different ploidy levels within *A. stolonifera.* The chimeras may eventually give rise to plantlets that become individual entities over time. However, Bjorkman (1954) may have been observing plants displaying Kik's aneusomaty in *A. stolonifera*.

Kik, et al. (1992) observed some relationship between ploidy level and ecological niche. Higher ploidy levels occurred with greater frequency in stable environments where clones could develop over a period of years and the role of sexual reproduction in survival was reduced. Non-tetraploids were not observed in highly unstable environments where high levels of individual turnover in the population relied on a seed bank for survival of a colony. Bradshaw (1958a,b) noted that the higher ploidy forms of creeping bentgrass are rare and almost wholly sterile which would tend to favor them in intensively grazed or undisturbed sites. Because *Agrostis* hybrid plant morphology (on which most of the taxonomy of the genera is based) is so similar to *A. stolonifera*, creeping bentgrass, the hybrids are difficult if not impossible to distinguish from creeping bentgrass. Therefore, it is possible that reports of higher ploidy level for *A. stolonifera* by Bjorkman and Kik may have been either observations of hybrid plants or cytotypes of *A. stolonifera* with varying ploidy levels.

Because of the similarity of bentgrasses and their hybrids in general and the lack of any reported or recorded serious impact of the hybrids, the study of *Agrostis* cytology and hybridization is more out of curiosity and an academic exercise rather than an environmental concern.

II.D. Related species

Other cultivated species of *Agrostis* include colonial bentgrass, redtop, velvet bentgrass, dryland bentgrass and Idaho redtop. Like creeping bentgrass, all of these species are perennial, wind-pollinated grasses that flower once per year and set seed during late summer. None of the cultivated, introduced or native *Agrostis* species are listed as noxious weeds in United States agriculture. None of the *Agrostis* species appear on the USDA, APHIS, PPQ, Federal Noxious Weed list or on any state noxious weed list[2].

Cultivated *Agrostis* Species:

The *Agrostis* species used for turf culture have been selected for their ability to persist from one year to the next. These species are also selected to tolerate regular mowing at a low height of cut and regular traffic yet still provide a dense and uniform sward that can recuperate from wear. In addition, the species must be able to produce an economical seed yield for production, distribution and establishment.

II.D.1. Colonial bentgrass

Colonial bentgrass, *A. capillaris* (previously *A. tenuis*) (Hitchcock, 1950; Widen, 1971; McNeill and Dore, 1976), is generally regarded as having 28 chromosomes (Bjorkman, 1954; Sokolovskaya, 1938). Jones (1956b) identified colonial bentgrass as a segmental allotetraploid with one of its diploid ancestors in common with *A. stolonifera* and the other belonging to *A. canina*. Stuckey and Banfield (1946) reported an almost complete aneuploid series of 28 to 42 among seeds collected from pasture plants they first identified as *A. tenuis*.

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[2] http://plants.usda.gov/java/noxComposite [Accessed October 22, 2015]

However, they do conclude that most of the 2n=42 plants resembled *A. alba,* even though they were first thought to be *A. tenuis*. Jones (1956b) is of the opinion that Stuckey and Banfield were observing hybrid material and that these seedling chromosome numbers have little bearing on what the chromosome number of their parent plants may have been. Stuckey and Banfield made no conclusion regarding the origin of the aneuploid plants.

Colonial bentgrass is predominantly cultivated for lawns and recreational turf. This species is more commonly used in Europe than in the United States. Colonial bentgrass characteristically has a lighter green color compared to creeping bentgrass and produces short rhizomes and stolons (Christians, 1998). Fewer than 10 colonial bentgrass commercial cultivars are eligible for certified seed production in Oregon (OSU, 1999).

II.D.2. Velvet bentgrass

Hitchcock (1950) lists velvet bentgrass as being introduced into the United States. This species, *A. canina* (previously *A. canina* subspecies *canina* or with *A. canina* subspecies *fascicularis* (Hubbard, 1984)) is a diploid (2n=14, ×=7) that usually forms 7 bivalents at meiosis (Bjorkman, 1951; Jones, 1956a). Jones (1956a) believes that brown velvet bentgrass (*A. canina* L. ssp. *montana* (Hartm.) Hartm.) (now recognized as *A. vinealis*), is most likely an autotetraploid (2n=4x=28) possibly derived from chromosome duplication within or between ecotypes of the diploid subspecies *canina*.

Velvet bentgrass has been used on a very limited basis on putting greens in the Pacific Northwest and New England other than its inclusion in imported South German bentgrass seed mixtures. The species is stoloniferous with a slow and low growing habit with high shoot density. It is reported to be more tolerant of lower mowing heights, cold, heat, drought and shade than creeping bentgrass, but can produce excessive thatch when managed under high maintenance conditions. Velvet bentgrass is competitive at low clipping heights, however its slow spreading rate and low growth habit is easily out-competed in most natural environments. Velvet bentgrass has a narrow niche adaptation and has intensive maintenance requirements (Turgeon, 1985). Three commercial varieties (Kingstown, Vesper and SR7200) are eligible for Certified seed production in Oregon with fewer than 500 total acres in production (Oregon State University, 2001a).

II.D.3. Redtop

Redtop, *A. gigantea* (preferred to *A. alba L.*,), is a hexaploid (2n=6x=42) (Jones, 1956c). Jones also concluded that the hexaploid redtop shares some homology with both the tetraploids: creeping and colonial bentgrass (Jones, 1956c). Hitchcock (1950) lists redtop as a perennial species introduced into the United States. Redtop produces only rhizomes, no stolons (Christians, 1998).

During the 1940s and 1950s redtop was often used as a nurse grass for turf establishment. In seed mixtures, redtop seedlings would germinate quickly but would yield over time to the desired turfgrasses in the mixture such as Kentucky bluegrass and fine fescue. Redtop inclusion as a nurse grass in improved seed mixtures was replaced by improved perennial ryegrasses in the 1970s. The species is rarely used in the turf industry today. Redtop is

considered to be a short-lived perennial in turf (Beard, 1973) however, persistent patches of unknown age of redtop have been observed in cool-season turf areas (Christians, 1998). One variety was eligible for Certified seed production in 2000 (Oregon State University, 1999). It is estimated that Certified redtop seed is currently produced on less than 500 acres in Oregon.

II.D.4. Dryland bentgrass

Dryland bentgrass, *A. castellana* Boiss. & Reut., is considered a hexaploid (2n=6x=42) (Darlington and Ammal, 1945). Reports of 2n=28 are also given for *A. castellana* (Bjorkman, 1954). 'Highland' dryland bentgrass, until recently, was treated as a cultivar of colonial bentgrass in the U.K. (Shildrick, 1976), but Highland is on the borderline of the morphological characters described for colonial bentgrass and is very distinctive in turfgrass performance (Shildrick, 1976). The features of Highland bentgrass are quite distinctive from other cultivars of colonial bentgrass and include vigorous spread by rhizomes, formation of aerial tillers under high mowing height, a prominent, jagged ligule twice the size of normal colonial bentgrass and similar in size to creeping bentgrass, blue-green leaf color, tall open growth habit and good winter color (Shildrick, 1976). Hitchcock (1950) lists Highland as an aberrant form of A. *tenuis* that may be a distinct species belonging to *A. castellana* based on seed and plant examination. The description of dryland bentgrass, particularly leaf color and heading records suggest that dryland bentgrass is very similar to Highland bentgrass (Shildrick, 1976).

The species has limited application in turf or forage due to its high susceptibility to several common turf diseases including *Rhizoctonia* brown patch; however; it is recognized for its heat and drought tolerance. There were 2815 acres of certified Highland bentgrass seed produced in Oregon in 2001 (Oregon State University, 2001a).

II.D.5. Idaho redtop

Idaho redtop or Idaho bentgrass, *A. idahoensis* (may be syn. with *A. clavata auct. non Trin.* and *A. borealis* Hartman var. *recta*.) (USDA, NRCS. 2001) is a perennial tetraploid (2n=4x=28). Furthermore, Idaho redtop is classified as non-creeping, without stolons or rhizomes. The species is native to the western U.S. and is adapted to wet meadows or bogs at medium to high elevations (Hitchcock, 1950).

One variety, GolfStar, establishes well in turf plots, but has a dull green color and an upright growth habit that is less attractive than creeping, colonial or velvet bentgrass. In New Jersey turf trials this variety has shown excellent resistance to dollar spot (Bonos, et al. 1998). GolfStar is eligible for Certified seed production in Oregon in 2001 with 87 acres planted on 3 farms (Oregon State University, 2001b). It is estimated that less than 500 acres of Idaho redtop are in production in Idaho.

II.D.6. Minor Agrostis species

Any significant level of hybridization or unexpected impact of potential hybrids among and within less common *Agrostis* species is unlikely. This conclusion is based on the limited

number of literature citations for naturalized hybrids among the *Agrostis* species selected for widespread perennial turf cultivation and seed production. In addition, there have been no reports of adverse agricultural impact by any *Agrostis* hybrid documented in the literature.

Carlbom (1967) did an exhaustive survey of native *Agrostis* species of the western United States. More recently, MacBryde (2005) compiled a list of 37 Agrostis species existing in the United States. Narrow adaptation and limited distribution of many of the *Agrostis* species listed in Appendix I will make hybridization with *A. stolonifera* unlikely in seed production areas and in turf use. For example, Carlbom noted that *A. howellii*, *A. diegoensis* and *A. perennans* were all adversely affected by wilt when temperature exceeded 85^0F in his experimental garden in Corvallis, which provides strong evidence of the narrow endemic adaptation of these *Agrostis* species. Glyphosate tolerance is unlikely to increase the frequency of hybridization that may already occur with *A. stolonifera*. Differences in chromosome number between many *Agrostis* species and creeping bentgrass will further limit F1 generation fertility and fecundity of any potential hybrids.

II.E. Life history

Life history refers to the patterns of establishment, growth, fecundity and survival in a given species or genotype (Kik et al., 1990a). An understanding of species life history can be useful for predicting invasiveness or weediness in a particular ecological niche (Barrett, 1988).

II.E.1. Introduction

Agrostis stolonifera is native to or introduced and naturalized in a variety of coastal, lowland and upland settings (Kik et al., 1990a,b) and is known to tolerate transient flooding (Davies and Singh, 1983). Even though a moist, fertile soil environment is a common characteristic of *A. stolonifera* habitat (Hunt et al., 1987), it also survives in sand dune environments with poorer water and nutrient retention characteristics (Kik, 1989). In those areas where *A. stolonifera* prevails despite a lack of high soil moisture, high humidity appears to play a significant role in its survival. When grown for golf course use and seed production, *A. stolonifera* receives generous applications of water and nitrogen to ensure its survival and recuperation under adverse conditions of foot and equipment traffic as well as heavy play (Beard, 2002; Meyer and Funk, 1989).

Agrostis stolonifera plant stands may originate from either seeds or stolon nodes. Sexual reproduction and a seed source are more important in unstable sand dune environments where a high degree of individual plant mortality is prevalent in the population (Kik et al., 1990a,b). This phenomenon contrasts with very stable grasslands where a higher incidence of vegetative propagation was reported. New *A. stolonifera* seedling establishment has not been found to significantly contribute to stand augmentation in established vegetated areas (Lush, 1989; Jonsdottir, 1991; Bullock et al., 1994; Howe and Snaydon, 1986; Rossi, 1999; Eriksson, 1989).

II.E.2. Survival

Hunt et al. (1987) reported that *A. stolonifera* colonized and prospered in environments with a high degree of disturbance and very limited environmental stress. Disturbance in this context refers to bare soil, which increases the probability of direct and firm contact of a seed or node with soil. Soil disturbance in an *A. stolonifera* environment can result from any of the following: tillage in seed production or turfgrass establishment sites, sod removal, burrowing animals, livestock grazing damage, golf course traffic or club damage, soil introduction through turfgrass topdressing or alluvial deposition. In a favorable environment, Crick and Grime (1987) demonstrated that *A. stolonifera* can rapidly produce roots. Once established, the plants spread primarily through clonal expansion via stolons (Jonsdottir, 1991; Kik et al., 1990b). Low environmental (light, moisture, nutrition, air, temperature) stress allows for extensive root development, the accumulation of carbohydrate reserves, production of vegetative tillers and increased survivability during temporary stress periods. Areas of high disturbance but high environmental stress tend to be populated by small colonies with more reliance on the seed bank for survival than vegetative mechanisms (Kik et al., 1990a,b).

Several studies suggest that rapid leaf succession rate in newly emerged seedlings contributes to the successful establishment of *A. stolonifera* and its ability to compete with surrounding vegetation. In a controlled environment study, Cattani (2001) reported that the second leaf must fully expand for an *A. stolonifera* seedling to persist longer than 14 days after emergence. Since tillering in *A. stolonifera* is not initiated until after the third leaf has at least partially emerged under non-competitive conditions (Cattani, 1999), the attainment of this stage is critical for persistence as it doubles the number of vegetative axes. Cattani (1999) also reported that reduced light duration was shown to lead to a delay in tillering until after the full emergence of the third leaf, thus further jeopardizing seedling survival in competitive environments.

Survival of newly germinated seedlings from a heterotrophic to an autotrophic state, which is critical for survival, is most successful during initial colonization of a newly disturbed site (Whalley et al., 1966). Consequently, successful augmentation of *A. stolonifera* populations by seedlings has been reported to occur in situations optimal for germination and establishment to occur. These include either major disturbance events in which the seed to soil contact is high (Sheldrick et al., 1990) or when seed is introduced into turf maintained at very low clipping heights (Cattani, 2001).

Conversely, successful establishment can be limited in existing swards due to either insufficient disturbance or excessive competition from the existing population. Howe and Snaydon (1986); Jonsdottir (1991) and Bullock et al. (1994) noted that seedling recruitment into natural stands and pastures, respectively, was unsuccessful in augmenting *A. stolonifera* populations. Sweeney and Danneberger (1998) were unable to demonstrate with molecular markers the success from direct interseeding *A. stolonifera* into an existing *A. stolonifera* stand utilized for golf putting green turf. The difficulty in establishment of *Agrostis* seedlings may be a consequence of the extremely small seed (0.07 mg $seed^{-1}$) size. Small seed requires a long duration of disturbance (reduced competition) and low stress for establishment and survival after germination (Cattani, 2001). Small seed may also preclude

direct contact with soil due to root competition from the existing vegetative stand (Kendrick and Danneberger, 2002).

Ahrens et al. conducted a large scale study to determine bentgrass distribution and habitat suitability that covered 8.5 km2 and included the detailed evaluation of 289 circular, 100 m2 plots in the northeastern U.S. (Ahrens et al., 2011). Creeping bentgrass presence was positively correlated with herbaceous plant cover and mowing, but negatively correlated with tree or shrub cover, poorly drained soils and leaf litter. Specifically, creeping bentgrass was most commonly found in managed areas such as home lawns and railroad right-of-ways.

In a study intended to simulate glyphosate treatment around glyphosate tolerant creeping bentgrass plants, Ahrens and Auer covered transplanted creeping bentgrass plants (3 cm by 3 cm at intial planting), applied glyphosate to surrounding plants, and measured impact on survival, growth and population dynamics (Ahrens and Auer, 2012b). No creeping bentgrass plants survived if glyphosate was not utilized to remove competition despite mature 3 cm2 plants being used to initiate the study. Only 56% of the creeping bentgrass in glyphosate treated plots survived. The surviving creeping bentgrass plants in plots treated with glyphosate showed more growth and flowers than control non-glyphosate treated plots.

Hart et al (2009) evaluated the competitiveness of Agrostis interspecific hybrids, including interspecific hybrids with creeping bentgrass, in a managed Kentucky bluegrass lawn and in a low maintenance roadside stand of mixed species. The hybrids' growth was intermediate relative to the parental species in both habitats.

Garrison et al. (2009) conducted a survey of two abandoned golf courses with creeping bentgrass putting greens in Michigan to document the impact of management cessation on creeping bentgrass survival Creeping bentgrass was nearly absent from Matheson Greens Golf Course (Northport, MI) five years after operations ceased. A similar trend took place at the Four Winds Golf Course (East Lansing, MI) in which creeping bentgrass putting greens contained less than 25% creeping bentgrass only two years after maintenance stopped. This study provided an analysis of the relevant fitness of creeping bentgrass in an environment that transitioned from being highly managed to unmanaged.

Garrison and Stier (2010) evaluated the fate of transplanted creeping bentgrass plants. They transplanted plugs grown in 3.8 by 14-cm cone-tainers into prairies located in Monroe and Wausau, WI. By year 2, creeping bentgrass was essentially eliminated at one site (Monroe, WI) and was significantly reduced in size at the second location (Wausau, WI). These results are very much consistent with work by Arhens and Auer (2012) and the results from abandoned Michigan golf courses (Garrison et al., 2009). The reduction in size and death of the creeping bentgrass plants was attributed to herbivory, environmental stresses and competition with surrounding vegetation.

II.E.3. Growth

A. stolonifera is perennial in nature and can exhibit enhanced stolon growth (Eriksson, 1989; Watschke, 1995) under favorable conditions. The species prefers areas with high disturbance but low environmental stress levels, e.g. good mineral nutrition and water

availability (Hunt et al., 1987). Kik et al. (1990a) reported that vegetative tillering and subsequent vegetative colonization were favored by stable environments rather than unstable and/or environmentally stressful ones. Although Kik et al. (1991) reported that *A. stolonifera* mortality occurred under three environments varying in the level of environmental stress, clonal survival was highest in the lowest stress environment.

Tillers arise from axillary buds that are most active in the late summer to early autumn time frame and may function vegetatively or reproductively according to environmental stimuli (Turgeon, 2002). Tillers are growth and expansion mechanisms. Attached tillers lead to the in-place expansion of the plant and stolons lead to the production of independently rooted daughter plants at stolon nodes (Cattani and Struik, 2001). Stolons generally develop as competition within the plant necessitates better light for growth (Cattani and Struik, 2001). Kik et al. (1992) reported that long-lived vegetative clones developed significant numbers of vegetative tillers. These tillers produced larger vegetative organs with a higher probability of survival than fertile individuals.

Seed producers enhance *A. stolonifera* reproductive tillering for seed production by planting in rows and removing top-growth after harvest. Golf courses manage *A. stolonifera* with frequent close mowing, aggressive cultivation, overseeding and sufficient agronomic inputs to achieve the highest shoot density and shortest internode length. Limited numbers of large, clonal colonies are less favored under these conditions than are large numbers of individual plants that contribute to visual and physical uniformity across the entire sward.

II.E.4. Fecundity

Fecundity refers to both reproductive and/or vegetative success of an organism in nature (Barbour, 1987). Both aspects of fecundity are important when considering *A. stolonifera* because of its predominantly clonal expansion activity in stable environments and seed production under environmental stress.

Collections of *A. stolonifera* from a sand dune with water and nutritional deficiencies were exclusively tetraploid and potentially fertile (Kik et al., 1990b). In 1992, Kik et al. further reported that as the stability of the environment of origin increased, higher ploidy levels increased as well and produced more robust vegetative tillers. Ploidy levels exceeding four were previously reported to be highly infertile (Bradshaw, 1958a; Bjorkman, 1954; Kik et al., 1992). These higher ploidy level individuals would rely on vegetative success rather than seed and seedling recruitment under low disturbance to survive. Aldrich (1984) notes that increased ploidy levels and fertility reduction are common to a wide range of perennial species in forage and other stable production systems.

Factors influencing reproductive *A. stolonifera* tillering include age of the tillers (Jonsdottir, 1991), environmental conditions (Turgeon, 2002) and cytotype (Kik et al., 1992). Jonsdottir (1991) reported that tillers arising after July in a natural stand are more likely to produce inflorescences in the following year and that second season tillers were the most productive. Studies of seed production methods suggest that renovation and associated tiller stimulation increase yields and are most effective when they occur shortly after harvest (Cattani et al., 1997).

Seed yields for *A. stolonifera* vary with environmental conditions. *Agrostis* species are considered to have low seed bank populations in nature (Frame, 1990), which may be due to poor seed production and/or poor seed persistence. Pastures and golf course turfgrass favor vegetative propagation over sexual reproduction because the environment is usually stable and the reproductive organs are routinely removed by clipping (Lush, 1988a). Williams (1984) found clipping reduced *A. capillaris* seed bank presence by about 18% per year. Golf course superintendents attempt to maintain the seed bank and high shoot density with a program of routine overseeding and cultivation.

Pollen-mediated gene flow on golf courses is considered unlikely due to regular mowing and the low probability of seedhead formation when maintained at a low cutting height. This consensus was drawn from a diverse working group of scientists (including academics and industry representatives) regarding gene flow from herbicide resistant bentgrasses and bluegrasses (Quemada, 1999) and is consistent with the findings of Johnson and Riordan (1999) and Lush (1988a). However, Quemada reported the workgroup's conclusion that minimal gene flow could not be completely prevented, but that herbicide resistance was unlikely to make creeping bentgrass or other turf species more invasive since they are "ill-adapted" for unmanaged ecosystems. Ahrens and Auer (2012a) utilized a retrospective population genetics approach to study gene flow from an 8 year old golf course to five feral creeping bentgrass populations. Four of the five populations were shown to be genetically homogenous, and therefore devoid of significant hybridization or progeny introduction including from the golf course. The fifth population showed more genetic variation, however, the authors commented the history of the site may have included diverse plantings. The authors suggested that two of the plants may have resulted from a hybridization event with the golf course varieties. While the data was not definitive, it showed higher correlation to commercial cultivars but not to cultivars planted on the golf course. Ahrens and Auer also noted that the frequency of identifying creeping bentgrass was less than suggested by their modeling. Competition from the existing vegetation was proposed as the reason for the lower than expected numbers and the lack of creeping bentgrass establishment. This study supports that pollen-mediatd gene flow is negligible from creeping bentgrass on routinely mowed golf courses to feral populations of creeping bentgrass.

Vegetative expansion of *A. stolonifera* is related to carbohydrate storage in vegetative tillers (Kik et al., 1992). Studies of *A. stolonifera* forage productivity in a monoculture (Haggar, 1976) and in a mixed stand (Frame, 1990) with white clover (*Trifolium repens* L.) reported that dry matter production is less than that of most other common forage grasses. While the volunteer occurrence of *A. stolonifera* colonies in pasture and some unmanaged settings is testimony to its vegetative success, its preference for generous water and nutrients limits that success in mixed populations to well-defined habitats.

II.E.5. Seed viability

The minimum germination percentage for Oregon Certified creeping bentgrass seed under the Association of Official Seed Analysts (AOSA, 1998) standard germination test is 85%. In addition, there appears to be some post-harvest ripening requirement, as the AOSCA (2001) requires a 7-day chilling before germination testing.

Seed persistence data on creeping bentgrass from controlled laboratory or field studies are limited. However, Cattani (pers. comm., 2002) has observed volunteer germination of A. stolonifera when sod was stripped from a seeded putting green five years after initial establishment. Bekker et al. (2000) estimated seed persistence for Agrostis spp. as long as 25 years. Garrison and Stier (2010) reported creeping bentgrass seed viability to be 34% after 22 months of being buried at 5 cm.

Seed production takes place early in the overall *Agrostis spp.* life span. Bekker et al. (2000) report that *Agrostis spp.* add little seed to the seed bank after the first few years of growth and seed bank presence is found primarily below the 5 cm level later in stand life. This finding suggests that seeds above 5 cm may germinate and then either contribute to the establishment of an initial sward or succumb to competition from mature grasses in established swards. Seeds below 5 cm are apparently not stimulated to germinate.

Zapiola and Mallory-Smith (2010) placed sterilized creeping bentgrass panicles in irrigation canals under six different conditions and determined the panicles moved at an average rate of 19 m min^{-1}. They also showed creeping bentgrass seeds that were stored in water at 20°C for 17 weeks maintained germination (88%), but germination was 46% if the water was at 4°C. Seeds stored in water at 4°C for 17 weeks were confirmed to be viable and the lack of germination was probably due to secondary dormancy.

II.E.6. Pollen movement

Wipff and Fricker (2001) measured bentgrass pollen traveling up to 292 m (958 feet) from the source during 1999 where they recovered a single plant among more than a thousand with the transgene. Wipff and Fricker (2001) modeled their 1998 and 1999 results. The best R^2 values from these models predicted transgene flows of 0.1% at 808 ft and 0.02% at 1,022 feet from 1999 data. R^2 values of data fit from these regression models were 0.1781 to 0.3817 in 1998 and 0.4516 to 0.8662 in 1999. The practical significance of their observations is unclear given that pedigreed seed isolation distances in place for decades have been sufficient to maintain accepted purity standards assigned to the various seed classes.

Belanger et al. (2003) examined intraspecific glufosinate tolerant transgene flow and interspecific transgene flow to non-transgenic *A. stolonifera, A. capillaris*, *A. castellana*, *A. gigantea* and *A. canina.* One plant of each species was planted at each sample point and 3 meters separated each sample point. The maximum distance from the centrally located transgenic source plants was 15 meters. The frequencies of transgenic hybrid recovery for *A. capillaris*, *A. castellana*, *A. gigantea* and *A. canina* within 15 meters in all directions were 0.044% (44,967 seedlings screened), 0.0015% (663,778 seedlings screened), 0.0% (2,298,418 seedlings screened) and 0.0% (7,556 seedlings screened), respectively for interspecific flow. Frequency of recovery for intra-specific transgene flow was 0.631% (155,773 seedlings screened). They further stated that although transgene flow can occur, the herbicide resistant transgenes would be unlikely to confer any competitive advantage to *Agrostis spp.* in natural ecosystems where herbicides are unlikely to be used.

It is important to note that pollen-mediated gene flow experiments conducted with isolated receptor plants (e.g., Wipff and Fricker, 2001; Belanger et al. 2003) will have a tendency to overestimate the amount of pollen movement with inter-varietal crossing under production field conditions since isolated or small populations of pollen receptor plants are more apt to be pollinated by their nearest neighbor than a remote pollen source. Consequently, pollination from distant source plants will be significantly less than in a non-competitive situation as used in this experiment. A number of factors will impact cross fertilization between grasses. These include: (1) synchrony of pollen shed and receptive stigmas (date and time of day); (2) proximity of the plants; (3) wind speed and direction; (4) pollen viability and longevity; (5) temperature and relative humidity and (6) compatibility between pollen and stigmas or styles (Burton, 1992).

Davies (1953) recorded anthesis dates and time of day for pollen shed in several *Agrostis* species from the British Isles (Table II.3). Whereas anthesis dates did overlap, it is apparent that there may be some isolation from pollen-mediated gene flow between some species provided by the pollination time of day. Belanger et al. (2003) were able to force hybridization in the greenhouse between *A. stolonifera* and *A. gigantea* or *A. canina* by artificially synchronizing anthesis and bagging panicles together, but they did not recover the hybrids in field studies conducted over two years. Belanger's group concluded that differences in anthesis dates for *Agrostis spp.* may limit gene flow between them.

Table II-2. Time of day for pollen shed in several *Agrostis* species (Davies, 1953)

Agrostis spp.	**Pollen shed**
A. stolonifera	10:00 – 11:30 am
A. tenuis (*A. capillaris*)	1:00 – 5:00 pm
A. gigantea	2:30 – 3:30 pm
A. canina var. *arida*	4:00 – 5:00 am
A. canina var. *fascicularis*	4:30 – 5:30 am

Size of the plant population and planting density have a major impact on outcrossing frequency due to intra-varietal pollen competition for receptive stigmas. In a series of pollination studies designed to examine isolation distance and competing pollen sources in perennial ryegrass, Griffiths (1951) concluded that the effects of intra-varietal (within a single field) pollen competition were highly effective in reducing inter-varietal crossing (between fields) and were similar to the effects of distance. Pollen flow decreases rapidly with distance. However, over short distances, intra-varietal pollen competition was more effective than distance because the nearest neighboring plants are likely to provide the biggest pollen contribution. These results help provide the basis for Griffiths' recommendation for (and the currently common practice of) cutting border rows from large

Certified fields after pollination where adequate isolation cannot be provided by distance. Similar conclusions were drawn by Heribert-Nilsson as cited in Griffiths (1951) for rye, by Knowles (1966) in smooth bromegrass and by Copeland and Hardin (1970) in perennial ryegrass.

Isolation distances prescribed for pedigree creeping bentgrass seed production vary depending on the class of Certified seed and the size of the field (Oregon State University, 2001a). Certified fields of less than 5 acres require 300 feet isolation from other *Agrostis spp.* while fields of more than 5 acres require only 165 feet isolation. Isolation distance required for Foundation seed fields of any size is 900 feet. The percentage of observable other-variety or off-type plants permitted is 2% in a Certified class eligible seed production field and 0.1% in a Registered class eligible seed production field. No visible off-type plants are permitted in a Foundation class eligible seed production field. These maximum thresholds only apply to the plants that are present in the field producing the particular class of Certified seed.

Jones and Newell (1946) confirmed the efficacy of distance isolation by studying the distribution of pollen at distance from source populations of several grass species. They found that the average amount of pollen captured at 990' from the source was <1% of that found at the source, however, there was no attempt to measure the viability of the pollen. They attributed the rapid decline in pollen concentration to gravity and dispersion. Pollen competition from adjacent fields of the same species would further reduce the potential that this pollen would find unfertilized and receptive stigmas.

II.E.6.1. Pollen viability

No specific literature reference documents the pollen viability characteristics of *A. stolonifera*. The Environmental Assessment of Pure Seed Testing's USDA APHIS Field Release Permit (97-087-02r) for glufosinate-tolerant / putative disease resistant bentgrass (Koehler, 1997) cites a pollen viability period of three hours but does not supply a reference. This assumption is most likely drawn from data on other species. Teare et al. (1970) determined that 98.6% of Kentucky bluegrass pollen began losing viability within three hours between 6 AM and 9 AM, as temperature was increasing and relative humidity was decreasing. No pollen was viable after 11 AM. They believed that factors other than temperature and humidity may be important for Kentucky bluegrass pollen longevity, but did not elaborate on what those may be. Knowlton (1922) determined that desiccation is the normal cause of death in corn pollen. Jones and Newell (1948) concluded that low temperatures and high relative humidity were best for maintaining grass pollen longevity.

These studies suggest that pollen longevity is probably greatest in cool and humid environments. Weather conditions during *A. stolonifera* pollination in Jefferson County and Malhuer County, Oregon, Canyon County, Idaho, and other inland locations such as the Willamette Valley are typically hot and dry, and should rapidly reduce pollen viability over time and distance. These adverse climatic conditions contribute to reducing the potential for hybridization.

II.E.6.2. Hybridization with other species

II.E.6.2.1. Interspecific crossing

There have been varying reports of interspecific crossing within the genus *Agrostis* (Davies, 1953; Jones 1956a,b,c; Bradshaw, 1958a; Hegi, 1935; Tutin, 1980; Welsh et al. 1987; Wipff and Fricker, 2001; Belanger, 2003). However, many of these hybrids were artificially produced or not confirmed to be hybrids. The hybrids described by Davies, Jones and Bradshaw were usually morphologically intermediate between the two parents and had a complete loss or much reduced fertility (Bradshaw, 1958a; Jones, 1956a,b,c). Differences were also noted for cytological characteristics of interspecific crossing. Finally, there is incomplete agreement among researchers regarding the genetic origin of plants with increased ploidy level, which have been attributed to both intra- and interspecific hybridization and intraplant/intraclone mutations.

Early assumptions of putative interspecific hybridization (Davies, 1953; Jones 1956a,b,c; Bradshaw, 1958a) that were based on increased ploidy levels may be questionable in light of Kik et al. (1989, 1990b, 1992, 1993). They found that *A. stolonifera* clones collected from divergent ecosystems had differing cytological make-up including ploidy levels and related distributions of fertility. Kik et al. (1993) reported on within-plant somaclonal ploidy level variation and suggests this as a source of increased reproductive ploidy levels and associated sterility. This finding is an alternative interpretation for earlier assumptions of the link between interspecific crossing and elevated ploidy level.

II.E.6.2.2. Intergeneric hybridization

Like *Agrostis*, most *Polypogon* spp. have similar niche adaptation and limited distribution. Putative hybrids have been reported between *Agrostis* and some *Polypogon* species (Bjorkman,1960; Parodi, 1951; Welsh *et al*., 1987). Bjorkman (1954) states that *Polypogon* species are often placed in the genus *Agrostis* (ex. *P. semiverticillatus* (Forskal) Hyl., syn. *A. verticillata* Villars) as they are very similar in appearance and adaptation. Sokolavskaya (1938) believes that *P. litoralis* is synonymous with *A. verticillata*. Bjorkman (1960) mentions sterile putative hybrids between *A. stolonifera* and *P. fugax* or *P. semiverticillatus* (syn. *A. verticillata* Villars). He also notes (Bjorkman, 1954) the species *P. fugax* Nees. is *P. litoralis* Sm. (a name that was originally given to the hybrid *A. stolonifera* × *P. monspeliensis* L. Desf.). However, Sokolovskaya (1938) mentions that early researchers felt that *P. litoralis* is a cross between *P. monspeliensis* and *A. alba*. Bjorkman (1960) later states that hybrids resulting from *A. stolonifera* crosses with *P. monspeliensis* L. are sterile.

Hitchcock (1950) lists other *Polypogon* species that may be found in the U.S. including:

- *P. interruptus* HBK, Ditch Polypogon. This perennial tufted non-creeping species has a limited adaptation to wet areas and ditches and would be of limited threat to agriculture. The species has been reported in CA, OR, WA and east to LA, NE and OK.

- *P. australis* Brongn. is listed as a perennial introduced in WA.

- *P. maritimus* Willd. is listed as an introduced annual occurring in GA, NE, CA.
- 2n = 14, 28.
- *P. elongatus* HBK is a perennial found in wet places in AZ.

Confusion in distinguishing *Polypogon* and *Agrostis* is widespread and there is disagreement on the relationships (Sokolovskaya, 1938). On the rare occasions that hybrids have been noted, the hybrids have been sterile (Bjorkman, 1960).

Koehler (1997) further summarized the impact and potential for *A. stolonifera* to form inter-generic hybrids. The following is extracted from the Environmental Assessment performed to approve USDA permit number 97-087-02r for glufosinate-tolerant / putative disease resistant bentgrass:

"Intergeneric hybrids are known to naturally occur between A. stolonifera and Polypogon monspeliensis (L) Desf., but the hybrids are sterile (Björkman,1960). Sterile hybrids have also been obtained with P. fugax and P. semiverticillatus (= P. viridis) (Björkman,1960). Many of these Polypogon species have a high degree of self-fertility, which would not favor the formation of hybrids in nature. P. monspeliensis has been described as a common weed in the western U.S. (Hitchcock, 1935). P. interruptus H. B. K (Chase, 1950), described as existing in ditches and wet places at low altitudes including from British Columbia to California, is listed as a weed of unspecified status in the continental U.S. by Holm et al. (1979). Based on its similarity to P. fugax as reported by Björkman (1960), it is unlikely to form fertile hybrids with A. stolonifera."

The USDA concluded that inter-generic hybrids between *Agrostis* and *Polypogon* are likely to be infrequent, sterile and of no significant impact (Koehler, 1997).

II.E.6.2.3. F1 survivability and growth

Bradshaw (1958b) concluded that the sterility of the F_1 hybrid between *A. tenuis* Sibth. and *A. stolonifera* L. is not important to its persistence in the environment in which it arose because it is well-adapted vegetatively. Sterile or partly sterile vegetative F1 hybrid clones would spread and persist only in conditions of high disturbance and low environmental stress. Perennial success of a colony should decline during periods when these conditions are not met. This suggests that the long-term survivability of sterile clones is limited in nature. This notion is supported by the ploidy levels reported in Kik et al. (1993), where fertile tetraploids ranged from 23-100% of the population regardless of the ecological setting.

II.E.6.2.4. F1 fertility

Most researchers have referred to sterility as a defining characteristic of interspecific hybrids and have attributed the phenomenon to reduced genome homology between parents that leads to sterile gametes or aneuploidy among F2 progeny (Jones, 1956a,b,c). While fertile hybrid crosses have occasionally been reported, they remain rare in the literature.

II.F. Weediness of A. stolonifera

II.F.1. Weed development potential

Agrostis stolonifera may be considered an economic weed in a limited number of circumstances: (1) as an escape from and/or volunteer in seed production fields in regions of diverse grass species production for pedigreed seed and (2) when growing or spreading into areas planted to other turfgrass types. Although the species may be a colonizer of nonagricultural habitats, its presence in these areas results from disturbances related to human activities, i.e., its use in animal-based agriculture and residential or commercial lawns. *A. stolonifera* is not considered a major weed in other nonagricultural habitats or agricultural situations.

II.F.2. Characteristics of weedy and invasive species

Holt (1988) reviewed the characteristics of weed success as opposed to evolutionary success because agricultural practice does not mimic evolution in nature. She identifies three components of weed success: rapid colonization of disturbed sites, difficulty in removal and suppression of crop plants. The first two measures are functions of a plant's adaptive strategy while the third relates to its competitiveness.

In general, for weeds to succeed in a given ecological niche, they must exhibit advantageous characteristics that include survivability, growth and reproductive fitness. The ecological niche, in this case, is typically defined by an agricultural production system rather than nature. Holt adapts a stress-disturbance model to her discussion of strategic adaptation. She argues that weeds can succeed based on their adaptation to system disturbances, such as tillage-induced germination or to environmental stresses, which include summer dormancy of cool season grasses, but not to both. Disturbance-tolerators thrive on disturbance in the absence of stress and are typically annuals with high seed production and rapid life cycles. Environmental stress-tolerators are most typically perennials in undisturbed areas. Species that tolerate neither disturbance nor stress are competitors because of their success in populating the most desirable and competitive ecological niche. Booth and Swanton (2002) note that selection within an environment is for characteristics that are required for survival. The authors also concluded that herbicide tolerance does not confer (and may reduce) the ability to out-compete other species in the absence of the herbicide.

Many of the worst agricultural weeds (Holm et al., 1979) are disturbance-tolerators because annual cropping is prevalent over perennial plants in most settings (Janick et al., 1974). In annual cropping, system disturbance is high (tillage, cultivation, traffic) and environmental stress is minimized (water, nutrient, pH management), favoring strong germinators and fast maturing individuals (Zimdahl, 1999; Aldrich 1984). Many perennials in nature thrive on minimal system disturbance so they can build extensive water and nutrient collecting systems for survival under adverse environmental conditions. However, perennials that are stress-tolerators in nature can behave as disturbance-tolerators in agricultural systems if water and nutrient inputs are sufficient to offset the effects of system disturbances (Aldrich, 1984), e.g., johnsongrass in soybean and cotton culture in the south. This is especially true of vegetatively propagating colonizers that survive or benefit from mechanical

fragmentation, which mimics seed as a dispersal mechanism for increasing population numbers.

Perennial weeds tend to be more significant pests in perennial crops than annuals, except for herbaceous species with fecund vegetative propagules (Ross and Lembi, 1999). The most successful examples store large carbohydrate reserves that provide regenerative capacity under field conditions or permit long dormancy, enable repeated recovery from defoliation and are stimulated by mechanical fragmentation.

II.F.3. General status of *A. stolonifera* as a weed

As noted previously, *A. stolonifera* thrives in moist, nutrient-rich habitats and is found naturally in pastures and meadows, stream and ditch banks and coastal environments. While observed in less ideal habitats, the species does not thrive in these instances and is not particularly competitive with better-adapted species (Crick and Grime, 1987; Haggar, 1976; Marshall, 1990; Smith, 1972).

A. stolonifera is not listed as a serious, principal or common weed in the continental U.S. by Holm et al. (1979). In addition, Agrostis species are not listed in Weeds of the Northeast (Uva et al., 1997), although they are known to occur in the wild there. None of the Agrostis species appear on the USDA, APHIS, PPQ, Federal Noxious Weed list or on any state noxious weed list3 and the USDA has determined that A. stolonifera will not be listed as a Federal Noxious Weed4.

II.F.3.1. *A. stolonifera* as a weed in grass seed production

Agrostis stolonifera seed has been produced in the Willamette Valley of Oregon for more than 85 years (Schoth, 1930). In general, cross-pollination or mixing of grass seed with off-types in fields and post-harvest processing operations has been minimized by the seed certification standards of the Oregon Seed Certification Service. All volunteer grasses, including *A. stolonifera,* are considered weeds when they occur in production fields of other grass types and are undesirable due to the negative impact on crop value in light of certification standards.

Assuming some limited establishment in grass seed production fields, *A. stolonifera* exhibits a number of inherent characteristics that minimize its potential to intermix with harvested seed of other grass types:

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[3] http://plants.usda.gov/java/noxComposite [Accessed October 22, 2015]

[4] https://www.aphis.usda.gov/plant_health/plant_pest_info/weeds/downloads/wra/agrostis_stolonifera.pdf [Accessed October 22, 2015]

A. stolonifera develops at a slower rate than other grasses and requires an earlier fall planting date compared to most other turfgrass species. *A. stolonifera* that germinates with another commercial species is less likely to mature physically for winter survival, to exhibit floral induction and initiation the following spring or to successfully produce seed[5].

A. stolonifera is the last grass to mature in the Willamette Valley of Oregon and therefore the last harvested, usually in late July or August. *Lolium perrene*, *Lolium multiflorum, Festuca arundinacea, Festuca longifolia and Festuca rubra spp.* are each harvested 3 to 5 weeks earlier, when most *A. stolonifera* seed is still immature. Thus, *A. stolonifera* seed is unlikely to be viable even when it is present as an impurity.

A. stolonifera is a shallow rooted species that requires more consistent soil moisture than other species in order to persist and produce seed. Summers are very dry in the Willamette Valley of Oregon and supplemental irrigation is required for the production of viable *A. stolonifera* seed. Other grasses typically are produced without irrigation, so *A. stolonifera* do not thrive in their midst (Meyer and Funk, 1989).

A. stolonifera seed is approximately 1/20 the size of *Lolium multiflorum, L. perenne, Festuca arundinaceae, and F. rubra*. Most *A. stolonifera* seed that might intermix with seed of these other species is removed routinely in combines and various seed cleaners in downstream conditioning operations. It is not listed as a common impurity of grass seed by the OSCS (Oregon State University, 2001a).

These facts are confirmed by reports listing the top 10 most frequently found impurities of grass seeds identified during routine seed purity tests. *A. stolonifera* is consistently absent from these reports indicating that it is not persisting in fields of other grass species. The tenth most frequently found impurity is found in about 1 in 100 seed lots. (Oregon State University, 2001a.).

II.F.3.2. *A. stolonifera* presence in OR

Agrostis stolonifera is present as a naturalized component of the North American flora. Most references, however, point to human disturbances and animal-based agriculture relationship to its occurrence (USDA-NRCS 1998; Mueller-Warrant et al. 2002). It is important to note that Hitchcock (1950) suggests *A. stolonifera* may be native to North America. Questions as to the origin of the naturalized *A. stolonifera* reported in USDA-NRCS (1998) and Mueller-Warrant (2002) are not easily answered. The presence of this species pre-dates the practice of seedsmanship in Oregon (see the cultivar description of 'Seaside' creeping bentgrass). There is an assumption by many that this species is entering natural areas; however, it would be more accurate to say that *A. stolonifera's* presence in an ecosystem is the result of human disturbance and use of the habitat for animal-based agricultural purposes (Frenot et al., 2001). In many cases, *A. stolonifera* has provided

[5] *Agrostis stolonifera* has a longer juvenile stage (greater time required to achieve an appropriate size for reproductive induction) and therefore requires an earlier fall planting date than most other turfgrass species for optimum economic seed production.

ecological benefits, i.e., the reduction of soil erosion due to the impacts of animals utilized in agriculture and the animals altering the ecosystem, including soil nutrient content.

II.F.3.3. *A. stolonifera* as a weed in managed turfgrass

Agrostis stolonifera has been grown in the U.S. on golf putting greens for over 110 years and for over 60 years for fairways and has never been considered an unusually serious weed of other turfgrasses. When it is considered a weed of other turfgrass species, it is due to the combination of its creeping habit and unique texture that combine to create non-uniform patches that deteriorate in quality and aesthetics when mowed above one-half inch (Watschke, 1995). The most common scenarios are lateral growth of *A. stolonifera* off of golf putting greens into surrounding turfgrass and the presence in other turfgrass seed that leads to patches in residential and commercial lawns. Nonetheless, *A. stolonifera* rarely earns the attention of professional turfgrass managers to the extent of crabgrass or dandelion because it is a relatively uncommon and localized concern.

II.F.3.4. *A. stolonifera* occurrence in general agriculture

None of the cultivated or native *Agrostis* species are listed as noxious or serious weeds in U.S. agriculture, except in turfgrass swards and seed production fields of other grass species (Holm et al., 1979). Tillage operations, herbicide programs and economical water management in most systems prohibit establishment and spread of the species. No mention of *A. stolonifera* is made in three prominent weed science texts (Zimdahl, 1999; Ross and Lembi, 1999; Aldrich, 1984).

Agrostis stolonifera does occur in pastures of moist and humid environments in the temperate zones. However, it has been naturally occurring in many pastures for centuries and is not necessarily considered a weed there (Bradshaw, 1958a). It is, in fact, one of the few cool season grass species that can tolerate frequent and continuous grazing because of its low growing and stoloniferous growth habit. Frame (1990) and Haggar (1976) both note that *A. stolonifera* is often less productive than other forages and may be less desirable for this reason, but it is seldom referred to as a problem weed.

II.F.3.5. *A. stolonifera* in natural systems

Agrostis stolonifera has been introduced to and naturalized in many locales around the world. The species has not developed into an ecological problem in the vast majority of them. In one very unusual occurrence, Gremmen et al. (1998) reported on the remarkable colonization and competitive success of *A. stolonifera* introduced in the 1950s to sub-Antarctic Marion Island. In this isolated occurrence, the species established in mesic to wet habitats on Marion Island and became predominant in these niches. Marion Island's sub-Antarctic location and uniform climate do not provide for an early spring flush of competitors. Rather, the ability to compete on equal terms throughout the year eliminates an early season advantage that many typical competitors enjoy. In the absence of grazing, human interventions and pests of *A. stolonifera*, the colonization of areas of Marion Island appears to be a significant anomaly for this species.

II.F.4. Weed performance and economic aspects

Generally, *A. stolonifera* has exhibited better success at establishing and colonizing newly disturbed sites than at expansion in stable environments (Sweeney and Danneberger, 1998; Jonsdottir, 1991). Seedling establishment is only marginally successful in highly competitive environments. Lush (1988a) found no *A. stolonifera* seedlings in an *A. stolonifera/P. annua* turf even though *A. stolonifera* seed was present in the seed bank (Lush 1988b). Vegetative colonization is most effective when moisture and disturbance conditions are optimal and competition is reduced.

Sweeney and Danneberger (1998) observed very low *A. stolonifera* establishment rates based on DNA analysis of 28 plants after four consecutive years of inter-seeding with core aeration into an *A. stolonifera* putting green. Howe and Snaydon (1986) reported less than 5% of the *A. stolonifera* seed sown directly into perennial rye grass pasture survived, regardless of nutrition level or season of establishment. This is in comparison to the much larger-seeded *Festuca rubra*, which exhibited seedling survival of up to 38.5%.

The difficulty in seedling establishment may be a consequence of the extremely small seed size (0.07 mg $seed^{-1}$) of *A. stolonifera*, which restricts establishment to a relatively short period after germination. Efficient transition from a heterotrophic to an autotrophic state is critical (Whalley et al., 1966) and may be prolonged in competitive settings. Bernston and Wayne (2000) reported that while below ground competition is linearly related to the size of the root system, above ground competition was asymmetric with respect to light capture, with plant height and leaf area being important positive factors. Plant height, in particular, is an obvious advantage of the existing vegetation over newly emerging vegetation (Bernston and Wayne, 2000). Jonsdottir (1991) and Bullock et al. (1994) both worked with canopies above 5 cm when unsuccessfully attempting to recruit seedlings in natural stands and pastures. Successful interseeding typically requires aggressive scalping of existing turf (Cattani and Struik, 2001; Kendrick and Danneberger, 1998; Sweeney and Danneberger, 1998).

Established seedlings will expand laterally through stolons, which root and form daughter plants at the nodes where moisture conditions are favorable for rooting. Attached daughter plants are less sensitive than seedlings to competition for light, nutrients and water during establishment because they derive resources from the mother plant (Ross and Lembi, 1999). However, observations from pastures and golf course putting greens describe colonies of limited size and suggest that vegetative colonization is also sensitive to competition and tends to be successful in localized areas of ideal conditions (Lush, 1988a). *Agrostis stolonifera* is late to green up and mature among forage grasses and may suffer an early season disadvantage in competing for resources and expanding geographically (Haggar, 1976).

Once established, *A. stolonifera* resists competition effectively and colonies in mixed swards can persist. Effective competitors tend to be aggressive disturbance-tolerators adapted to the same environment such as annual bluegrass or more aggressive creeping species such as bermudagrass (Aldrich, 1984; Ross and Lembi, 1999).

II.F.4.1. Economic weediness of *A. stolonifera* in grass seed production

The potential for *A. stolonifera* to become a serious weed of other grass species grown for seed is limited not only by certification-driven field management practices but also by differences in crop production conditions and practices.

Agrostis stolonifera plants are most likely to be introduced into other grass seed production fields either through lapses in field sanitation procedures, e.g., viable stolons transported on tillage equipment, or as an impurity of stock seed. Its creeping habit does encourage lateral growth, but border plants are removed as part of routine field sanitation. Successful establishment of *A. stolonifera* by either seed or stolon in seed fields of other grasses is discouraged because it requires high soil moisture or humidity to survive, and most other grass seeds in the region are grown under arid summer conditions without irrigation. Dryland production of these other grasses is facilitated by their larger seed size, which provides a larger carbohydrate reserve for initial establishment, deeper root system to tap deep soil moisture, and earlier maturity for harvest before soil moisture becomes limiting. Introduced *A. stolonifera* stolons are unlikely to root under dryland conditions and its very small seed size provides little energy for establishment under drought stress. Similarly, an occasional bentgrass seed germinating among a high population of large seeded grasses would be less fit to successfully compete for resources.

A stolonifera establishment from seed in other grass seed production fields would be further discouraged because of the widespread use of soil active herbicides used to control germinating seedlings (Oregon State University, 2001b). Turfgrass seed production fields are routinely treated with soil active, long residual herbicides such as pronamide, diuron, ethofumesate and others to control a range of grassy and broadleaf seedlings.

As noted in previous sections, *A. stolonifera* that manages to colonize production fields of other grass species is unlikely to produce viable seed due to differences in maturity timings, existing herbicide programs and harvest schedules. *Agrostis stolonifera* matures later than other grasses grown in the region and so will often be immature if harvested with other grasses. Dryland seed production systems should favor seed production over vegetative propagation of *A. stolonifera* (Kik et al., 1990a; Kik et al., 1992), so unsuccessful seed production should constitute the bulk of *A. stolonifera* activity and discourage aggressive vegetative expansion in the field.

Seed production fields are routinely scouted for genetic off-types, broadleaf and grassy weeds in keeping with certification standards. Hand or chemical roguing eliminates unwanted plants. This level of management intensity makes an uncontrollable or economically devastating infestation of *A. stolonifera* highly unlikely when certification practices are followed. *Agrostis stolonifera* possesses no unique competitive characteristics in the presence of other seed grasses that would constitute an overwhelming advantage and result in serious crop loss. In a worst case scenario, small areas of crop might be lost to localized herbicide application or tillage operations to eliminate problem colonies, but large scale infestations are very unusual. Control of *Agrostis sp.* in a turfgrass seed production field can be achieved with a variety of chemicals geared for seedling control. Crop rotation

is also part of an integrated weed control program and contributes to control of volunteer *Agrostis* plants (Glenn Jacklin, 2002. personal communication).

II.F.4.2. Economic weediness of *A. stolonifera* in managed turfgrass

The most likely mechanisms of *A. stolonifera* establishment in other turfgrasses are lateral encroachment from golf putting green or fairway cultures, escape from a previous golf course renovation or as a seed impurity in new plantings (Beard, 2002; Dernoeden, 2002; Oregon State University, 2001a). Inputs to the turfgrass, such as water and nutrients, can favor the establishment and initial expansion of *A. stolonifera* in both golf course and seed production scenarios even in the face of competition from desirable grasses. *Agrostis stolonifera* is grown in a concentrated geographic area relative to total U.S. grass seed production (Oregon Department of Agriculture, 2001). The small seed size of *A. stolonifera* facilitates exclusion from larger seeds in processing so the presence of *A. stolonifera* seed in other turfgrass seed is unusual. Oregon Seed Certification Service (Oregon State University, 2001a) does not identify *A. stolonifera* as among the top 10 foreign impurities of other turfgrass seed.

Agrostis stolonifera seed is rarely an impurity in established stands of other turfgrasses. Numerous researchers have reported on the difficulty of successful seedling establishment in mature turfgrass stands, presumably due to competitive barriers that were explained earlier in this Section. Canopy height and resource competition appear to be critical issues. Lush (1988a) was unsuccessful in finding seedlings of *A. stolonifera* in a golf course putting green study managed with a cutting height of 6 mm (0.24 inches), even though *Poa annua* seedlings established readily. Little success was also reported from interseeding *A. stolonifera* into an existing *A. stolonifera* stand utilized for golf course putting green turf (Sweeney and Danneberger, 1998). The presence of *A. stolonifera* seed occurs almost exclusively through overseeding since natural *A. stolonifera* seedbank populations are low (Lush, 1988b) and cannot occur under typical mowing regimes because reproductive tillers are routinely removed (Lush, 1988a). Therefore, natural seed dispersal from *A. stolonifera* stands to adjacent turfgrasses is essentially of no practical concern.

Vegetative invasion from golf course putting green or fairway *A. stolonifera* stands is generally limited to short distances from well-defined edges because of competition from existing stands (Beard, 2002). The impact is almost purely aesthetic. Establishment typically occurs when growth conditions favor *A. stolonifera* over the adjacent species. In southern climates where *A. stolonifera* may be grown on golf course putting greens next to warm season grass fairways, encroachment is more typically from bermudagrass or zoysiagrass fairways into *A. stolonifera* greens (Dernoeden, 2002). Established colonies of *A. stolonifera* can persist but do not necessarily spread over large areas due to competition from other grass species.

II.F.4.3. *A. stolonifera* weediness in general agriculture

Agrostis stolonifera infestations in annual or perennial food or fiber crops are not routinely reported, presumably because standard integrated weed control programs are effective at disrupting the plant life cycle and/or eliminating it. System disturbances associated with

cropping systems such as mechanical tillage might seem to favor the establishment of *A. stolonifera.* Further, the supplemental water needed by *A. stolonifera* is not provided in dry land management, which removes the ability of bentgrass to establish and persist. In perennial systems such as orchards, *A. stolonifera* is not differentiated from other sod-forming species because it poses no unique risk to orchard crops.

Agrostis stolonifera has been demonstrated to establish in disturbed and abandoned agricultural fields and in pastures. However, *A. stolonifera* is not desired in pastures due to its low dry matter production, poor nitrogen use efficiency (Sheldrick et al., 1990) and low digestibility (Frame 1990). However, on continuously and intensively grazed areas of low stress, bentgrass is competitive (Bradshaw, 1958a). The presence of *A. stolonifera* observed by Schulte (2001) resulted from a feeding preference by grazers for other grass species, which enabled *A. stolonifera* to persist. However, Jonsdottir (1991) and Bullock et al. (1994) noted that seedling recruitment into natural stands and pastures, respectively, was unsuccessful in augmenting *A. stolonifera* populations. In general, *A. stolonifera* is recognized as a colonizer of pastures but not with serious economic consequences.

II.F.4.4. *A. stolonifera* invasiveness in natural systems

Much of the biological information that is pertinent to invasiveness in natural systems was presented earlier in this Section. That information is used here to characterize invasiveness in natural systems directly.

It is difficult to predict where commercial seed might be introduced, but the literature clearly indicates that introduced *A. stolonifera* colonizes and naturalizes in habitats similar to its origin, i.e., moist and/or humid meadows and early succession forests, but not widely beyond these habitats (Collett *et al*., 1996). Further, since *Agrostis* species are considered to have low seed bank populations (Frame, 1990), the most likely means of introduction in a natural system outside of the geographic origin of *A. stolonifera* is through commercial seed (Gremmen *et al*., 1998). Stolon introduction could occur as a stowaway on tillage or excavating equipment but is highly unlikely in an isolated ecosystem. There are no reports of ecosystem disturbance by *A. stolonifera* outside of these conditions.

As discussed previously, Hunt et al. (1987) reported that *A. stolonifera* prefers environments with a high degree of disturbance and very limited environmental stress. Such environments are rarely unpopulated or noncompetitive in nature. In fact, these are exactly the conditions preferred by highly aggressive disturbance-tolerating species. This suggests that *A. stolonifera* would be unable to monopolize sites in the presence of significant competition from other plant species. Slow recovery from winter dormancy in the spring (Collet et al., 1996) is a competitive disadvantage, which is likely responsible for limiting *A. stolonifera* colonization in most natural settings. The unusual success of *A. stolonifera* on Marion Island (Gremmen et al., 1998) supports this conclusion. As previously noted, Marion Island's sub-Antarctic location and uniform climate do not provide for an early spring flush of competitors. Rather, the ability to compete on equal terms throughout the year eliminates the early season advantage that many typical competitors enjoy. In the absence of grazing, human interventions and pests of *A. stolonifera*, the colonization of areas of Marion Island appears to be a significant anomaly for this species.

II.F.4.5. Weed implications of *A. stolonifera* outcrossing

Throughout the more than 85 years of *A. stolonifera* seed production in the Willamette Valley of Oregon (Schoth, 1930), there has been no demonstration that random recombinations within *A. stolonifera* or sexually compatible species have either increased aggressiveness or been detrimental in that environment. Outcrossing is nearly impossible in managed turfgrass settings because normal mowing practices remove reproductive tillers and prevent flowering and pollination (Lush, 1988a). The flowering period for *A. stolonifera* coincides with peak golf play periods of the year that necessitates very frequent clippings, which essentially eliminates any reproductive growth as it appears. *Agrostis stolonifera* in the wild or in general agricultural settings could potentially mature and cross with a compatible individual. However, there is no reason to expect any more aggressive behavior than that observed in the more than 85 years of continuous commercial seed production in the Willamette Valley.

Various works (Jones, 1956b,c; Bradshaw 1958a; Wipff and Fricker, 2001; Belanger, 2003) have demonstrated that interspecific hybridization with *A. stolonifera* is possible. However, the potential for increased risk of weediness has not been demonstrated nor is it a conclusion of most of these studies. Hybrids have also been occasionally observed to be more vegetatively adapted than their parents in habitats intermediate of those preferred by their respective parents (Bradshaw, 1958b). However, the persistence of any hybrid offspring through sexual reproduction is unlikely due to the overwhelming sterility of F1 hybrids (Jones, 1956b) and even in the rare case of a fertile F1 hybrid, the fitness of the F2 hybrid is highly questionable (Bradshaw, 1958b).

Higher ploidy cytotypes as would be characteristic of infertile hybrids were not observed to be present in hostile environments while lower ploidy cytotypes were fertile and present in all environments (Kik et al., 1993). The implication is that there is a survival advantage to sexual reproduction regardless of environment. Since strategic adaptability is a foundation of weed success (Holt, 1988), sterile hybrids are highly unlikely to persist in nature or establish themselves as new weed pests.

II.G. Summary

Agrostis stolonifera is both a native and naturalized species in the U.S., has been grown on golf courses for over 110 years, and has been grown continuously for commercial seed production in the Willamette Valley of Oregon for more than 85 years in the proximity of several other *Agrostis* species. While it is an undesirable species in some situations, *A. stolonifera* is not considered a serious economic pest in any setting except in pedigreed seed fields of other grass species in the U.S. Pacific Northwest. Even in the latter case, it has not been reported as one of the 10 most common weed or crop seed impurities of turfgrass seed tested by the Oregon State University Seed Lab (Oregon State University, 2001a). *A. stolonifera* is not unique in this aspect because many grass species are considered weeds when they occur in turfgrass seed production fields. Even genetic off-types of the turfgrass seed species being commercially produced are considered serious and must be removed to qualify for Certified production. Seed producers employ integrated field management

programs to maintain the purity of their product and weed management is a critical part of those efforts.

Agrostis stolonifera is not considered a serious weed of agriculture or in the environment. While it is a perennial species, it is not a relentless colonizer because of its need for both low stress and high disturbance environments. Consequently, *A. stolonifera* typically establishes in only modest areas within mixed plant communities. Therefore, the impact of *A. stolonifera* presence within other turfgrass species is primarily an aesthetic rather than a functional or economic issue. Finally, although *A. stolonifera* can cross with related species to form hybrids, they are largely sterile, typically intermediate to the parent species and pose essentially no risk to agriculture or the environment.

III. DESCRIPTION OF THE TRANSFORMATION SYSTEM

III.A. Characteristics of the recipient plant material

The creeping bentgrass plant tissue that received DNA conferring tolerance to glyphosate was embryogenic plant callus derived from a single seed of the creeping bentgrass cultivar Backspin. This plant and tissue was chosen for insertion of the *cp4 epsps* gene because it responds well to particle bombardment transformation and tissue culture regeneration. B99061R was developed from randomly selected, non-transgenic embryogenic callus regenerate in tissue culture and further maitained by vegetative propagation.

III.B. Description of the transformation system

Biolistic transformation (microprojectile or particle bombardment) was used to produce event ASR368. This DNA delivery system is well documented to transfer and integrate new DNA into a plant genome (Klein et al., 1987; Lee; 1996; Sanford et al., 1993). Prior to bombardment, DNA containing the *cp4 epsps* gene was precipitated onto microscopic gold particles using calcium chloride and spermidine. The precipitated DNA and particles were then placed onto a plastic macrocarrier and accelerated at high velocity such that a stopping screen retained the macrocarrier. The particles with DNA were permitted to continue their flight and eventual penetration and incorporation into the creeping bentgrass plant cells. These cells were transferred to a selective media containing glyphosate and only those cells transformed with the *cp4 epsps* gene continued to grow. Event ASR368 was selected among these transformed plant cells. The flowchart in Figure III.1 illustrates the development of event ASR368.

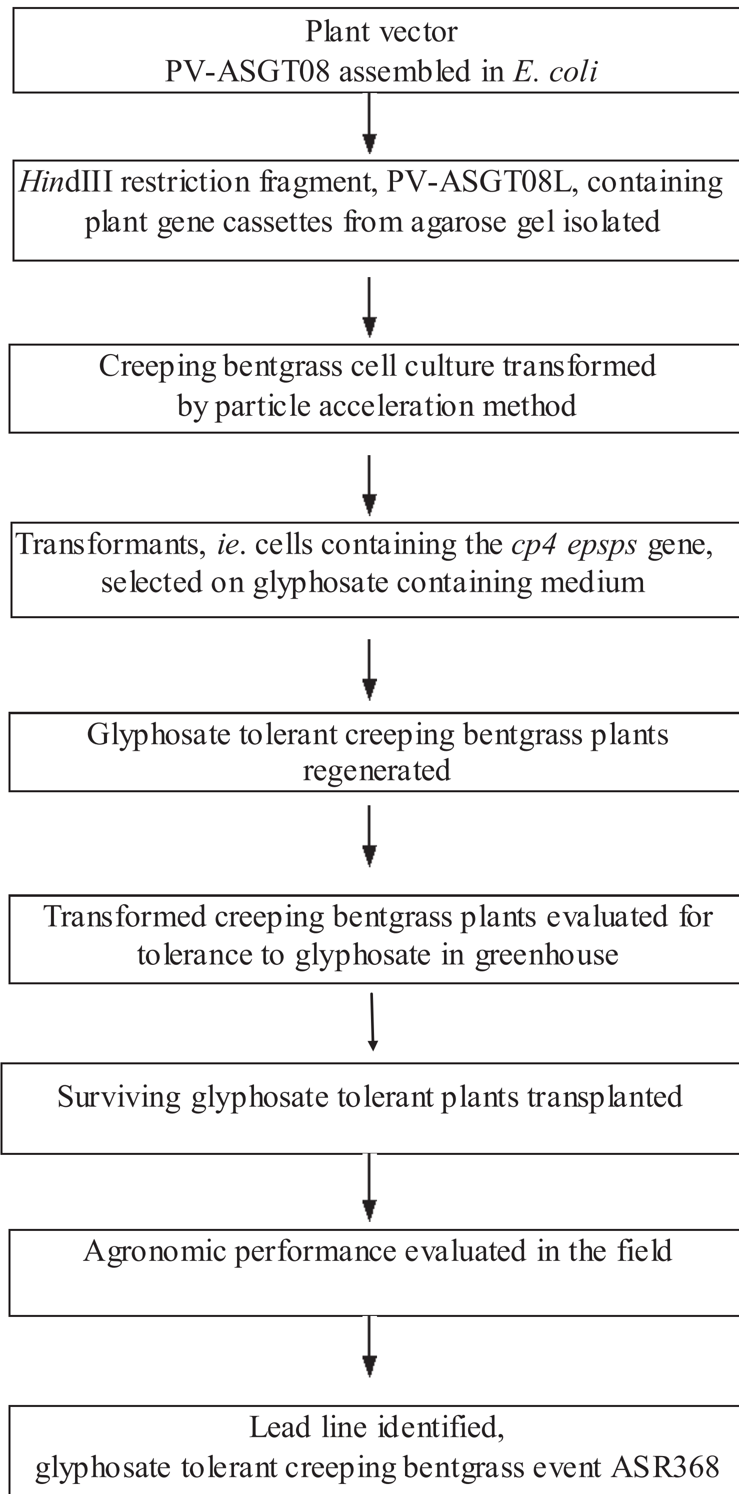


Figure III-1. Schematic of the Development of ASR368

IV. DONOR GENES AND REGULATORY SEQUENCES

IV.A. Vector PV-ASGT08L

Event ASR368 was produced by transformation of non-transgenic bentgrass tissue with a ~ 6.7 kb linear *Hin*d III DNA derived from the plasmid vector PV-ASGT08 (Figure V-1) developed by Monsanto Company (St. Louis, MO). This linear segment, PV-ASGT08L (Figure V-2), contained two *cp4 epsps* gene expression cassettes. The first *cp4 epsps* gene expression cassette contained the *cp4 epsps* coding sequence under the regulation of the rice actin promoter, a rice actin intron, a chloroplast transit peptide (CTP2) sequence and a nopaline synthase (NOS) 3' polyadenylation sequence. The second *cp4 epsps* gene expression cassette contained the *cp4 epsps* coding sequence under the regulation of the cauliflower mosaic virus (CaMV) enhanced 35S plant promoter (e35S), a maize heat-shock protein 70 (*Zm*HSP70) intron, CTP2 and the NOS 3' polyadenylation sequence. The *ctp2 cp4 epsps* coding region used to produce event ASR368 is the same as that employed in several other Roundup Ready crops such as soybean, which have been previously reviewed and granted nonregulated status by the USDA. A description of the elements in the linear DNA segment PV-ASGT08L is provided in Table V-1.

IV.B. The *cp4 epsps* gene and CP4 EPSPS protein

Event ASR368 plants contain two copies of the *cp4 epsps* gene that encode the CP4 EPSPS protein, which imparts tolerance to glyphosate. The *cp4 epsps* gene was isolated originally from *Agrobacterium* sp. strain CP4 and produces an enzyme, 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) (Padgette et al., 1995). This enzyme, unlike most native plant and microbial EPSPS enzymes, is naturally tolerant to glyphosate (Padgette et al., 1995). EPSPS catalyzes the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) in both microorganisms and plants. EPSP is an intermediate required for the production of aromatic amino acids (Herrmann, 1983; Haslam, 1974).

The native *Agrobacterium* gene sequence was modified to create a synthetic gene that allows greater production of the CP4 EPSPS protein in plants (Padgette et al., 1995). Bacterial genes, like those from *Agrobacterium,* have several features that reduce their ability to function efficiently in plants. Therefore, plant-preferred versions of these genes were synthesized and used in developing the plasmid vectors (Della-Cioppa et al., 1986 and 1987; Shah et al., 1986).

The *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The *ctp*2 *cp4 epsps* gene sequence, present in event ASR368, is approximately 1.7 kb in size. The deduced amino acid sequence of the CP4 EPSPS protein with the CTP2 transit peptide is shown in Figure V.3. The target for glyphosate in plants, the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme, is located in the chloroplast. Many chloroplast-localized proteins, including EPSPS, are expressed from nuclear genes as precursors and are targeted to the chloroplast by a chloroplast transit peptide (CTP) that is removed during the import process. It has been

demonstrated *in vivo* (Timko et al., 1988) and *in vitro* (Della-Cioppa et al., 1986 and 1987) that non-chloroplast proteins may be targeted to the chloroplast by use of protein hybrids containing a CTP and that a CTP amino acid sequence is sufficient to target a protein to the chloroplast. The *ctp* coding sequence from the *Arabidopsis thaliana epsps* coding region (Klee et al., 1987) was joined to the *cp4 epsps* coding sequence to achieve chloroplast localization of CP4 EPSPS protein. The CP4 EPSPS protein, as a bacterial protein, contains no CTP. The *Arabidopsis ctp* DNA sequence was modified by site-directed mutagenesis to place a *Sph* I restriction site at the CTP processing site to accomplish this joining. This change replaced the Glu-Lys at this location with Cys-Met. The DNA sequence of this CTP peptide is designated as *ctp2*. The CTP2 CP4 EPSPS hybrid protein was demonstrated to allow import into chloroplasts isolated from *Lactuca sativa*, using methods described previously (Della-Cioppa et al., 1986 and 1987).

IV.C. The chloroplast transit peptide (CTP2)

In both plant gene expression cassettes, the *cp4 epsps* coding sequence is fused to a chloroplast transit peptide (designated CTP2) whose sequence is based on the CTP isolated from *Arabidopsis thaliana* EPSPS (Klee et al., 1987). This CTP directs the CP4 EPSPS protein to the chloroplast, the location of EPSPS in plants and the site of aromatic amino acid biosynthesis (Kishore and Shah, 1988). CTPs are typically cleaved from the "mature" protein following delivery to the plastid (Della-Cioppa et al., 1986).

IV.D. Regulatory sequences

In the first gene cassette, the *cp4 epsps* coding sequence is under the control of the 5' non-coding end of the rice actin 1 sequence (ract1) containing the promoter and first intron (McElroy et al., 1990), approximately 1.4 kb in size, introduced upstream of the CTP sequence. The second cassette contains the *cp4 epsps* coding sequence under the control of the enhanced CaMV 35S promoter (e35S) (Kay et al., 1987; Odell et al., 1985), which is approximately 0.6 kb in size. Located between the e35S promoter and the *cp4 epsps* sequence is the 0.8-kb intron from the corn *hsp*70 (heat shock protein), present to increase the levels of gene transcription (Rochester et al., 1986). In each cassette, the *cp4 epsps* sequence is joined to the 0.3-kb nopaline synthase 3' nontranslated sequence, NOS 3', from *Agrobacterium tumefaciens* (Fraley et al., 1983), which provides the transcription termination and the mRNA polyadenylation signal.

An origin of replication sequence (*ori*) is also present in PV-ASGT08 to allow for the replication of the plasmid in *E. coli* (Viera and Messing, 1987). Following the *ori* region is the DNA sequence encoding the enzyme *neomycin phosphotransferase type II* (*nptII*). This enzyme confers resistance to aminoglycoside antibiotics (e.g., kanamycin and neomycin) and was used for selection of bacteria during the construction of the plasmid. The coding sequence for the *nptII* gene was derived from the prokaryotic transposon Tn5 (Beck et al., 1982) and is present with its own bacterial promoter. The fragment, used for the transformation of event ASR368, contains neither the *ori* or the *nptII* sequences.

The plasmid PV-ASGT08 was amplified in *E. coli* and purified from bacterial lysates. The *cp4 epsps* gene expression linear DNA fragment was isolated from the plasmid prior

to creeping bentgrass transformation experiments by digesting PV-ASGT08 with the restriction enzyme *Hind* III. The plasmid backbone (~2.6 kb) and the *cp4 epsps* expression cassettes (~6.7 kb) were separated by gel electrophoresis and the *cp4 epsps* expression cassette fragment was electro-eluted from a gel slice. The agarose gel-isolated *Hind* III restriction fragment utilized in the transformation of glyphosate-tolerant creeping bentgrass event ASR368 was designated PV-ASGT08L. This fragment contains neither the *ori* or *nptii* sequences.

V. CHARACTERIZATION OF THE GENETIC MODIFICATION

V.A. Molecular characterization of glyphosate tolerant creeping bentgrass event ASR368

Southern blot, PCR and nucleotide sequence analyses were performed to characterize the DNA integrated into the genome of event ASR368. These analyses support the following conclusions: (1) the genome of event ASR368 contains a single DNA insertion comprised of a single copy of the DNA segment used for transformation; (2) both *cp4 epsps* gene expression cassettes within the single insert are intact; (3) transcription of the first *cp4 epsps* gene expression cassette contains the *cp4 epsps* coding sequence under the regulation of the rice actin promoter, a rice actin intron, a chloroplast transit peptide (CTP2) sequence from *A. thaliana* and a nopaline synthase (NOS) 3' polyadenylation sequence from *A. tumefaciens* and the second *cp4 epsps* gene expression cassette contains the *cp4 epsps* coding sequence under the regulation of the cauliflower mosaic virus (CaMV) enhanced 35S plant promoter (e35S), a maize heat-shock protein 70 (*Zm*HSP70) intron, CTP2 and the NOS 3' polyadenylation sequence; and (4) the genome of event ASR368 does not contain any detectable plasmid backbone DNA.

The 5' and 3' ends of the event ASR368 insert and the *cp4 epsps* coding regions were verified by PCR and DNA sequencing. These data support the conclusion that only the full-length CTP2-CP4 EPSPS protein should be encoded by the insert in event ASR368. The genetic stability of the transgene was also demonstrated by Southern blot analysis on genomic DNA from the R0, F1 and F2 generations of event ASR368.

V.A.1. Materials and methods

V.A.1.1. Test substance

The test substance for the study was glyphosate tolerant creeping bentgrass event ASR368. In addition, leaf material from event ASR368 F1 and F2 generations was used to analyze the stability of the inserted DNA across generations.

V.A.1.2. Control substances

The control substance for the molecular characterization of glyphosate tolerant creeping bentgrass events ASR368 was the non-transformed bentgrass line 99061R/990028 (B99061R). In addition, leaf material from the bentgrass line V13-2-2, the maternal parental line of the F1 and F2 generations, was used as a control in analyzing the stability of the inserted DNA across generations.

V.A.1.3. Reference substances

Plasmid PV-ASGT08, the source plasmid, served as the primary reference substance in these analyses. The plasmid, mixed with DNA from the 99061R/990028 non-transformed control substance, was used as a size indicator and a positive hybridization control in

Southern blot analysis. Additionally, molecular size markers from Gibco BRL [1 Kb DNA extension ladder (40 Kb-0.4 Kb) catalog #10511] were used for size estimations.

V.A.1.4. Southern blot strategy

Genomic DNA from event ASR368 was digested with restriction enzymes and subjected to Southern blot hybridization analysis to characterize the DNA that was integrated into the bentgrass genome. A map showing the linear DNA segment, PV-ASGT08L, that was used to generate the event ASR-368, along with the locations of the restriction sites utilized for Southern analysis, is shown in Figure V-2.

DNA from the test substance was digested with *Hin*d III or *Nde* I/*Sma* I on each Southern blot to characterize event ASR368. *Hin*d III does not cleave within the PV-ASGT08L segment and, therefore, gives an indication of the number of insertions in event ASR368. *Nde* I and *Sma* I cleave at the 5' and 3' ends of the PV-ASGT08L segment, respectively. This enzyme combination gives an indication of the number of copies of the PV-ASGT08L segment in event ASR368. The conventional and plasmid DNAs were also digested with *Nde* I/*Sma* I for all blots. Only the P-ract and P-ract/intron blots contained an additional *Hin*d III conventional control digest in the long and short runs, as specified in the protocol. These Southern blots were probed with all of the elements of PV-ASGT08 and with two overlapping probes that collectively span the entire PV-ASGT08L sequence.

The genetic stability of the event ASR368 insert over multiple generations was assessed by digesting genomic DNA from the test substances (R0, F1 and F2) with *Sph* I. This enzyme generates a unique fingerprint for event ASR368 when probed with the *ctp2 cp4 epsps* coding region.

V.A.1.5. DNA isolation

DNA extracted from leaf tissue was used for all of the analyses in this report. Leaf tissue was frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. Approximately 2 g of the processed leaf was transferred to a 50 mL conical tube and ~16 mL of CTAB extraction buffer [1.5% (w/v) CTAB, 75 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 1.05 M NaCl and 0.75% (w/v) PVP (MW 40,000)] were added to the processed leaf. The samples were incubated at 65°C for approximately 30 minutes with intermittent mixing and then allowed to cool to room temperature. An equal volume (~15 mL) of room temperature chloroform:isoamyl alcohol [24:1 (v/v)] was added to the samples. The suspension was mixed for 5 minutes and the two phases separated by centrifugation at ~16,000 × *g* for 5 minutes at room temperature. The aqueous (top) layer was removed and placed into a clean 50 mL conical tube. Approximately 1/10 volume (~1.5 mL) of 10% CTAB buffer [10% (w/v) CTAB and 0.7 M NaCl] and an equal volume of chloroform:isoamyl alcohol [24:1 (v/v)] were added to the aqueous phase, which was then mixed by inversion for 5 minutes. The samples were centrifuged at ~16,000 × *g* for 5 minutes at room temperature to separate the phases. The aqueous (upper) phase was removed, mixed with an equal volume (~15 mL) of CTAB precipitation buffer [1% (w/v) CTAB, 50 mM Tris pH 8.0 and 10 mM EDTA pH 8.0] and allowed to stand at room

temperature for approximately 1 hour. The samples were centrifuged at ~10,000 × *g* to pellet the DNA, the supernatant was decanted and the pellet was dissolved in approximately 2 mL of high salt TE [10mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 1 M NaCl] by incubating at 37°C with gentle swirling for approximately 2 hours or by sitting in a 4°C refrigerator overnight. Centrifugation was performed at ~23,000 × *g* to pellet any remaining impurities. The supernatant was removed, placed into a clean 15 mL tube and approximately 1/10 volume (~150 µl) of 3M NaOAc, pH 5.2 and 2 volumes (~4 mL relative to the supernatant) of 100% ethanol were added to precipitate the DNA. The precipitated DNA was spooled into a microfuge tube containing 70% ethanol. The DNA was pelleted in a microfuge at maximum speed (14,000 rpm) for ~ 5 minutes, dried and re-dissolved in TE, pH 8.0 in a 4°C refrigerator overnight.

V.A.1.6. DNA quantitation

The purified genomic DNA was quantitated using a Hoefer DyNA Quant™ 200 Fluorometer (San Francisco, CA) with Roche Molecular Weight Marker IX (catalog #1449 460) used as a calibration standard.

V.A.1.7. Restriction enzyme digestion

Approximately 10 µg of genomic DNA from the test event and control lines were used for the restriction enzyme digests. Overnight digests were performed at 37°C in a total volume of 500 µl using 100 units of each restriction enzyme. Some of the control digests were spiked with either 10 or 20 pg of plasmid PV-ASGT08 instead of ½ and 1 copy number equivalents due to limited information about the genome size of *Agrostis stolonifera*. After digestion, the samples were precipitated by adding 1/10 volume (~50 µl) of 3M NaOAc and 2 volumes (~1 mL relative to the original digest volume) of 100% ethanol, followed by incubation at -20°C for at least one hour. The digested DNA was pelleted by centrifugation, washed with 70% ethanol, vacuum dried for approximately 4 minutes in a vacufuge and re-dissolved at room temperature in water.

V.A.1.8. Agarose gel electrophoresis

Digested DNAs were separated on 0.8% agarose gels in 1X TBE buffer. A 'long run' and a 'short run' were performed for each Southern blot analysis, except for the generational stability blot that only contained a single run. The long run facilitated greater resolution of the higher molecular weight DNAs while the short run ensured that all smaller molecular weight DNAs were retained on the gel. The long run/short run involved a 4-6 hour electrophoresis at 80-85 V and an overnight (15.5-19 hours) run at 35-38 V. After electrophoresis, the gels were stained in 0.5 µg/mL ethidium bromide for 20-30 minutes and photographed.

V.A.1.9. DNA probe preparation

Plasmid PV-ASGT08 DNA was isolated from *E. coli* cultures. DNA probe templates homologous to the P-ract, plasmid backbone, NOS 3' polyadenylation sequence, insert probe #1 (containing the P-ract1/ract1 intron, *ctp2-cp4 epsps*, NOS 3' polyadenylation

sequence, and the e35S promoter), and insert probe #2 (containing the e35S promoter, *Zm*HSP70 intron, *ctp2-cp4 epsps*, the NOS 3' polyadenylation sequence) were prepared by PCR using plasmid PV-ASGT08 as a template. A single plasmid backbone probe was prepared instead of two to three overlapping probes as specified in the protocol. The probe templates for the P-ract1/ract1 intron, e35S promoter/*Zm*HSP70 intron and *ctp2-cp4 epsps* were prepared by PCR using gel purified restriction fragments from PV-ASGT08.

Approximately 25 ng of each probe template was labeled with ^{32}P-CTP using the random priming method (RadPrime DNA Labeling System, Gibco BRL). The radiolabeled probe was purified prior to hybridization using a Sephadex G-50 column (Roche).

V.A.1.10. Southern blot analyses

Southern blot analyses (Southern, 1975) were performed according to SOP GEN-PRO-025-02. Following electrophoresis, the gel was placed in depurination solution (0.125 N HCl) for ~10 minutes followed by denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 minutes, and then neutralizing solution (0.5 M Tris-HCl pH 7, 1.5 M NaCl) for ~30 minutes. The DNA from the agarose gels was transferred to Hybond-N nylon membranes (Amersham) using a Turboblotter™ (Schleicher & Schuell). The DNA was allowed to transfer for 4 hours to overnight (in 20X SSC) and covalently cross-linked to the membrane with a UV Stratalinker™ 1800 (Stratagene) set to autocrosslink. The blots were prehybridized for approximately 45 minutes-5 hours in an aqueous solution of 0.5 M sodium phosphate, 7% SDS (w/v) and 0.1 mg/mL *E. coli* tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 15.5-22.5 hours at approximately 65°C. Membranes were washed in an aqueous solution of 0.1% (w/v) SDS and 0.1X SSC for two 15 minute intervals, followed by two 20 minute intervals at 65°C. Multiple exposures of the blots were generated using Kodak Biomax MS film in conjunction with one Kodak Biomax MS intensifying screen.

V.A.1.11. Verification of genomic DNA sequences flanking the 5' and 3' ends of the insert

The sequences of the 5' and 3' insert to plant genomic DNA junctions were determined using Clontech's Universal Genome Walker™ Kit and the RAGE method (Rapid Amplification of Genomic DNA Ends) and verified by PCR. The 5' junction was verified using one primer designed to the genomic DNA sequence flanking the 5' end of the insert paired with a second primer in the rice actin promoter of the inserted DNA. The 5' junction was verified using 50 ng of leaf genomic DNA (1 µl) as a template, 15 pmol of each primer (1.5 µl each) and the Expand High Fidelity PCR system (Roche) in a 50 µl reaction volume. The amplification of the reactions was performed under the following cycling conditions: 1 cycle at 94°C for 2 minutes; 10 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute; 25 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute + 5 additional seconds per cycle; 1 cycle 72°C for 7 minutes.

The 3’ junction was verified using one primer designed to the genomic DNA sequence flanking the 3’ end of the insert paired with a second primer located in the NOS 3’ polyadenylation sequence of the inserted DNA. The PCRs were conducted using 211 ng of leaf genomic DNA (1 µl) as a template, 15 pmol of each primer (1.5 µl each), and the Expand Long Template PCR system (Roche) in a 50 µl reaction volume. The amplification of the reactions was performed under the following cycling conditions: 1 cycle at 94°C for 2 minutes; 35 cycles at 94°C for 10 seconds, 60°C for 30 seconds, 68°C for 30 seconds; 1 cycle at 68°C for 10 minutes.

All PCR products were separated on a 1% TAE agarose gel and visualized by staining with ethidium bromide. Separated PCR products were purified from the agarose matrix and subjected to DNA sequencing using dye-terminator chemistry (Monsanto Genomics Sequencing Center) to confirm the sequences.

V.A.1.12. PCR analysis and sequence confirmation of the *cp4 epsps* coding regions

The linkage of the elements contained within the event ASR368 insert was confirmed by generating two overlapping PCR products that spanned the length of the insert. The PCR analyses were conducted multiple times using 192 ng of genomic DNA as a template in a 50 µl reaction volume containing a final concentration of 1.75 mM $MgCl_2$, 0.3 µM of each primer, 350 µM each dNTP and 2.5 units of Expand Long Template enzyme mix (Roche). The reactions for the 5’ end of the insert were performed under the following cycling conditions: 94°C for 2 minutes; 10 cycles at 94°C for 10 seconds, 65°C, decreasing 1°C per cycle, for 30 seconds, 68°C for 4 minutes; 25 cycles at 94°C for 10 seconds, 56°C for 30 seconds, 68°C for 4 minutes increasing 20 seconds per cycle; 1 cycle at 68°C for 10 minutes. The reactions for the 3’ end of the insert were performed under the following cycling conditions: 94°C for 2 minutes; 10 cycles at 94°C for 10 seconds, 65°C, decreasing 1°C per cycle, for 30 seconds, 68°C for 2 minutes; 25 cycles at 94°C for 10 seconds, 56°C for 30 seconds, 68°C for 2 minutes increasing 20 seconds per cycle; 1 cycle at 68°C for 10 minutes. The PCR products were either separated using 1.0% agarose gel electrophoresis according to SOP # GEN-PRO-003-01 or purified directly from the PCR reaction mixture using the QIAquick PCR Purification Kit (Qiagen). The PCR products derived from event ASR368 that were separated using agarose gel electrophoresis were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen) following the procedure supplied by the manufacturer. The purified PCR products were then sequenced with the initial PCR primers as well as primers designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using dye-terminator chemistry. A consensus sequence was created from interpretable sequence runs using DNAstar/SeqManII version 5.00.

V.A.2. Results and discussion

V.A.2.1. Insert and copy number

V.A.2.1.1. Insert probe #1

A probe containing the first *cp4 epsps* expression cassette and the e35S promoter (Figure V-1) was used to probe event ASR368. The results are shown in Figure V.4. The 99061R/990028 control *Nde* I/*Sma* I long run (lane 1) showed a low level of background hybridization. Plasmid PV-ASGT08 mixed with the 99061R/990028 control *Nde* I/*Sma* I short run digests (lanes 4 and 5) produced the expected size band at approximately 6.3 kb, in addition to the low background hybridization. The event ASR368 *Hin*d III long and short run digests (lanes 2 and 6) each produced one band at approximately 10 Kb, in addition to the low level background hybridization (also seen in Figures V.7 and V.8, lanes 1 and 7). The event ASR368 *Nde* I/*Sma* I long and short run digests (lanes 3 and 7) each produced a single band not present in the 99061R/990028 control lanes at approximately 6.3 Kb. The presence of a single band in each lane suggests that event ASR368 contains a single, intact copy of integrated DNA located on a 10 Kb *Hin*d III restriction segment.

The predicted 321 bp band from the *Nde* I to *Nde* I sites, corresponding to the 5' portion of the rice actin promoter, is not detected on this blot. This is presumably due to the low percentage of the total probe template that this portion represents.

V.A.2.1.2. Insert probe #2

A probe containing the second *cp4 epsps* expression cassette (Figure V-1) was used to probe event ASR368. The results are shown in Figure V-5. The 99061R/990028 control *Nde* I/*Sma* I long run digest (lane 1) did not produce any detectable background bands. Plasmid PV-ASGT08 mixed with the 99061R/990028 control *Nde* I/*Sma* I short run digests (lanes 4 and 5) produced the expected size band at approximately 6.3 kb with no detectable background bands. The event ASR368 *Hin*d III long and short run digests (lanes 2 and 6) each produced one band at approximately 10 kb. The event ASR368 *Nde* I/*Sma* I long and short run digests (lanes 3 and 7) each produced one band at approximately 6.3 kb. These results also suggest that event ASR368 contains a single, intact copy of integrated DNA located on a 10 kb *Hin*d III restriction segment.

V.A.2.2. *ctp2-cp4 epsps* coding region intactness

A probe containing the *ctp2*-*cp4 epsps* coding region (Figure V-1) was used to probe event ASR368. The results are shown in Figure V-6. The 99061R/990028 control *Nde* I/*Sma* I long run digest (lane 1) did not produce any detectable background bands. Plasmid PV-ASGT08 mixed with the 99061R/990028 control *Nde* I/*Sma* I short run digests (lanes 4 and 5) produced the expected size band at approximately 6.3 kb with no detectable background bands. The event ASR368 *Hin*d III long and short run digests (lanes 2 and 6) each produced one band at approximately 10 kb. The event ASR368 *Nde* I/*Sma* I long and short run digests (lanes 3 and 7) each produced one band at

approximately 6.3 kb. These results suggest that event ASR368 contains a single segment of integrated DNA located on a 10 kb *Hin*d III restriction segment; no additional detectable DNA segments were detected using this probe.

V.A.2.3. Rice actin promoter/intron probe

A probe containing the rice actin promoter/intron (Figure V-1) was used to probe event ASR368. The results are shown in Figure V-7. The 99061R/990028 control long run digests [lanes 1 (*Hin*d III) and 2 (*Nde* I/*Sma* I)] showed a high level of background hybridization. Plasmid PV-ASGT08 mixed with the 99061R/990028 control *Nde* I/*Sma* I short run digests (lanes 5 and 6) produced the expected size bands at approximately 6.3 kb and 0.32 kb (very faint), in addition to the high background hybridization. The 99061R/990028 control *Hin*d III short run digest (lane 7) also showed a high level of background hybridization. The event ASR368 *Hin*d III long and short run digests (lanes 3 and 8) each produced one band not present in their respective 99061R/990028 control lanes at approximately 10 kb. The event ASR368 *Nde* I/*Sma* I long and short run digests (lanes 4 and 9, respectively) each produced one band not present in their respective 99061R/990028 control lanes at approximately 6.3 kb. The expected, faint, 0.32 kb band can be seen in the event ASR368 *Nde* I/*Sma* I short run sample. These results confirm data suggesting that event ASR368 contains a single, intact segment of integrated DNA located on a 10 kb *Hin*d III restriction segment and also establish that event ASR368 contains the intact rice actin promoter/intron with no additional detectable DNA segments detected using this probe.

V.A.2.4. Rice actin promoter probe

High levels of background hybridization were observed when probing with the rice actin promoter/intron. Similar high levels of background hybridization have been reported to the USDA in other monocots using the same probe (USDA Petition 00-011-01p). Due to the high level of background hybridization found (Figure V-7) a similar blot was probed with the rice actin promoter alone. The results are shown in Figure V-8. The 99061R/990028 control long run digests [lanes 1 (*Hin*d III) and 2 (*Nde* I/*Sma* I)] showed a moderate level of background hybridization. Plasmid PV-ASGT08 mixed with the 99061R/990028 control *Nde* I/*Sma* I short run digests (lanes 5 and 6) produced the expected size bands at approximately 6.3 kb and 0.32 kb (very faint), in addition to the moderate background hybridization. The 99061R/990028 control *Hin*d III short run digest (lane 7) also showed a moderate level of background hybridization. The event ASR368 *Hin*d III long and short run digests (lanes 3 and 8) each produced one band not present in their respective 99061R/990028 control lanes at approximately 10 kb. The event ASR368 *Nde* I/*Sma* I long and short run digests (lanes 4 and 9) each produced one band not present in their respective 99061R/990028 control lanes at approximately 6.3 kb. The expected 0.32 kb band can be seen hybridizing very faintly in the event ASR368 *Nde* I/*Sma* I short run sample. These results confirm data suggesting that event ASR368 contains a single, intact segment of integrated DNA located on a 10 kb *Hin*d III restriction segment and also establish that event ASR368 contains the intact rice actin promoter with no additional detectable DNA segments detected using this probe.

V.A.2.5. NOS 3' polyadenylation sequence probe

A probe containing the NOS 3' polyadenylation sequence (Figure V-1) was used to probe event ASR368. The results are shown in Figure V-9. The 99061R/990028 control *Nde* I/*Sma* I long run digest (lane 1) did not produce any detectable background bands. Plasmid PV-ASGT08 mixed with the 99061R/990028 control *Nde* I/*Sma* I short run digests (lanes 4 and 5) produced the expected size band at approximately 6.3 kb with no detectable background bands. The event ASR368 *Hin*d III long and short run digests (lanes 2 and 6) each produced one band at approximately 10 kb. The event ASR368 *Nde* I/*Sma* I long and short run digests (lanes 3 and 7) each produced one band at approximately 6.3 kb. These results confirm data suggesting that event ASR368 contains a single segment of integrated DNA located on a 10 kb *Hin*d III restriction segment and also establish that event ASR368 contains the intact NOS 3' polyadenylation sequences, with no additional detectable DNA segments detected using this probe.

V.A.2.6. Enhanced 35S promoter/*Zm*HSP70 intron probe

A probe containing the enhanced 35S promoter and *Zm*HSP70 intron (Figure V-1) was used to probe event ASR368. The results are shown in Figure V-10. The 99061R/990028 control *Nde* I/*Sma* I long run digest (lane 1) did not produce any detectable background bands. Plasmid PV-ASGT08 mixed with the 99061R/990028 control *Nde* I/*Sma* I short run digests (lanes 4 and 5) produced the expected size band at approximately 6.3 kb with no detectable background bands. The event ASR368 *Hin*d III long and short run digests (lanes 2 and 6) each produced one band at approximately 10 kb. The event ASR368 *Nde* I/*Sma* I long and short run digests (lanes 3 and 7) each produced one band at approximately 6.3 kb. These results confirm data suggesting that event ASR368 contains a single segment of integrated DNA located on a 10 kb *Hin*d III restriction segment and also establish that event ASR368 contains the intact enhanced 35S promoter and *Zm*HSP70 intron sequences, with no additional detectable DNA segments detected using this probe.

V.A.2.7. Analysis for backbone fragments

A probe containing the PV-ASGT08 backbone sequence (Figure V-1) was used to analyze event ASR368. The results are shown in Figure V-11. The 99061R/990028 control *Nde* I/*Sma* I long run digest (lane 1) did not produce any detectable background bands. Plasmid PV-ASGT08 mixed with the 99061R/990028 control *Nde* I/*Sma* I short run digests (lanes 4 and 5) produced a band that appears to migrate slightly above 3.1 kb with no detectable background bands. The apparent shift in migration of the plasmid can be attributed to the method used to record the molecular weight markers on the autoradiograph. The molecular weight markers were cut from the membrane prior to hybridization. After exposure of the membrane to film, the removed markers were realigned with the membrane and the sizes of the markers were indicated on the films. A small error occurred when the markers were realigned with the membrane. The event ASR368 *Hin*d III long and short run digests (lanes 2 and 6) showed no hybridization. The event ASR368 *Nde* I/*Sma* I long and short run digests (lanes 3 and 7) showed no

hybridization. This result establishes that event ASR368 does not contain any detectable plasmid backbone sequence as none was detected using this probe.

V.A.2.8. Genetic stability

Genomic DNA from the test and control substances was digested with *Sph* I and probed with the *ctp2-cp4 epsps* coding region. The results are shown in Figure V-12. The V13-2-2 and 99061R/990028 control lanes did not produce any detectable background bands (lanes 4 and 5, respectively). Plasmid PV-ASGT08 mixed with the 99061R/990028 control digest (lane 6) produced the expected size bands at approximately 3.35, 3.23 (closely migrated) and 2.72 kb with no detectable background bands. The R0, F1 and F2 test substance lanes (lanes 1-3, respectively) produced three bands at 8.3, 6.2, and 3.35 kb. The 3.35 kb band represents the internal *Sph* I sequence, while the 8.3 and 6.2 kb bands represent border segments. The 8.3 kb band is a result of the border segment at the 3' end of the insert. The less intense, 6.2 kb band is a result of the border segment at the 5' end of the insert. This 6.2 kb band is less intense than the others because a smaller portion of the target sequence is located on this segment. This result establishes the stability of the insert in event ASR368 over multiple plant breeding generations.

V.A.2.9. Genomic sequence flanking the insert

PCR was performed on genomic DNA to confirm the insert to plant junction sequences at the 5' and 3' ends of the event ASR368 insert. The results of these PCRs are shown in Figure V-13. Panel A shows the result of the PCR confirmation of the 5' insert to plant junction. As expected, the non-transformed control and the no template control (lanes 4 and 5, respectively) did not yield PCR products. The event ASR368 (lanes 1-3) produced the expected 896 bp band. The PCR products from these reactions were subjected to DNA sequencing. The combined overlapping DNA sequences establish the sequence of the 5' insert to plant junction (Figure V-14).

Figure V-13, Panel B, shows the result of the PCR confirmation of the 3' insert to plant junction. As expected, the non-transformed control and the no template control (lanes 4 and 5, respectively) did not yield PCR products. The event ASR368 (Figure V-13, Panel B, lanes 1-3) produced the expected 474 bp band. The PCR products from these reactions were subjected to DNA sequencing. The combined overlapping DNA sequences confirm and establish the sequence of the 3' insert to plant junction (Figure V-15).

V.A.2.10. PCR analysis and sequence confirmation of the genetic element organization and the *cp4 epsps* coding regions

The overlapping PCR products generated from the DNA insert in event ASR368 were subjected to DNA sequencing to further confirm the organization of the elements within the insert and the *ctp2-cp4 epsps* coding regions. The consensus sequence representing the insert in event ASR368, generated by compiling numerous sequencing reactions performed on the two PCR products that spanned the length of the insert, is shown in Figure V-16. The DNA sequence of the insert contains 6639 bases with base one equal

to base 212 of PV-ASGT08, and base 6639 equal to 6850 in plasmid PV-ASGT08 (Figure V-1). Both of the PCR products also contained creeping bentgrass genomic DNA flanking the insert, which is identical to that reported in Figures V-14 and V-15.

The deduced amino acid sequences of the CTP-CP4 EPSPS proteins encoded in event ASR368 were identical to the deduced amino sequences of the CTP-CP4 EPSPS proteins encoded in plasmid vector PV-ASGT08.

V.A.2.11. Conclusions

Glyphosate tolerant creeping bentgrass event ASR368 was produced by particle acceleration technology using a linear DNA segment from plasmid PV-ASGT08 containing two *cp4 epsps* gene expression cassettes. Event ASR368 contains one insertion of the integrated DNA located on a 10 kb *Hin*d III restriction fragment. This insert contains one copy of the segment used in transformation. The individual genetic components in each of the two CP4 EPSPS gene expression cassettes in the integrated DNA are intact. The genome of event ASR368 does not contain any detectable plasmid backbone DNA including, *ori* or the *nptII* coding sequences. Sequences of the 5' and 3' ends of the insert were determined by genome walking and confirmed by PCR amplification and nucleotide sequencing. In addition, the *ctp2-cp4 epsps* coding regions were confirmed to be identical to those in plasmid PV-ASGT08. These data establish that only the expected full-length CTP2-CP4 EPSPS protein should be encoded by the insert in event ASR368. In addition, the genetic stability of the inserted DNA was demonstrated by Southern blot analysis on genomic DNA of the R0, F1 and F2 generations of event ASR368.

V.B. Segregation data

V.B.1. Methods

A flowchart depicting the development and breeding history of event ASR368 and the progeny used to investigate genetic inheritance are presented in Figure V-17. Segregation data for nineteen populations derived from the reciprocal crosses made between F1 plants hemizygous for the *cp4 epsps* gene and derived from event ASR368 plants and elite parental plants are presented in Table V.2 (USDA # 00-22401n). Paired reciprocal crosses were conducted using pollination shoot bags (Lawson #411) to isolate 3-5 heads each of an F1 GT plant (hemizygous for the *cp4 epsps* gene) with an equal number of heads from a single elite parent plant.

The inheritance of the introduced DNA in the progenies from the reciprocal F1 crosses was monitored phenotypically at the whole plant level by application of glyphosate at the two to three leaf stage in a greenhouse. Data from these analyses provide evidence of the number of loci, as well as the stability of the introduced DNA.

V.B.2. Results and discussion of segregation study

Statistical significance for the segregation data was determined using Chi square analysis. For these analyses a Chi square value (χ^2) was determined as follows: $\chi^2 = \sum [(|o-e|-0.5)^2/e]$ where o = observed frequencies for each class, e = expected frequencies for each class and 0.5 = Yates correction factor for Chi square analysis with one degree of freedom (df) (Little and Hills, 1978). The calculated Chi square value was compared to a table of Chi square values to determine whether the observed frequencies fit the expectation for a single insert at $p = 0.05$ and/or $p = 0.01$.

The Chi square analysis indicates that a single T-DNA insert in event ASR368 is integrated in the plant genome and is inherited as a single locus following a Mendelian one-locus model in a stable manner through several plant populations. These results are also consistent with the genetic analysis described in Section V.A.2.8., which demonstrated the genetic stability of the transgene by Southern blot analysis of the R_0, F_1 and F_2 generations of event ASR368.

Creeping bentgrass is essentially an obligate outcrossing species as it is more likely to cross-pollinate than self-pollinate when exposed to other compatible pollen sources. The 1:1 glyphosate tolerant (GT) to glyphosate sensitive (GS) progeny ratios identified among reciprocal cross progeny indicate that cross-pollination between both non-transgenic plants and plants containing the *cp4 epsps* gene is more likely than self-pollination within these creeping bentgrass plants. The fact that reciprocal crosses provided similar 1:1 ratios indicates that both non-transgenic and transgenic plants are selfing and crossing in similar fashion. These results also support the conclusion that F1 GT progeny are hemizygous for the *cp4 epsps* gene.

Further discussion of the open-pollinating nature of creeping bentgrass can be found in Section VIII.E of this petition. In greenhouse evaluations, viable seed set among all self-pollinated bentgrass genotypes was negligible in comparison to the number of viable seeds set upon open-pollination. These results reflect the self-incompatibility systems known to exist in the *Agrostis* genus.

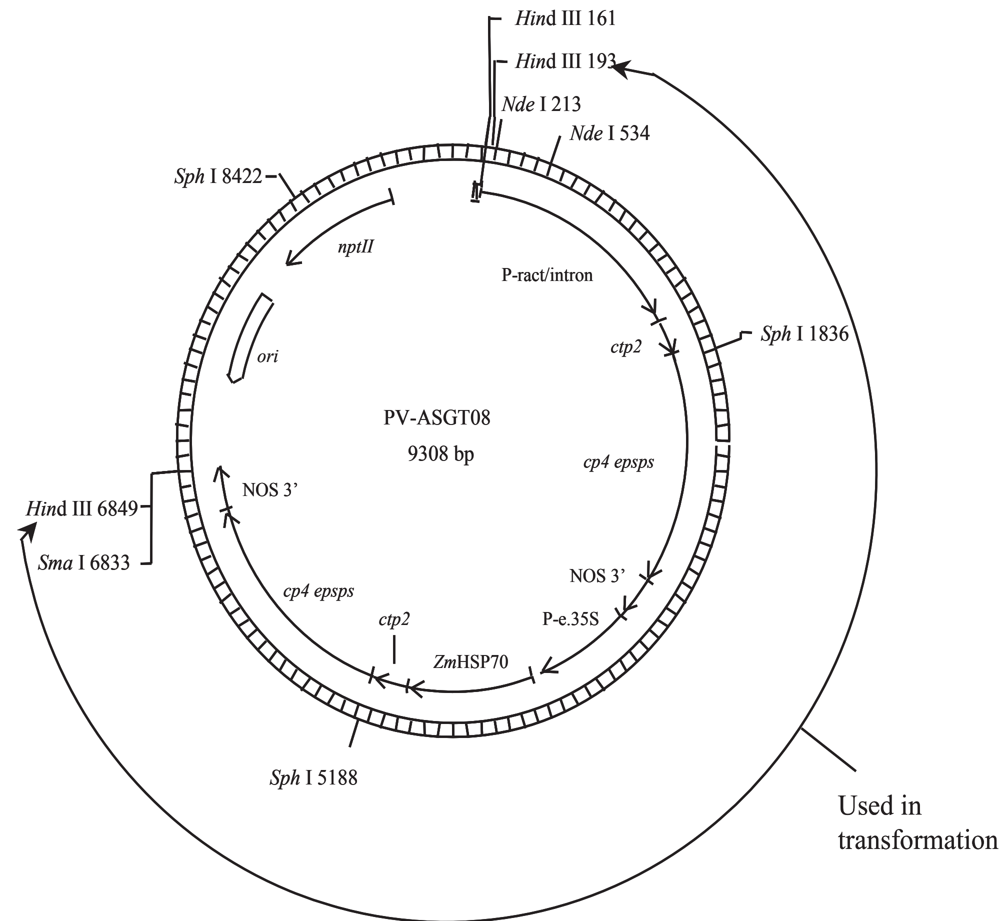
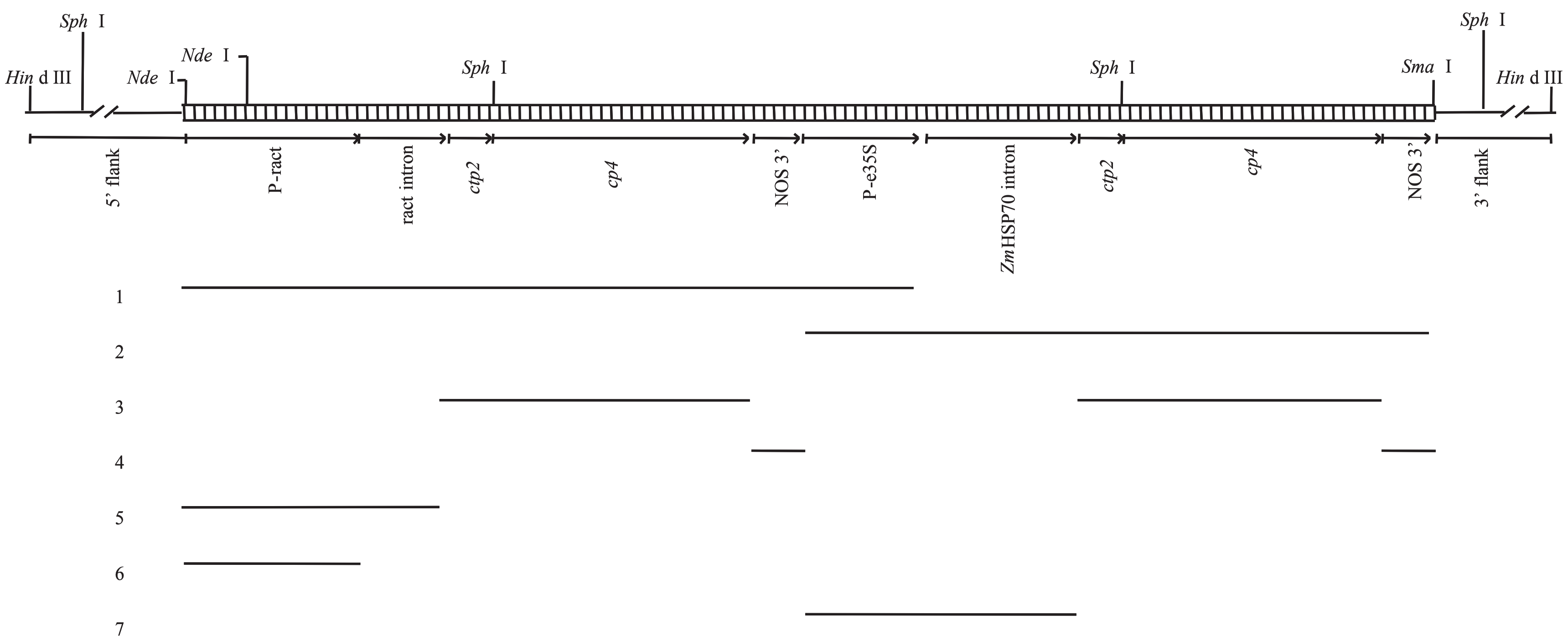


Figure V-1. Plasmid Map of PV-ASGT08

Circular map of the plasmid vector PV-ASGT08 is shown with genetic elements annotated. Restriction sites with positions relative to the size of the plasmid vector for enzymes used in the Southern analysis are shown. Probes used in the Southern analysis are detailed in the accompanying table.



Probe Number	Probe Name	Start Position	End Position	Total Length (bp)
1	Insert Probe #1	180	4062	3883
2	Insert Probe #2	3446	6874	3409
3	*ctp2 -cp4 epsps*	1608, 4958	3167, 6517	1560
4	NOS 3'	3217, 6567	3472, 6822	256
5	P-ract + ract1 intron	266	1582	1317
6	P-ract	175	1150	976
7	P-e35S+ *Zm* HSP70 intron	3474	4936	1463

Figure V-2. Map of the insert in glyphosate tolerant creeping bentgrass event ASR368

A schematic of the predicted DNA insert in glyphosate tolerant creeping bentgrass event ASR368 based on Southern blot analysis, PCR and nucleotide sequencing

Table V-1. Summary of Genetic Elements in PV-ASGT08

Genetic Element	Position in PV-ASGT08	Function
P-ract1/ract1 intron	199-1592	The 5' region of rice (*Oryzae sativa*) actin1 gene containing the promoter, transcription start site and first intron. (McElroy *et al*., 1990)
ctp2	1609-1836	The DNA sequence for chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS; transit peptide directs the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid biosynthesis.
cp4 epsps	1837-3204	The coding sequence for the native 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium sp*. strain CP4.
NOS 3'	3217-3472	The 3' nontranslated region of the nopaline synthase (NOS) coding sequence from *Agrobacterium tumifaciens* which terminates transcription and directs polyadenylation (Fraley *et al*., 1983).
P-e35S	3489-4101	The cauliflower mosaic virus (CaMV) promoter (Odell *et al*., 1985) with the duplicated enhancer region (Kay *et al*., 1987) used to drive expression of the *ctp2-cp4 epsps* coding region.
Zm HSP70	4131-4934	The intron from the maize (*Zea mays*) *hsp70* gene (heat shock protein) present to stabilize the level of gene transcription.
ctp2	4959-5186	The DNA sequence for chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS; transit peptide directs the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid biosynthesis.

Table V-1. Summary of the genetic elements in plasmid PV-ASGT08 (continued).

Genetic Element	Position in PV-ASGT08	Function
cp4 epsps	5187-6554	The coding sequence for the native 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium sp*. Strain CP4.
NOS 3'	6567-6822	The 3' nontranslated region of the nopaline synthase (NOS) coding sequence from *Agrobacterium tumifaciens,* which terminates transcription and directs polyadenylation (Fraley *et al*., 1983).
ORI	7251-7905	The minimum pBR322 sequence required for plasmid replication. Sequence downstream of this region is known to affect copy number.
NPTII	7986-8969	The gene for the enzyme neomycin phosphotransferase type II from Tn5, a transposon isolated from *Escherichia coli (*Beck *et al.,* 1982). The *npt*II gene also contains a 0.153 kb portion of the 0.378 kb *ble* gene from Tn5.

```
  1  MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51  LKKSGMTLIG SELRPLKVMS SVSTACMLHG ASSRPATARK SSGLSGTVRI
101  PGDKSISHRS FMFGGLASGE TRITGLLEGE DVINTGKAMQ AMGARIRKEG
151  DTWIIDGVGN GGLLAPEAPL DFGNAATGCR LTMGLVGVYD FDSTFIGDAS
201  LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP ITYRVPMASA
251  QVKSAVLLAG LNTPGITTVI EPIMTRDHTE KMLQGFGANL TVETDADGVR
301  TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR
351  TGLILTLQEM GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM
401  IDEYPILAVA AAFAEGATVM NGLEELRVKE SDRLSAVANG LKLNGVDCDE
451  GETSLVVRGR PDGKGLGNAS GAAVATHLDH RIAMSFLVMG LVSENPVTVD
501  DATMIATSFP EFMDLMAGLG AKIELSDTKA A
```

Figure V-3. Deduced amino acid sequence of the CP4 EPSPS protein

Sequence includes the CTP2 transit peptide (amino acids 1-76 are the transit peptide).

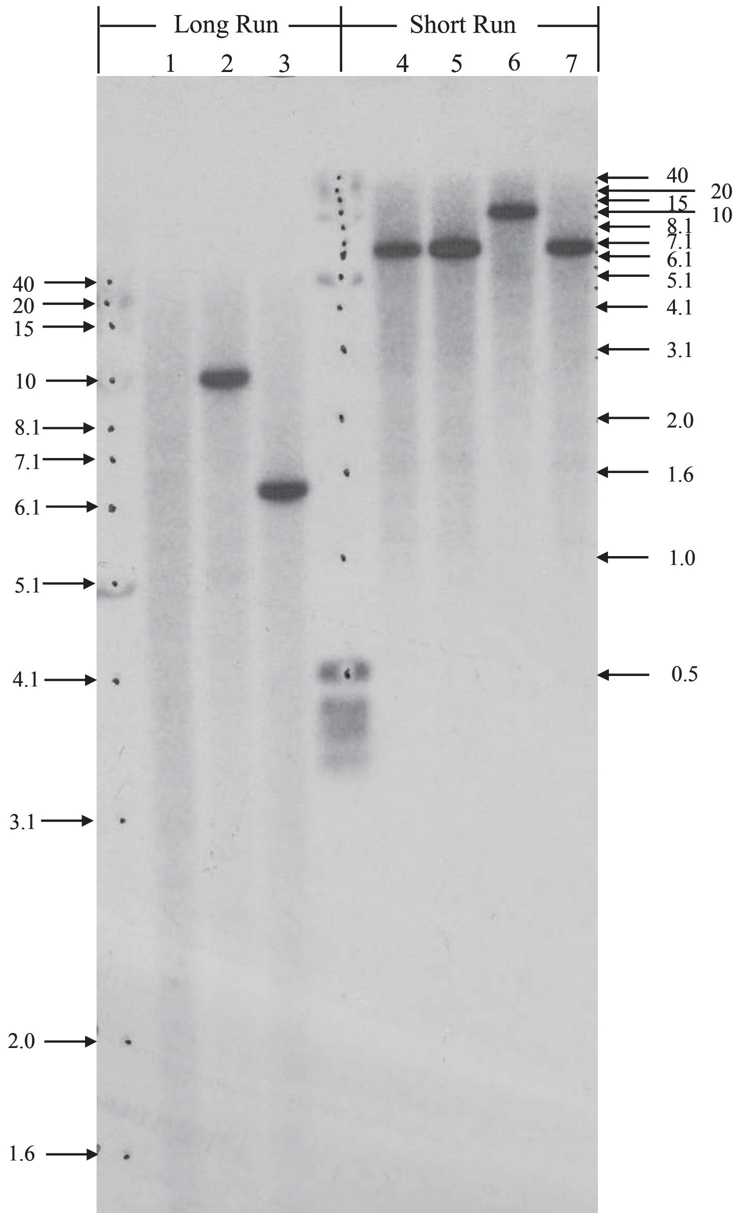


Figure V-4. Southern blot analysis of event ASR368: insert probe #1

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with restriction enzymes. The blot was probed with 32P-labeled insert probe #1. Lane designations are as follows: Lane 1 99061R/990028 (Nde I/Sma I); Lane 2 ASR368 (Hind III); Lane 3 ASR368 (Nde I/Sma I); Lane 4 99061R/990028 + 10 pg of PV-ASGT08 (Nde I/Sma I); Lane 5 99061R/990028 + 20 pg of PV-ASGT08 (Nde I/Sma I); Lane 6 ASR368 (Hind III); Lane 7 ASR368 (Nde I/Sma I).

⟶ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers

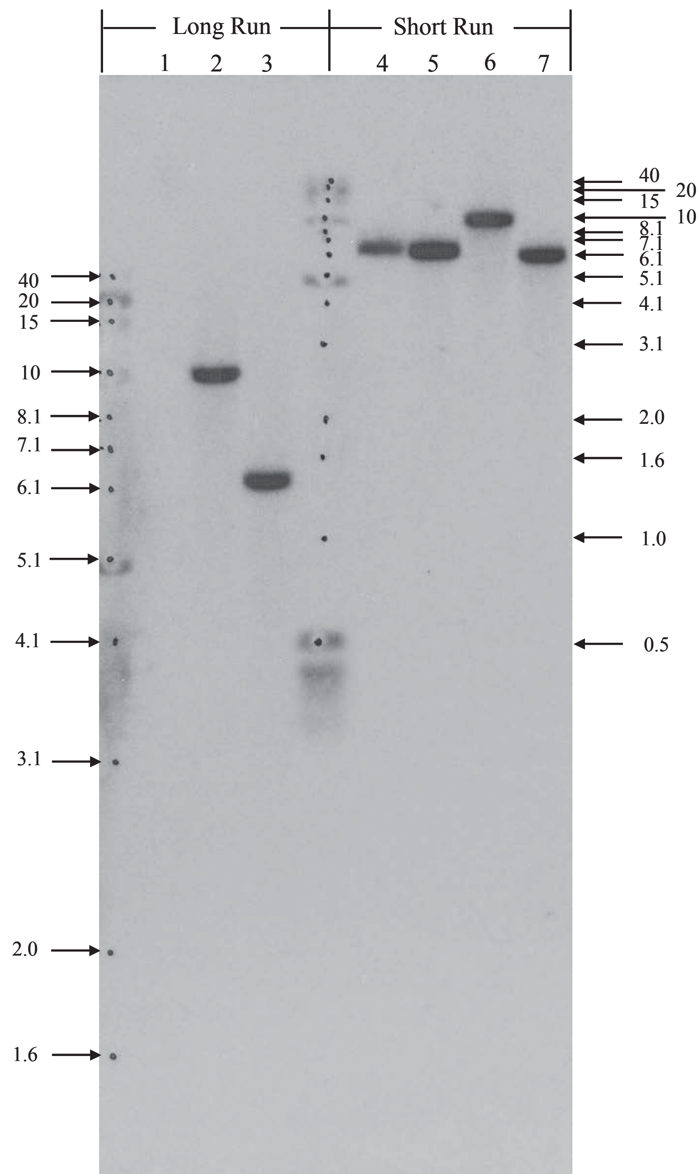


Figure V-5. Southern blot analysis of event ASR368: insert probe #2

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with restriction enzymes. The blot was probed with ^{32}P-labeled insert probe #2. Lane designations are as follows: Lane 1 99061R/990028 (*Nde* I/*Sma* I); Lane 2 ASR368 (*Hin*d III); Lane 3 ASR368 (*Nde* I/*Sma* I); Lane 4 99061R/990028 + 10 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 5 99061R/990028 + 20 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 6 ASR368 (*Hin*d III); Lane 7 ASR368 (*Nde* I/*Sma* I).

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

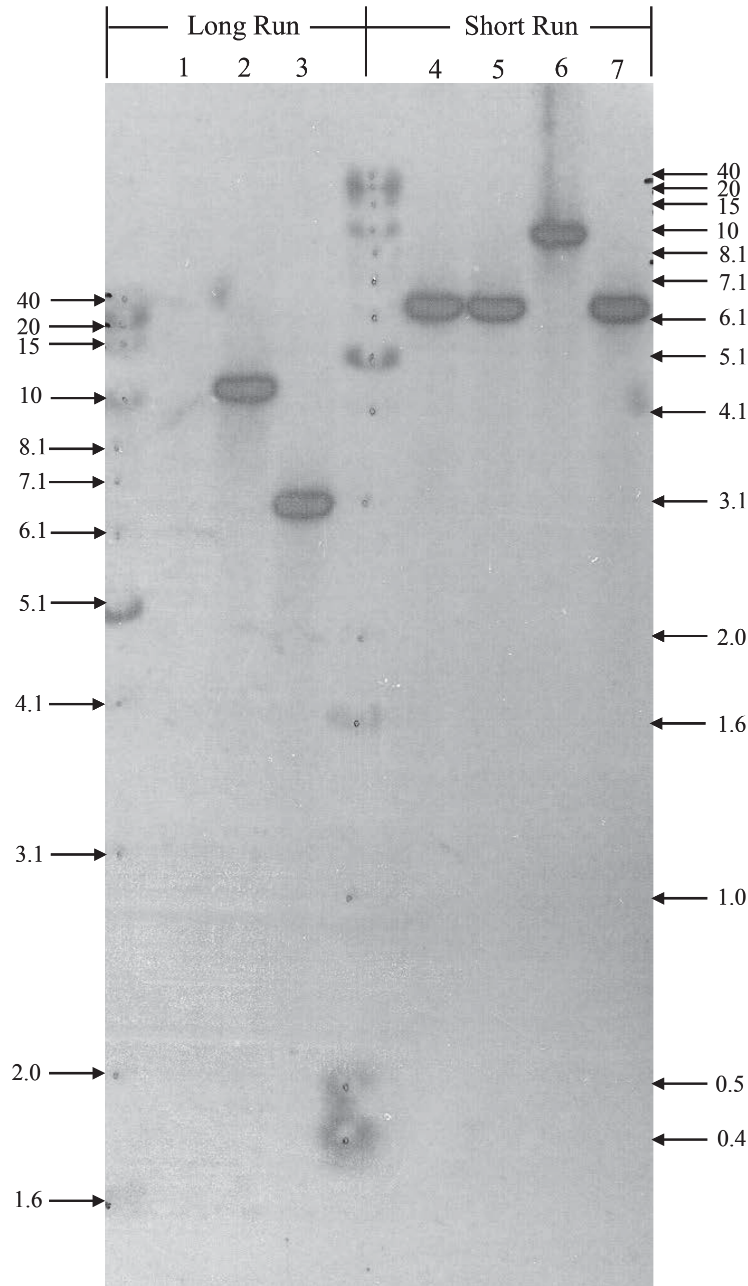


Figure V-6. Southern blot analysis of event ASR368: ctp2-cp4 epsps probe

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with restriction enzymes. The blot was probed with ^{32}P-labeled *ctp2-cp4 epsps*. Lane designations are as follows: Lane 1 99061R/990028 (*Nde* I/*Sma* I); Lane 2 ASR368 (*Hin*d III); Lane 3 ASR368 (*Nde* I/*Sma* I); Lane 4 99061R/990028 + 10 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 5 99061R/990028 + 20 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 6 ASR368 (*Hin*d III); Lane 7 ASR368 (*Nde* I/*Sma* I).

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

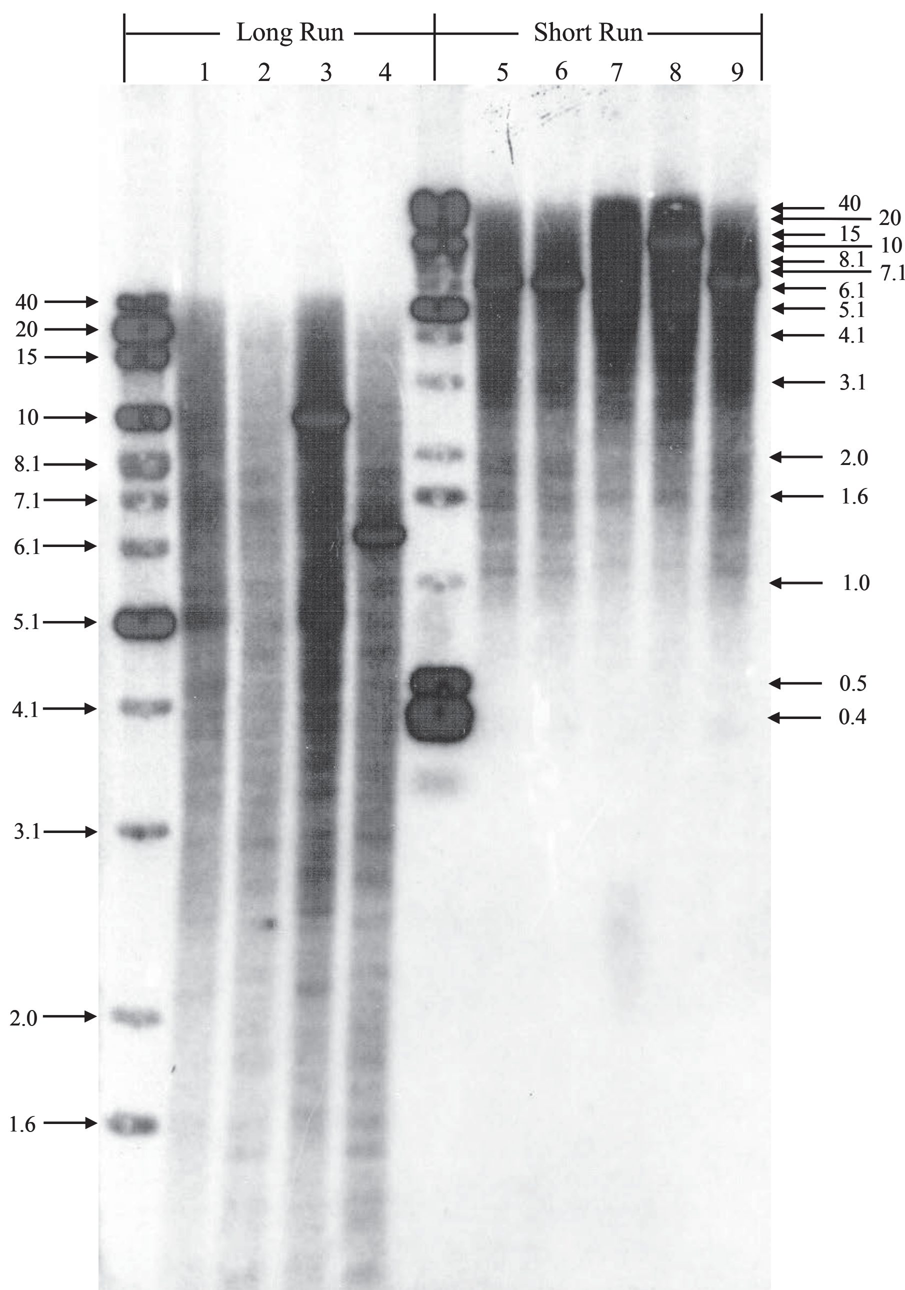


Figure V-7. Southern blot analysis of event ASR368: P-ract/intron probe

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with restriction enzymes. The blot was probed with ^{32}P-labeled rice actin promoter and intron. Lane designations are as follows: Lane 1 99061R/990028 (*Hin*d III); Lane 2 99061R/990028 (*Nde* I/*Sma* I); Lane 3 ASR368 (*Hin*d III); Lane 4 ASR368 (*Nde* I/*Sma* I); Lane 5 99061R/990028 + 10 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 6 99061R/990028 + 20 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 7 99061R/990028 (*Hin*d III); Lane 8 ASR368 (*Hin*d III); Lane 9 ASR368 (*Nde* I/*Sma* I).

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

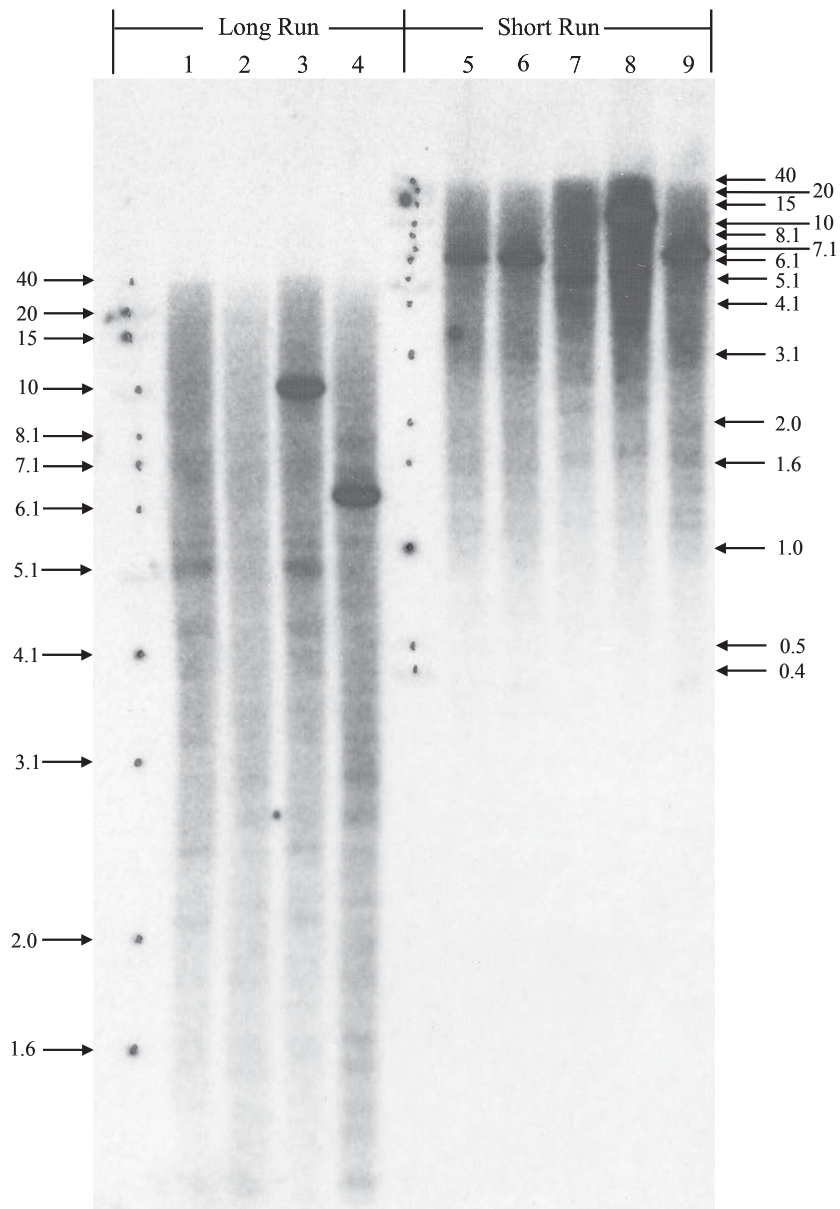


Figure V-8. Southern blot analysis of event ASR368: P-ract probe

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with restriction enzymes. The blot was probed with ^{32}P-labeled rice actin promoter and intron. Lane designations are as follows: Lane 1 99061R/990028 (*Hin*d III); Lane 2 99061R/990028 (*Nde* I/*Sma* I); Lane 3 ASR368 (*Hin*d III); Lane 4 ASR368 (*Nde* I/*Sma* I); Lane 5 99061R/990028 + 10 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 6 99061R/990028 + 20 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 7 99061R/990028 (*Hin*d III); Lane 8 ASR368 (*Hin*d III); Lane 9 ASR368 (*Nde* I/*Sma* I).

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

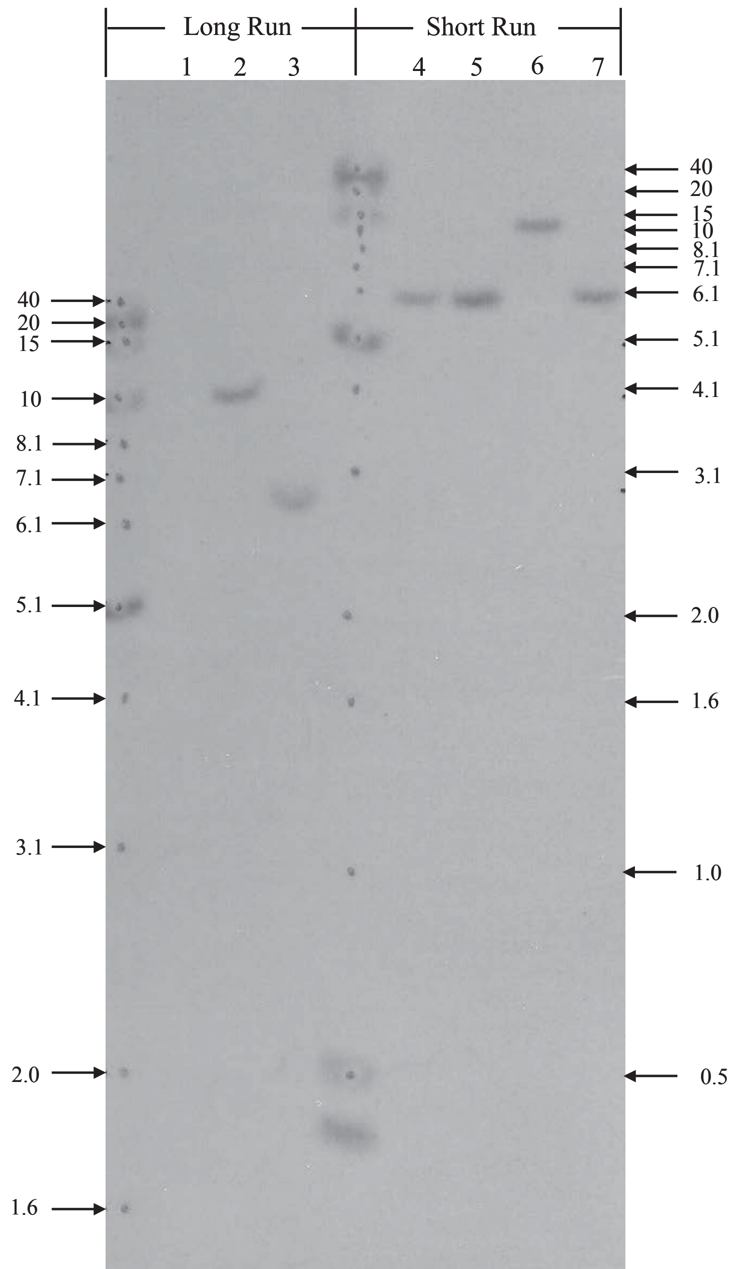


Figure V-9. Southern blot analysis of event ASR368: NOS 3' polyadenylation sequence probe

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with restriction enzymes. The blot was probed with ^{32}P-labeled NOS 3' polyadenylation sequence. Lane designations are as follows: Lane 1 99061R/990028 (*Nde* I/*Sma* I); Lane 2 ASR368 (*Hin*d III); Lane 3 ASR368 (*Nde* I/*Sma* I); Lane 4 99061R/990028 + 10 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 5 99061R/990028 + 20 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 6 ASR368 (*Hin*d III); Lane 7 ASR368 (*Nde* I/*Sma* I).

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

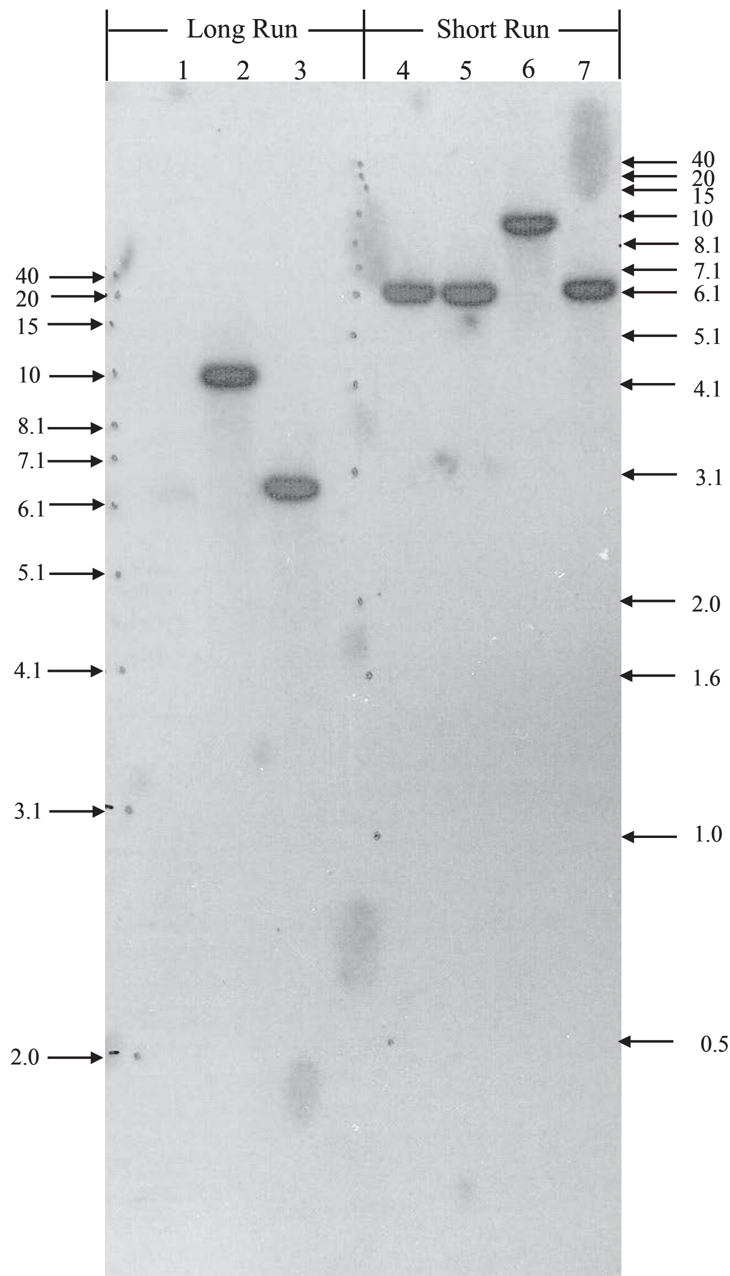


Figure V-10. Southern blot analysis of event ASR368: e35S/*Zm*HSP70 probe

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with restriction enzymes. The blot was probed with ^{32}P-labeled e35S/*Zm*HSP70 promoter. Lane designations are as follows: Lane 1 99061R/990028 (*Nde* I/*Sma* I); Lane 2 ASR368 (*Hin*d III); Lane 3 ASR368 (*Nde* I/*Sma* I); Lane 4 99061R/990028 + 10 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 5 99061R/990028 + 20 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 6 ASR368 (*Hin*d III); Lane 7 ASR368 (*Nde* I/*Sma* I).

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

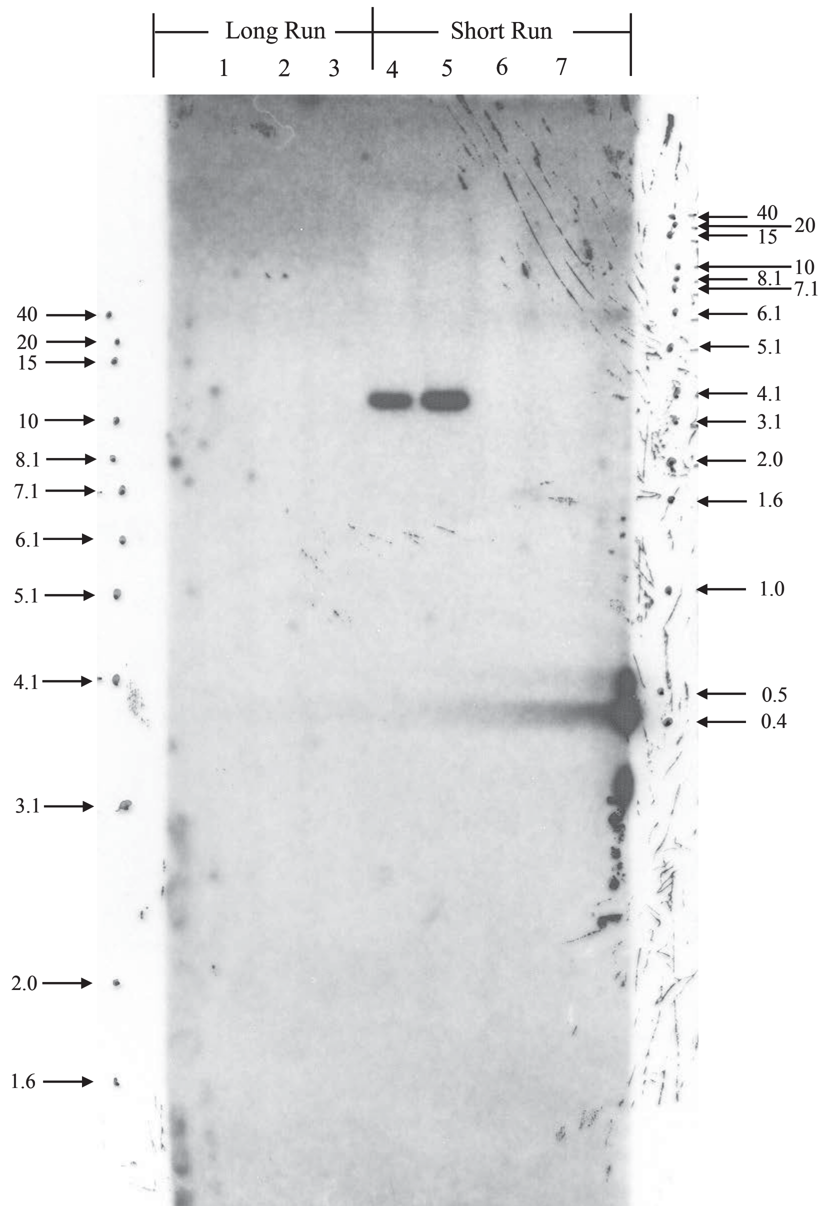


Figure V-11. Southern blot analysis of event ASR368: backbone probe

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with restriction enzymes. The blot was probed with ^{32}P-labeled backbone. Lane designations are as follows: Lane 1 99061R/990028 (*Nde* I/*Sma* I); Lane 2 ASR368 (*Hin*d III); Lane 3 ASR368 (*Nde* I/*Sma* I); Lane 4 99061R/990028 + 10 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 5 99061R/990028 + 20 pg of PV-ASGT08 (*Nde* I/*Sma* I); Lane 6 ASR368 (*Hin*d III); Lane 7 ASR368 (*Nde* I/*Sma* I).

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

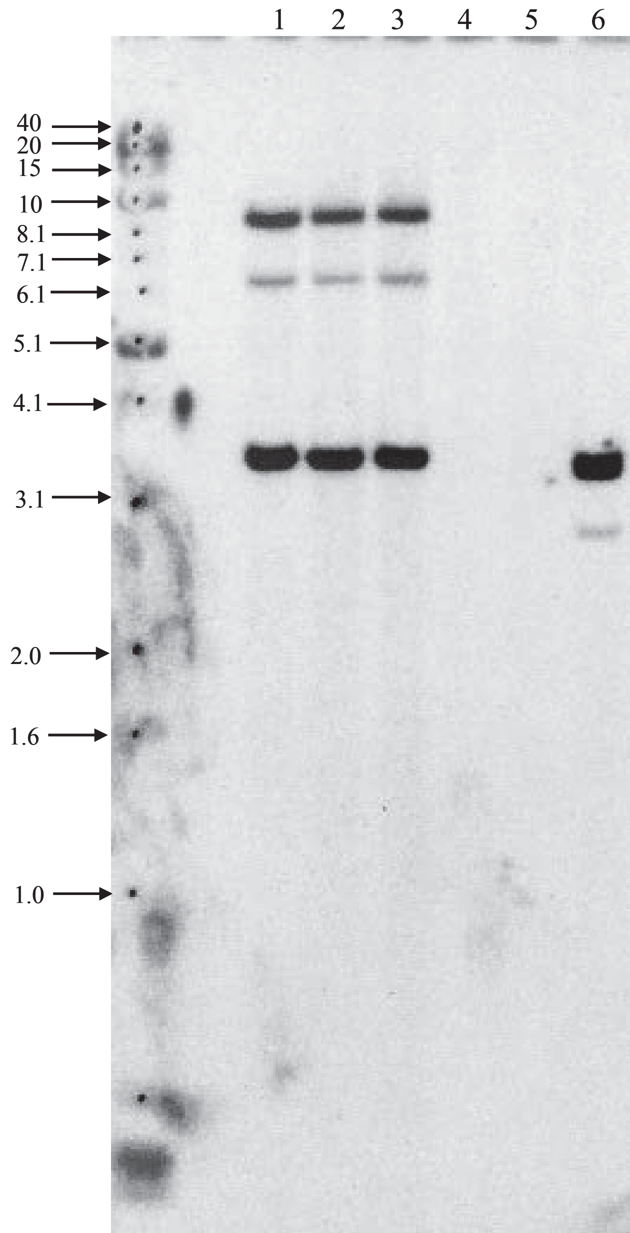


Figure V-12. Southern blot analysis of the genetic stability of event ASR368

Ten micrograms each of 99061R/990028 and ASR368 genomic DNA isolated from leaf tissue were digested with *Sph* I. The blot was probed with ^{32}P-labeled *ctp2-cp4 epsps*. Lane designations are as follows: Lane 1 ASR368 R0; Lane 2 ASR368 F1; Lane 3 ASR368 F2; Lane 4 V13-2-2; Lane 5 99061R/990028; Lane 6 99061R/990028 + 20 pg of PV-ASGT08.

→ Symbol denotes size of DNA, in kilobase pairs, obtained with MW markers.

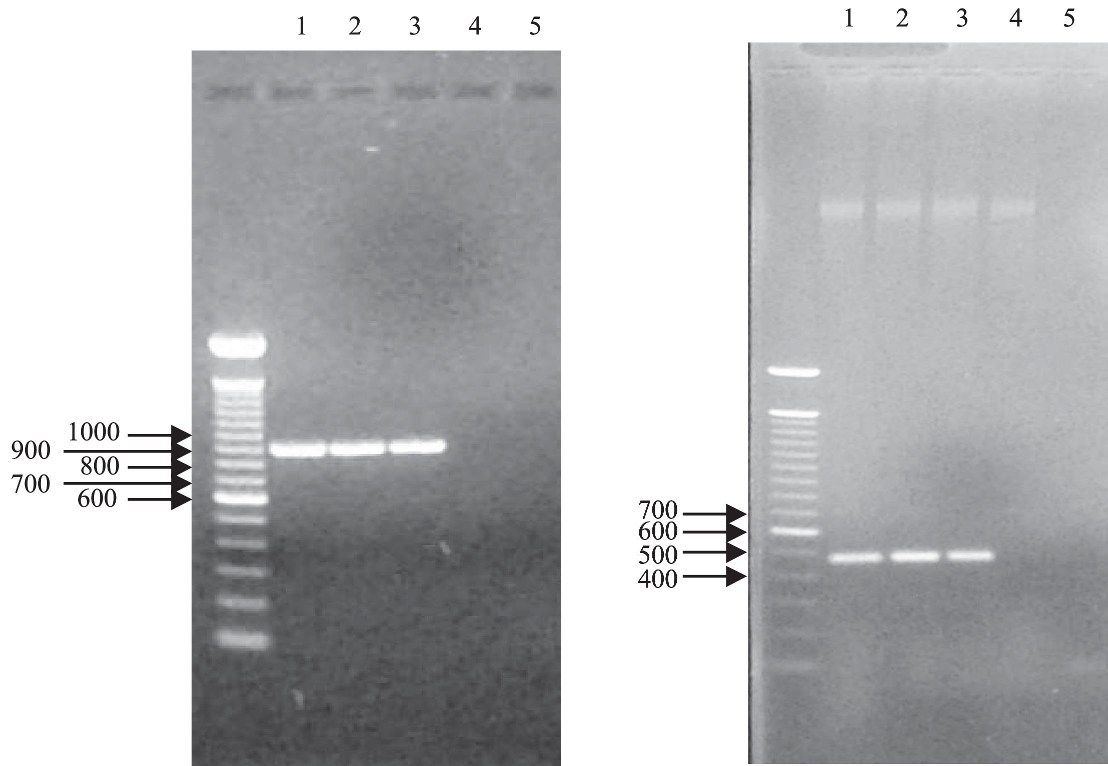


Figure V-13. PCR confirmation of the 5' and 3' border sequences of the event ASR368 insert

PCR was performed using primers specific to the 5' and 3' border sequences for event ASR368 on genomic DNA isolated from leaf tissue from 99061R/990028 (non-transformed control) and event ASR368. Panel A shows the results from the 5' border sequence confirmation. Panel B shows the results from the 3' border sequence confirmation. Lane designations are as follows:

Panel A – 5' border sequence verification

Lane 1 event ASR368
Lane 2 event ASR368
Lane 3 event ASR368
Lane 4 99061R/990028 non-transformed control
Lane 5 No template control

Panel B – 3' border sequence verification

Lane 1 event ASR368
Lane 2 event ASR368
Lane 3 event ASR368
Lane 4 99061R/990028 non-transformed control
Lane 5 No template control

→ Symbol denotes size of DNA, in base pairs, obtained with MW markers.

```
CBI  [ 1 aagcgagtat cctgATAAGA AAGGAAGAAG ACGATCGCTC TGTCTATGGG
      51 CGGGGCTCAG GGCGACGACA GAACCAGAGC TTTCGTCGTG AACAAAACAG
     101 GGAAGGACCA AAGCAGAGGA AGAGGAGAGG AAACAGAGAG AAAGAGGGGG
     151 TTGGTAGGTA CTTGGTGGTC CCTGCTACTT CTCCAACAGC AGCAGAAAGG
     201 AAAGAAGAAC GAACCAAGGC ACAAGTACGC TCCAACCGAG CCATCCCTTT
     251 CTTCCCTTTA TCATTGACTT TAATCATGAG AAATCTAATT AATTAATTAA
     301 ACTCTACGCA AAAGGCATAT AAAATTGTCA ATTATGCAAG GCAGTTGCCC
     351 TGTTTCTGGT AGCCGGTTAC AACACAGGAA GACAACCAAA AGCGTCGGAA
     401 AAGTGAGTTT AGTCGAATCT GAATTCAATG TGAAAGATTT TTGTAAAGAA
     451 TGAAATAAAT CCCGATAAAA AAAGAATGAA CAAAAGGAAA CTAAAAAACT
     501 GTGGATGTGA GTCCAACGTT TAAGCATATC GATGCAAACG TGATGAAGAA
     551 CCAAACGCGC CGGCGGAAGA CGGATTCCCG GAAGACCAAA TTAAAGACGA
     601 TAGTTGTCGA GCAAACGACC AAAAGAAGAA GATCCGACAT ATGCTTAAGA
     651 AGAGAGTCGG GATAGTCCAA AATAAAACAA AGGTAAGATT ACCTGGTCAA
     701 AAGTGAAAAC ATCAGTTAAA AGGTGGTATA AAGTAAAATA TCGGTAATAA
     751 AAGGTGGCCC AAAGTGAAAT TTACTCTTTT CTACTATTAT AAAAATTGAG
     801 GATGTTTTTG TCGGTACTTT GATACGTCAT TTTTGTATGA ATTGGTTTTT
     851 AAGTTTATTC GCTTTTGGAA ATGCATATCT GTATTTGagt cgggtt]
```

Figure V-14. 5' Flanking sequence of the insert in event ASR368

The underlined base pairs 1-637 represent the genomic DNA flanking the 5' end of the insert in event ASR368. Base pairs 638-896 are a portion of the rice actin promoter sequence. The PCR primers used to generate the PCR product are double underlined. The italicized, lower case sequence represents the oligonucleotide sequence that was not confirmed as part of this study because amplification and sequencing were performed with the same primer.

```
[1  agattgaatc ctGTTGCCGG TCTTGCGATG ATTATCATAT AATTTCTGTT
 51 GAATTACGTT AAGCATGTAA TAATTAACAT GTAATGCATG ACGTTATTTA
101 TGAGATGGGT TTTTATGATT AGAGTCCCGC AATTATACAT TTAATACGCG
151 ATAGAAAACA AAATATAGCG CGCAAACTAG GATAAATTAT CGCGCGCGGT
201 GTCATCTATG TTACTAGATC GGGGATATCC CCGGGGAATT CGGTACCATG
251 TACCACGGAA CAGAAAAAAG AAAGGCCCAC GGTTGTGCAG GAAACGGCCA
301 CCGCGCGAGC CAGCGCCTCA CGCCTCATCC GCCATTCCGT CGAGCACCCC
351 GCACGCGCCG CCGCTGCTAT GCTCCTCCGG CCGCGCCCCT TCCTCCTCCA
401 GGTCCTCACG CCGCTTCGCT CCTCCCGCGC CCCCCTCGCG GTCCGCCGCA
451 CGCTCTCAGC gcacgccgcg gcag]
```

Figure V-15. 3' Flanking sequence of the insert in event ASR368

The base pairs 1-248 represent a portion of the NOS 3' polyadenylation sequence and the polylinker sequence. The underlined base pairs 249-474 represent the genomic DNA flanking sequence at the 3' end of the insert in event ASR368. The PCR primers used to generate the PCR product are double underlined. The italicized, lower case sequence represents the oligonucleotide sequence that was not confirmed as part of this study because amplification and sequencing were performed with the same primer.

1 CATATGCTTA AGAAGAGAGT CGGGATAGTC CAAAATAAAA CAAAGGTAAG
51 ATTACCTGGT CAAAAGTGAA AACATCAGTT AAAAGGTGGT ATAAAGTAAA
101 ATATCGGTAA TAAAAGGTGG CCCAAAGTGA AATTTACTCT TTTCTACTAT
151 TATAAAAATT GAGGATGTTT TTGTCGGTAC TTTGATACGT CATTTTTGTA
201 TGAATTGGTT TTTAAGTTTA TTCGCTTTTG GAAATGCATA TCTGTATTTG
251 AGTCGGGTTT TAAGTTCGTT TGCTTTTGTA AATACAGAGG GATTTGTATA
301 AGAAATATCT TTAGAAAAAC CCATATGCTA ATTTGACATA ATTTTTGAGA
351 AAAATATATA TTCAGGCGAA TTCTCACAAT GAACAATAAT AAGATTAAAA
401 TAGCTTTCCC CCGTTGCAGC GCATGGGTAT TTTTTCTAGT AAAAATAAAA
451 GATAAACTTA GACTCAAAAC ATTTACAAAA ACAACCCCTA AAGTTCCTAA
501 AGCCCAAAGT GCTATCCACG ATCCATAGCA AGCCCAGCCC AACCCAACCC
551 AACCCAACCC ACCCCAGTCC AGCCAACTGG ACAATAGTCT CCACACCCCC
601 CCACTATCAC CGTGAGTTGT CCGCACGCAC CGCACGTCTC GCAGCCAAAA
651 AAAAAAAGAA AGAAAAAAAA GAAAAAGAAA AAACAGCAGG TGGGTCCGGG
701 TCGTGGGGGC CGGAAACGCG AGGAGGATCG CGAGCCAGCG ACGAGGCCGG
751 CCCTCCCTCC GCTTCCAAAG AAACGCCCCC CATCGCCACT ATATACATAC
801 CCCCCCCTCT CCTCCCATCC CCCCAACCCT ACCACCACCA CCACCACCAC
851 CTCCACCTCC TCCCCCCTCG CTGCCGGACG ACGAGCTCCT CCCCCCTCCC
901 CCTCCGCCGC CGCCGCGCCG GTAACCACCC CGCCCCTCTC CTCTTTCTTT
951 CTCCGTTTTT TTTTCCGTCT CGGTCTCGAT CTTTGGCCTT GGTAGTTTGG
1001 GTGGGCGAGA GGCGGCTTCG TGCGCGCCCA GATCGGTGCG CGGGAGGGGC
1051 GGGATCTCGC GGCTGGGGCT CTCGCCGGCG TGGATCCGGC CCGGATCTCG
1101 CGGGGAATGG GGCTCTCGGA TGTAGATCTG CGATCCGCCG TTGTTGGGGG
1151 AGATGATGGG GGGTTTAAAA TTTCCGCCGT GCTAAACAAG ATCAGGAAGA
1201 GGGGAAAAGG GCACTATGGT TTATATTTTT ATATATTTCT GCTGCTTCGT
1251 CAGGCTTAGA TGTGCTAGAT CTTTCTTTCT TCTTTTTGTG GGTAGAATTT
1301 GAATCCCTCA GCATTGTTCA TCGGTAGTTT TTCTTTTCAT GATTTGTGAC
1351 AAATGCAGCC TCGTGCGGAG CTTTTTTGTA GGTAGAAGTG ATCAACCATG
1401 GCGCAAGTTA GCAGAATCTG CAATGGTGTG CAGAACCCAT CTCTTATCTC
1451 CAATCTCTCG AAATCCAGTC AACGCAAATC TCCCTTATCG GTTTCTCTGA
1501 AGACGCAGCA GCATCCACGA GCTTATCCGA TTTCGTCGTC GTGGGGATTG
1551 AAGAAGAGTG GGATGACGTT AATTGGCTCT GAGCTTCGTC CTCTTAAGGT
1601 CATGTCTTCT GTTTCCACGG CGTGCATGCT TCACGGTGCA AGCAGCCGGC
1651 CCGCAACCGC CCGCAAATCC TCTGGCCTTT CCGGAACCGT CCGCATTCCC
1701 GGCGACAAGT CGATCTCCCA CCGGTCCTTC ATGTTCGGCG GTCTCGCGAG
1751 CGGTGAAACG CGCATCACCG GCCTTCTGGA AGGCGAGGAC GTCATCAATA
1801 CGGGCAAGGC CATGCAGGCG ATGGGCGCCC GCATCCGTAA GGAAGGCGAC
1851 ACCTGGATCA TCGATGGCGT CGGCAATGGC GGCCTCCTGG CGCCTGAGGC
1901 GCCGCTCGAT TTCGGCAATG CCGCCACGGG CTGCCGCCTG ACGATGGGCC
1951 TCGTCGGGGT CTACGATTTC GACAGCACCT TCATCGGCGA CGCCTCGCTC
2001 ACAAAGCGCC CGATGGGCCG CGTGTTGAAC CCGCTGCGCG AAATGGGCGT

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2051 GCAGGTGAAA TCGGAAGACG GTGACCGTCT TCCCGTTACC TTGCGCGGGC
2101 CGAAGACGCC GACGCCGATC ACCTACCGCG TGCCGATGGC CTCCGCACAG
2151 GTGAAGTCCG CCGTGCTGCT CGCCGGCCTC AACACGCCCG GCATCACGAC
2201 GGTCATCGAG CCGATCATGA CGCGCGATCA TACGGAAAAG ATGCTGCAGG
2251 GCTTTGGCGC CAACCTTACC GTCGAGACGG ATGCGGACGG CGTGCGCACC
2301 ATCCGCCTGG AAGGCCGCGG CAAGCTCACC GGCCAAGTCA TCGACGTGCC
2351 GGGCGACCCG TCCTCGACGG CCTTCCCGCT GGTTGCGGCC CTGCTTGTTC
2401 CGGGCTCCGA CGTCACCATC CTCAACGTGC TGATGAACCC CACCCGCACC
2451 GGCCTCATCC TGACGCTGCA GGAAATGGGC GCCGACATCG AAGTCATCAA
2501 CCCGCGCCTT GCCGGCGGCG AAGACGTGGC GGACCTGCGC GTTCGCTCCT
2551 CCACGCTGAA GGGCGTCACG GTGCCGGAAG ACCGCGCGCC TTCGATGATC
2601 GACGAATATC CGATTCTCGC TGTCGCCGCC GCCTTCGCGG AAGGGGCGAC
2651 CGTGATGAAC GGTCTGGAAG AACTCCGCGT CAAGGAAAGC GACCGCCTCT
2701 CGGCCGTCGC CAATGGCCTC AAGCTCAATG GCGTGGATTG CGATGAGGGC
2751 GAGACGTCGC TCGTCGTGCG TGGCCGCCCT GACGGCAAGG GGCTCGGCAA
2801 CGCCTCGGGC GCCGCCGTCG CCACCCATCT CGATCACCGC ATCGCCATGA
2851 GCTTCCTCGT CATGGGCCTC GTGTCGGAAA ACCCTGTCAC GGTGGACGAT
2901 GCCACGATGA TCGCCACGAG CTTCCCGGAG TTCATGGACC TGATGGCCGG
2951 GCTGGGCGCG AAGATCGAAC TCTCCGATAC GAAGGCTGCC TGATGAGCTC
3001 GAATTCCCGA TCGTTCAAAC ATTTGGCAAT AAAGTTTCTT AAGATTGAAT
3051 CCTGTTGCCG GTCTTGCGAT GATTATCATA TAATTTCTGT TGAATTACGT
3101 TAAGCATGTA ATAATTAACA TGTAATGCAT GACGTTATTT ATGAGATGGG
3151 TTTTTATGAT TAGAGTCCCG CAATTATACA TTTAATACGC GATAGAAAAC
3201 AAAATATAGC GCGCAAACTA GGATAAATTA TCGCGCGCGG TGTCATCTAT
3251 GTTACTAGAT CGGGGATAGC TTCTGCAGGT CCGATTGAGA CTTTTCAACA
3301 AAGGGTAATA TCCGGAAACC TCCTCGGATT CCATTGCCCA GCTATCTGTC
3351 ACTTTATTGT GAAGATAGTG GAAAAGGAAG GTGGCTCCTA CAAATGCCAT
3401 CATTGCGATA AAGGAAAGGC CATCGTTGAA GATGCCTCTG CCGACAGTGG
3451 TCCCAAAGAT GGACCCCCAC CCACGAGGAG CATCGTGGAA AAAGAAGACG
3501 TTCCAACCAC GTCTTCAAAG CAAGTGGATT GATGTGATGG TCCGATTGAG
3551 ACTTTTCAAC AAAGGGTAAT ATCCGGAAAC CTCCTCGGAT TCCATTGCCC
3601 AGCTATCTGT CACTTTATTG TGAAGATAGT GGAAAAGGAA GGTGGCTCCT
3651 ACAAATGCCA TCATTGCGAT AAAGGAAAGG CCATCGTTGA AGATGCCTCT
3701 GCCGACAGTG GTCCCAAAGA TGGACCCCCA CCCACGAGGA GCATCGTGGA
3751 AAAAGAAGAC GTTCCAACCA CGTCTTCAAA GCAAGTGGAT TGATGTGATA
3801 TCTCCACTGA CGTAAGGGAT GACGCACAAT CCCACTATCC TTCGCAAGAC
3851 CCTTCCTCTA TATAAGGAAG TTCATTTCAT TTGGAGAGGA CACGCTGACA
3901 AGCTGACTCT AGCAGATCTA CCGTCTTCGG TACGCGCTCA CTCCGCCCTC
3951 TGCCTTTGTT ACTGCCACGT TTCTCTGAAT GCTCTCTTGT GTGGTGATTG
4001 CTGAGAGTGG TTTAGCTGGA TCTAGAATTA CACTCTGAAA TCGTGTTCTG
4051 CCTGTGCTGA TTACTTGCCG TCCTTTGTAG CAGCAAAATA TAGGGACATG
4101 GTAGTACGAA ACGAAGATAG AACCTACACA GCAATACGAG AAATGTGTAA
4151 TTTGGTGCTT AGCGGTATTT ATTTAAGCAC ATGTTGGTGT TATAGGGCAC
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4201 TTGGATTCAG AAGTTTGCTG TTAATTTAGG CACAGGCTTC ATACTACATG
4251 GGTCAATAGT ATAGGGATTC ATATTATAGG CGATACTATA ATAATTTGTT
4301 CGTCTGCAGA GCTTATTATT TGCCAAAATT AGATATTCCT ATTCTGTTTT
4351 TGTTTGTGTG CTGTTAAATT GTTAACGCCT GAAGGAATAA ATATAAATGA
4401 CGAAATTTTG ATGTTTATCT CTGCTCCTTT ATTGTGACCA TAAGTCAAGA
4451 TCAGATGCAC TTGTTTTAAA TATTGTTGTC TGAAGAAATA AGTACTGACA
4501 GTATTTTGAT GCATTGATCT GCTTGTTTGT TGTAACAAAA TTTAAAAATA
4551 AAGAGTTTCC TTTTTGTTGC TCTCCTTACC TCCTGATGGT ATCTAGTATC
4601 TACCAACTGA CACTATATTG CTTCTCTTTA CATACGTATC TTGCTCGATG
4651 CCTTCTCCCT AGTGTTGACC AGTGTTACTC ACATAGTCTT TGCTCATTTC
4701 ATTGTAATGC AGATACCAAG CGGCCTCTAG AGGATCCAGG AGCAACCATG
4751 GCGCAAGTTA GCAGAATCTG CAATGGTGTG CAGAACCCAT CTCTTATCTC
4801 CAATCTCTCG AAATCCAGTC AACGCAAATC TCCCTTATCG GTTTCTCTGA
4851 AGACGCAGCA GCATCCACGA GCTTATCCGA TTTCGTCGTC GTGGGGATTG
4901 AAGAAGAGTG GGATGACGTT AATTGGCTCT GAGCTTCGTC CTCTTAAGGT
4951 CATGTCTTCT GTTTCCACGG CGTGCATGCT TCACGGTGCA AGCAGCCGGC
5001 CCGCAACCGC CCGCAAATCC TCTGGCCTTT CCGGAACCGT CCGCATTCCC
5051 GGCGACAAGT CGATCTCCCA CCGGTCCTTC ATGTTCGGCG GTCTCGCGAG
5101 CGGTGAAACG CGCATCACCG GCCTTCTGGA AGGCGAGGAC GTCATCAATA
5151 CGGGCAAGGC CATGCAGGCG ATGGGCGCCC GCATCCGTAA GGAAGGCGAC
5201 ACCTGGATCA TCGATGGCGT CGGCAATGGC GGCCTCCTGG CGCCTGAGGC
5251 GCCGCTCGAT TTCGGCAATG CCGCCACGGG CTGCCGCCTG ACGATGGGCC
5301 TCGTCGGGGT CTACGATTTC GACAGCACCT TCATCGGCGA CGCCTCGCTC
5351 ACAAAGCGCC CGATGGGCCG CGTGTTGAAC CCGCTGCGCG AAATGGGCGT
5401 GCAGGTGAAA TCGGAAGACG GTGACCGTCT TCCCGTTACC TTGCGCGGGC
5451 CGAAGACGCC GACGCCGATC ACCTACCGCG TGCCGATGGC CTCCGCACAG
5501 GTGAAGTCCG CCGTGCTGCT CGCCGGCCTC AACACGCCCG GCATCACGAC
5551 GGTCATCGAG CCGATCATGA CGCGCGATCA TACGGAAAAG ATGCTGCAGG
5601 GCTTTGGCGC CAACCTTACC GTCGAGACGG ATGCGGACGG CGTGCGCACC
5651 ATCCGCCTGG AAGGCCGCGG CAAGCTCACC GGCCAAGTCA TCGACGTGCC
5701 GGGCGACCCG TCCTCGACGG CCTTCCCGCT GGTTGCGGCC CTGCTTGTTC
5751 CGGGCTCCGA CGTCACCATC CTCAACGTGC TGATGAACCC CACCCGCACC
5801 GGCCTCATCC TGACGCTGCA GGAAATGGGC GCCGACATCG AAGTCATCAA
5851 CCCGCGCCTT GCCGGCGGCG AAGACGTGGC GGACCTGCGC GTTCGCTCCT
5901 CCACGCTGAA GGGCGTCACG GTGCCGGAAG ACCGCGCGCC TTCGATGATC
5951 GACGAATATC CGATTCTCGC TGTCGCCGCC GCCTTCGCGG AAGGGGCGAC
6001 CGTGATGAAC GGTCTGGAAG AACTCCGCGT CAAGGAAAGC GACCGCCTCT
6051 CGGCCGTCGC CAATGGCCTC AAGCTCAATG GCGTGGATTG CGATGAGGGC
6101 GAGACGTCGC TCGTCGTGCG TGGCCGCCCT GACGGCAAGG GGCTCGGCAA
6151 CGCCTCGGGC GCCGCCGTCG CCACCCATCT CGATCACCGC ATCGCCATGA
6201 GCTTCCTCGT CATGGGCCTC GTGTCGGAAA ACCCTGTCAC GGTGGACGAT
6251 GCCACGATGA TCGCCACGAG CTTCCCGGAG TTCATGGACC TGATGGCCGG
6301 GCTGGGCGCG AAGATCGAAC TCTCCGATAC GAAGGCTGCC TGATGAGCTC

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6351 GAATTCCCGA TCGTTCAAAC ATTTGGCAAT AAAGTTTCTT AAGATTGAAT
6401 CCTGTTGCCG GTCTTGCGAT GATTATCATA TAATTTCTGT TGAATTACGT
6451 TAAGCATGTA ATAATTAACA TGTAATGCAT GACGTTATTT ATGAGATGGG
6501 TTTTTATGAT TAGAGTCCCG CAATTATACA TTTAATACGC GATAGAAAAC
6551 AAAATATAGC GCGCAAACTA GGATAAATTA TCGCGCGCGG TGTCATCTAT
6601 GTTACTAGAT CGGGGATATC CCCGGGGAAT TCGGTACCA]
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Figure V-16. DNA sequence of the insert in event ASR368

Bases 1-6639 represent the DNA sequence of the transgene insert in creeping bentgrass event ASR368. Base one of the insert is equal to base 212 of plasmid PV-ASGT08, while base 6639 equals base 6850 in plasmid PV-ASGT08 (Figure V-1).

Table V-2. Segregation data and Chi square analysis of glyphosate tolerant (GT) and glyphosate susceptible (GS) phenotypes recovered from progeny of reciprocal crosses involving F1 GT progeny derived from event ASR368 and elite parent plants.

Population Designation		# of	GT[1]		deviation	-Yates	d^2	d^2	GS[2]		deviation	-Yates	d^2	d^2	Chi-	Signif.[3]
Female	male	Plants	obs.	expected	/(d)/	Corr. Fact.		expected	obs.	expected	/(d)/	Corr. Fact.		expected	Square	
10-1-8 x ASR368A	15-2-4	41	19	20.5	1.5	1	1	0.049	22	20.5	1.5	1	1	0.049	0.098	NS
15-2-4	10-1-8 x ASR368A	10	6	5	1	0.5	0.25	0.050	4	5	1	0.5	0.25	0.050	0.100	NS
10-1-8 x ASR368B	15-2-4	14	8	7	1	0.5	0.25	0.036	6	7	1	0.5	0.25	0.036	0.071	NS
15-2-4	10-1-8 x ASR368B	98	57	49	8	7.5	56.25	1.148	41	49	8	7.5	56.25	1.148	2.296	NS
14-2-6	13-2-2 x ASR368	282	131	141	10	9.5	90.25	0.640	151	141	10	9.5	90.25	0.640	1.280	NS
13-2-2 x ASR368	14-2-6	352	163	176	13	12.5	156.25	0.888	189	176	13	12.5	156.25	0.888	1.776	NS
14-2-6	13-2-2 x ASR368	64	28	32	4	3.5	12.25	0.383	36	32	4	3.5	12.25	0.383	0.766	NS
13-2-2 x ASR368	14-2-6	92	43	46	3	2.5	6.25	0.136	49	46	3	2.5	6.25	0.136	0.272	NS
13-2-2 x ASR368	15-2-9	37	20	18.5	1.5	1	1	0.054	17	18.5	1.5	1	1	0.054	0.108	NS
15-2-9	13-2-2 x ASR368	126	55	63	8	7.5	56.25	0.893	71	63	8	7.5	56.25	0.893	1.786	NS
15-2-9	13-2-2 x ASR368	51	23	25.5	2.5	2	4	0.157	28	25.5	2.5	2	4	0.157	0.314	NS
10-1-8 x ASR368C	10-1-7c	55	26	27.5	1.5	1	1	0.036	29	27.5	1.5	1	1	0.036	0.073	NS
10-1-7c	10-1-8 x ASR368C	66	35	33	2	1.5	2.25	0.068	31	33	2	1.5	2.25	0.068	0.136	NS
10-1-7c	13-2-2 x ASR368	25	11	12.5	1.5	1	1	0.080	14	12.5	1.5	1	1	0.080	0.160	NS
13-2-2 x ASR368	10-1-7c	91	47	45.5	1.5	1	1	0.022	44	45.5	1.5	1	1	0.022	0.044	NS
10-1-7v	15-2-5 x ASR368	22	10	11	1	0.5	0.25	0.023	12	11	1	0.5	0.25	0.023	0.045	NS
15-2-5 x ASR368	10-1-7v	94	41	47	6	5.5	30.25	0.644	53	47	6	5.5	30.25	0.644	1.287	NS
15-2-9	15-2-5 x ASR368	11	4	5.5	1.5	1	1	0.182	7	5.5	1.5	1	1	0.182	0.364	NS
15-2-5 x ASR368	15-2-9	39	27	19.5	7.5	7	49	2.513	12	19.5	1.5	1	0.5	0.026	2.538	NS

[1] GT= glyphosate tolerant, 2 GS= glyphosate susceptible, 3 NS= not significant at p=0.05 (chi-square=3.84, 1 df).

Creeping bentgrass cv. Backspin

Conventional creeping bentgrass cultivar developed through the crossing of Penncross and several other collections of creeping bentgrass from U.S. and French golf courses.

1998 **B99061R/99028**

Non-transgenic, single seed selection from nontransgenic creeping bentgrass cv. Backspin.

- Transformation with PV-ASGT08L (via.particle bombardment)
- Selection and regeneration in tissue culture (glyphosate amended media)
- Explants transferred to greenhouse

1999 **Event ASR368 R0**

- Greenhouse assay for glyphosate resistance, plant selection with glyphosate
- Ro designation of event ASR368
- Transplants established in fall

Figure V-17. Source of genetic materials and crosses used to develop and investigate the genetic inheritance of ASR368

*Footnote regarding the genetic similarity of these varieties with Penncross, Crenshaw, Backspin and Penn A-4.

VI. CHARACTERIZATION AND SAFETY ASSESSMENT OF THE CP4 EPSPS PROTEIN PRODUCED IN ASR368

Characterization of the introduced protein(s) in a biotechnology-derived crop is important to establishing food, feed, and environmental safety. ASR368 contains the *cp4 epsps* expression cassette that, when transcribed and translated, results in the expression of the CP4 EPSPS protein.

This section summarizes: 1) the identity and function of the CP4 EPSPS protein produced in ASR368; 2) assessment of equivalence between the plant-produced and *E. coli*-produced proteins; 3) the level of the CP4 EPSPS protein in plant tissues from ASR368; and 4) assessment of the potential allergenicity and toxicity of the CP4 EPSPS protein produced in ASR368. The data support a conclusion that the CP4 ESPSP protein is safe for human or animal consumption and safe for the environment based on several lines of evidence summarized below. These data and information were submitted to FDA as part of a food/feed safety and nutritional assessment summary document in September 2002, and FDA completed its consultation, identified under BNF No. 000079, on September 23, 2003.

VI.A. Identity and Function of the CP4 EPSPS Protein from ASR368

The enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), catalyzes one of the enzymatic steps of the shikimic acid pathway, and is the target for the broad spectrum herbicide glyphosate (Haslam 1993; Herrmann and Weaver 1999; Kishore, et al. 1988; Steinrücken and Amrhein 1980). The shikimic acid pathway and EPSPS enzymes are ubiquitous to plants and microorganisms, but absent in mammals, fish, birds, reptiles, and insects (Alibhai and Stallings 2001). EPSPS proteins have been isolated from both plant and microbial sources and their properties have been extensively studied (Harrison, et al. 1996; Haslam 1993; Schönbrunn, et al. 2001; Steinrücken and Amrhein 1984). The plant and microbial enzymes are mono-functional with a molecular weight of 44-51 kDa (Franz, et al. 1997; Kishore et al. 1988). EPSPS enzymes catalyze the transfer of the enolpyruvyl group from phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (EPSP) (Alibhai and Stallings 2001). Shikimic acid is a substrate for the biosynthesis of the aromatic amino acids (phenylalanine, tryptophan and tyrosine) and other aromatic molecules necessary for plant growth.

The EPSPS transgene in ASR368 is derived from *Agrobacterium* sp. strain CP4 (*cp4 epsps*). The *cp4 epsps* coding sequence encodes a 47.6 kD EPSPS protein consisting of a single polypeptide of 455 amino acids (Padgette et al. 1996). The CP4 EPSPS protein is similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in glyphosate agricultural herbicides, relative to endogenous plant EPSPS (Padgette et al. 1996). In conventional plants, including weeds, glyphosate blocks the biosynthesis of EPSP, thereby depriving plants of essential amino acids (Haslam 1993; Steinrücken and Amrhein 1980). In glyphosate tolerant plants, which are tolerant to glyphosate agricultural herbicides, requirements for aromatic amino acids and other metabolites are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette et al. 1996).

The CP4 EPSPS protein expressed in ASR368 is identical to the CP4 EPSPS protein expressed in other glyphosate tolerant products across several crops, including soybean, corn, canola, cotton, sugar beet, and alfalfa.

VI.B. Characterization and Equivalence of the CP4 EPSPS Protein from ASR368

The safety assessment of crops derived through biotechnology includes characterization of the protein(s) produced from the inserted DNA and confirmation of the safety of the protein(s). For safety data generated using proteins produced from a heterologous source (e.g., *Escherichia coli*-produced protein) to be applied to plant-produced protein(s), the equivalence of the plant and *E. coli*-produced proteins must be assessed. The CP4 EPSPS protein produced in ASR368 was demonstrated to be equivalent to both *E. coli*-produced CP4 EPSPS, used previously for human and animal safety studies, and the CP4 EPSPS produced in commercial Roundup Ready soybean (event 40-3-2) based on identical electrophoretic mobilities and detection using specific antibodies as established by western blot analysis (Figure VI-1). This demonstration of equivalence justifies the use of previously conducted protein studies using CP4 EPSPS protein from *E. coli* to establish the safety of the CP4 EPSPS protein expressed in ASR368, summarized in Section V.E.

In a western blot, using published analytical methods (Harrison et al., 1996), the *E. coli*-produced CP4 EPSPS was loaded at two concentrations in lanes 5 and 6 (2.5 ng and 1 ng, respectively) (Figure VI-1). Additionally, *E. coli*-produced CP4 EPSPS was spiked at 2.5 ng and 1 ng in 5.0 µg of control extract from non-transgenic creeping bentgrass (lanes 1 and 2, respectively) and at 2.5 ng in 5.0 µg of control extract from non-transgenic soybean (lane 8) matrix to account for any possible bias associated with the relative mobility of CP4 EPSPS in plant extracts. The protein extract prepared from ASR368 was loaded in lane 4 (5.0 µg), and the protein extract prepared from the Roundup Ready soybean variety (AG5602, 5.0 µg) was loaded in lane 9. Bands at the expected apparent molecular weight (~47 kD) were observed for the *E. coli*-produced CP4 EPSPS (whether alone or in plant matrix), the CP4 EPSPS in ASR368, and CP4 EPSPS in Roundup Ready soybeans. No bands were detected in the control, non-transgenic creeping bentgrass extract (lane 3). A low level of background is detected in the soybean extracts (lanes 7-9). A second, faint, immune-reactive band was detected below the primary band at ~37 kD in the *E. coli*-produced CP4 EPSPS (either alone or in plant matrix), the CP4 EPSPS in ASR368, and CP4 EPSPS in Roundup Ready soybean. This band is presumably a low abundance degradation product of the full-length CP4 EPSPS.

The CP4 EPSPS protein produced in ASR368 was demonstrated to be equivalent to both the *E. coli*-produced CP4 EPSPS protein used for the human and animal safety studies and the CP4 EPSPS produced from commercial Roundup Ready soybean. Equivalence was based on visually equivalent apparent molecular weights and immunological properties when detected using antibodies specific for CP4 EPSPS protein. This demonstration of equivalence justifies the application of the safety data generated using the *E. coli*-produced protein for the CP4 EPSPS protein produced in ASR368.

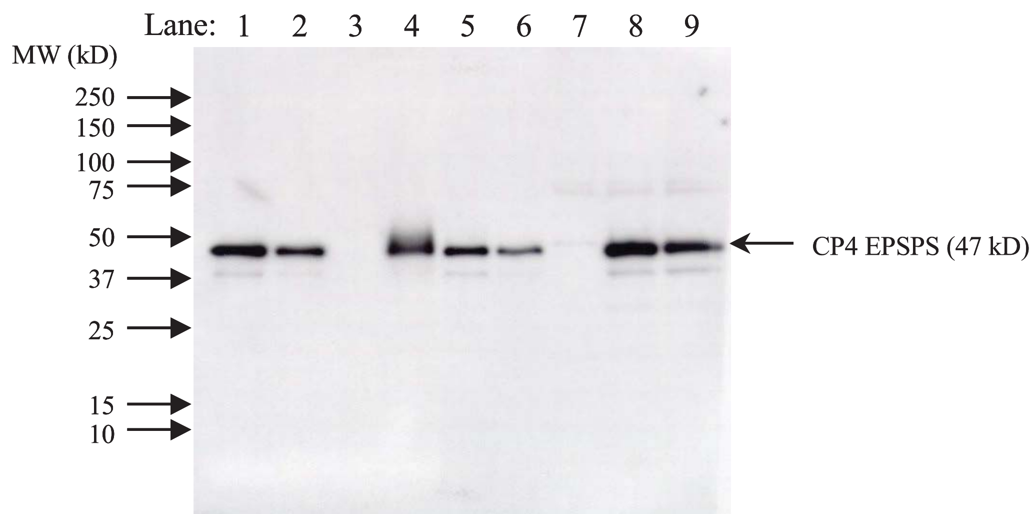

Figure VI-1. Western blot showing the equivalence of CP4 EPSPS protein expressed by *E. coli* Roundup Ready soybean and glyphosate tolerant creeping bentgrass event ASR368

Lane	Description	Amount of Total Protein
1	Control bentgrass extract spiked with 2.5 ng *E. coli* CP4 EPSPS standard	5.0 µg control extract and 2.5 ng standard
2	Control bentgrass extract spiked with 1 ng *E. coli* CP4 EPSPS standard	5.0 µg control extract and 1 ng standard
3	Control bentgrass extract (Backspin)	5.0 µg control extract
4	ASR368 bentgrass extract	5.0 µg
5	*E. coli* CP4 EPSPS standard	2.5 ng
6	*E. coli* CP4 EPSPS standard	1 ng
7	Control soybean extract (A1900)	5.0 µg
8	Control soybean extract spiked with 2.5 ng *E. coli* CP4 EPSPS standard	5.0 µg control extract and 2.5 ng standard
9	A5602 RR soybean extract	5.0 µg

An aliquot of the 1X Laemmli samples for creeping bentgrass and soybean were diluted 10-fold before analysis because of the high expression level of CP4 EPSPS.

VI.C. Expression Levels of CP4 EPSPS Proteins in ASR368

Levels of the CP4 EPSPS protein were estimated in event ASR368 forage samples collected from replicated field sites during the 2000 - 2001 growing season. The field release sites were: Marion County, OR; Union County, OH; Clinton County, IL; and Ottawa County, MI. Field production was conducted using agronomic practices typical of the commercial cultivation of creeping bentgrass and under environmental conditions representative of geographical regions where creeping bentgrass could be grown.

Grass forage samples collected from event ASR368 and the non-transformed parental control line (B99061R) consisted of all shoot tissue approximately one inch above the soil surface. The forage samples were analyzed using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Harlow and Lane, 1988) to estimate the level of CP4 EPSPS protein present in forage tissue. The ELISA consisted of a monoclonal anti-CP4 EPSPS antibody as the capture antibody and a polyclonal anti-CP4 EPSPS conjugated to horseradish peroxidase as the detection antibody. A horseradish peroxidase substrate, TMB (3,3',5,5' tetramethylbenzidene), was added for color development. The CP4 EPSPS protein levels in forage extracts were quantified by comparison of the sample absorbance (OD) to the absorbance produced by a range of concentrations of the *E. coli*-produced CP4 EPSPS reference standard. The CP4 EPSPS protein standard was purified from an *E. coli* strain expressing the *Agrobacterium* sp. strain CP4 EPSPS gene. The protein standard has been previously characterized (Harrison et al., 1993).

The CP4 EPSPS protein levels (corrected for assay bias) estimated in creeping bentgrass forage samples for event ASR368 are summarized in Table VI-1. The average CP4 EPSPS protein level in forage tissue, collected across the growing season from event ASR368 was 68.6 µg/g fwt, with a standard deviation of 16.9 µg/g fwt. Additionally, the CP4 EPSPS protein levels were comparable across the growing season, ranging from 77.1 µg/g fwt (standard deviation 12.7 µg/g fwt) as the average of the first sampling time point to 64.1 µg/g fwt (standard deviation 16.2 µg/g fwt) as the average of the final sampling time point. All of the control samples were below the LOD of 9.9 µg/g fwt. In summary, the grand average CP4 EPSPS protein level, across timepoints, was estimated to be 68.6 µg/g fwt, with a standard deviation of 16.9 µg/g fwt in event ASR368 forage.

In summary, the average CP4 EPSPS protein level, across timepoints, was estimated to be 68.6 µg/g fwt, with a standard deviation of 17.3 µg/g fwt in glyphosate tolerant creeping bentgrass forage generated from an n=60; five timepoints, four field sites and three replications.

Table VI-1. CP4 EPSPS protein levels in plant forage tissues collected from event ASR368 produced in U.S. field trials in the years 2000 and 2001.

Tissue Type	Forage[1] (Young Leaf) Timepoint 1	Forage[1] (OSL2) Timepoint 2	Forage[1] (OSL3) Timepoint 3	Forage[1] (OSL4) Timepoint 4	Forage[1] (OSL5) Timepoint 5
Average CP4 EPSPS Protein Level (µg/g fwt)[2, 3]	77.1	69.7	66.6	65.6	64.1
Standard Deviation[3]	12.7	20.4	13.6	21.4	16.2
Range[4]	63.6 – 105.2	33.6 – 104.5	39.8 – 86.5	25.9 – 97.1	42.9 – 92.9
B99061R	<LOD[5]	<LOD[5]	<LOD[5]	<LOD[5]	<LOD[5]

1 Forage samples consisted of the whole aerial portion of the plant, minus the roots, and harvested at the late vegetative growth (pseudo-erect) stage. Samples were collected at five sampling time-points labeled as: Young Leaf, OSL2 (~ 100 days after planting), OSL3 (~ 330 days after planting), OSL4 (~ 390 days after planting) and OSL5 (~480 days after planting).

2 Protein levels are expressed as micrograms (µg) of protein per gram (g) fresh weight of tissue (fwt) and have been corrected for overall method bias.

3 Forage Analyses for ASR368: the average and standard deviation were calculated for each timepoint from the analyses of three replicate from four field sites (n=12).

4 Minimum and maximum values from the analyses of samples across all sites for each tissue type.

5 The level of the CP4 EPSPS protein in the control line (B99061R) was below the limit of detection (LOD) for all forage samples (9.9 µg/g fwt).

VI.D. Assessment of Potential Allergenicity of Creeping Bentgrass and the CP4 EPSPS Protein

Creeping bentgrass varieties have been grown on U.S. golf courses for more than 110 years and for the past 85 years have been grown almost exclusively for seed production in the Willamette Valley of Oregon (Schoth, 1930). Hence, the plant has a long history of safe use in both golf course and seed production environments. There are no documented clinical reports of allergic reactions specific to *Agrostis stolonifera*. In addition, this species is not considered to be one of the grasses of clinical significance based on known allergens that cause clinical reactions (Suphioglu, 2000). Creeping bentgrass may have the ability to elicit an allergic reaction from a susceptible individual due to cross-reactive allergens from different grasses (Suphioglu, 1993). However, creeping bentgrass has been grown almost exclusively in the Willamette Valley of Oregon for more than 85 years and there has been no specific record of an occurrence in the scientific literature.

The allergenic potential of an introduced protein is assessed by comparing the physiochemical characteristics of the introduced protein to physiochemical characteristics of known allergens (Codex Alimentarius 2009). Using a weight of evidence approach, a protein is not likely to be associated with allergenicity if: 1) the protein is from a non-allergenic source; 2) the protein represents a small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence; and 4) the protein does not show resistance to pepsin digestion. The CP4 EPSPS proteins have been assessed for their potential allergenicity according to these safety assessment guidelines and conclusions are as follows.

1) The CP4 EPSPS protein originates from *Agrobacterium* sp. strain CP4, an organism that has not been reported to be a source of known allergens.

2) The CP4 EPSPS protein is present at a very low level of the leaf tissue of ASR368 (~69 µg/g of tissue on a fresh weight basis). Furthermore, creeping bentgrass is not consumed by humans, nor will ASR368 be commercialized. Thus, human exposure to CP4 EPSPS from ASR368 is negligible.

3) Bioinformatics analyses demonstrated that the CP4 EPSPS protein does not share amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes.

4) Finally, *in vitro* digestive fate experiments conducted with the CP4 EPSPS protein demonstrate that the protein is rapidly digested in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF).

Taken together, these data support the conclusion that the CP4 EPSPS protein does not pose an allergenic risk to humans or animals.

VI.E. Safety Assessment Summary of the CP4 EPSPS Protein

A comprehensive set of factors have been considered and assessed in the safety assessment of the CP4 EPSPS protein in food and feed and the environment. The results are summarized below along with the conclusions reached from each assessment.

VI.E.1. The *cp4 epsps* Donor Organism has a History of Safe Use

The donor organism for *cp4 epsps*, *Agrobacterium* sp. strain CP4, is not known for human or animal pathogenicity, and is not commonly allergenic (FAO-WHO 1991). The history of safe use of *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of this donor organism for USDA-APHIS deregulations, as well as completed consultations with the FDA, regarding Roundup Ready varieties of soybean (1995 and 2007), canola (1995, 2002, and 2012), maize (1998 and 2000), sugar beet (1998 and 2004), alfalfa (2004), and cotton (1995 and 2005).

VI.E.2. CP4 EPSPS Protein has a History of Safe Use

The CP4 EPSPS protein present in ASR368 is similar to EPSPS proteins consumed in a variety of food and feed sources. CP4 EPSPS protein is homologous to EPSPS proteins naturally present in plants, including food and feed crops (e.g., soybean and maize) and fungal and microbial food sources such as baker's yeast (*Saccharomyces cerevisiae*), all of which have a history of safe consumption (Harrison et al. 1996; Padgette et al. 1996). The similarity of the CP4 EPSPS protein to EPSPS proteins in a variety of foods and feeds supports extensive safe consumption of the family of EPSPS proteins and the lack of human or animal health concerns. The ubiquitous presence of homologous EPSPS enzymes in crops and common microorganisms establishes that EPSPS proteins, and their enzymatic activity, pose no hazards to humans, animals, or the environment. In addition, the CP4 EPSPS protein in ASR368 is identical to the CP4 EPSPS protein in numerous other Roundup Ready varieties of soybean, maize, canola, sugar beet, cotton and alfalfa. Further, the U.S. EPA has established an exemption from the requirement of a tolerance for residues of CP4 EPSPS protein and the genetic material necessary for its production in all plants (U.S. EPA 1996).

VI.E.3. CP4 EPSPS Catalyzes a Specific Enzyme Reaction

CP4 EPSPS, like other EPSPS enzymes, functions in the shikimate pathway that is integral to aromatic amino acid biosynthesis in plants and microorganisms (Levin and Sprinson 1964; Steinrücken and Amrhein 1980). Therefore, EPSPS and its activity are found widely in food and feed derived from plant and microbial sources. Genes for numerous EPSPS proteins have been cloned (Padgette et al. 1996) and the catalytic domains of this group of proteins are conserved. Bacterial EPSPS proteins have been well characterized with respect to their three dimensional X-ray crystal structures (Stallings, et al. 1991) and detailed kinetic and chemical mechanisms (Anderson and Johnson 1990).

VI.E.4. CP4 EPSPS Protein is Not Homologous to Known Allergens or Toxins

Bioinformatics analyses were performed to assess the potential for allergenicity, toxicity, or biological activity of CP4 EPSPS. The analyses demonstrated that the protein does not share amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins that could have adverse effects to human or animal health.

VI.E.5. CP4 EPSPS Protein is Labile in *in vitro* Digestion Assays

As has been described in previous regulatory submissions for a number of Roundup Ready crops (USDA-APHIS, 2015b), the CP4 EPSPS protein is readily digestible in simulated gastric (SGF) and simulated intestinal fluids (SIF) (Harrison et al., 1996). Rapid degradation of the CP4 EPSPS protein in SGF and SIF makes it highly unlikely that the protein would be absorbed in a form other than as component nutritional amino acids in the small intestine or have any adverse effects on human or animal health.

VI.E.6. CP4 EPSPS Protein is Not Acutely Toxic

The CP4 EPSPS protein produced in ASR368 is similar to native EPSPS proteins that are ubiquitous in plant and microbial tissues in the environment and is not known to be toxic to other organisms (ILSI-CERA 2010; 2011; USDA-APHIS 2015a). An acute oral toxicology study was conducted with the CP4 EPSPS protein. Results indicate that CP4 EPSPS did not cause any adverse effects in mice, with No Observable Adverse Effect Levels (NOAELs) for CP4 EPSPS at 572 mg/kg, the highest dose tested (Harrison et al., 1996). The highest dose level tested is many fold higher than the level of CP4 EPSPS that is present in tissues of ASR368.

VI.E.7. Human and Animal Exposure to CP4 EPSPS from ASR368

Humans do not consume creeping bentgrass and animal exposure would be negligible since Scotts and Monsanto have no intention to commercialize ASR368. Furthermore, the CP4 EPSPS protein in ASR368 is identical to the same protein in other glyphosate tolerant crops with a history of safe human and animal consumption.

VI.E.8. Non-Target Assessment for CP4 EPSPS Protein

The USDA-APHIS has previously determined that the gene imparting glyphosate tolerance, *cp4 epsps,* and the CP4 EPSPS protein that it encodes, pose no significant risk to non-target organisms due to its long history of use and no known toxicity to non-target organisms (Harrison et al. 1996; ILSI-CERA 2010; USDA-APHIS 2015b).

VI.F. CP4 EPSPS Protein Characterization and Safety Conclusion

The data and information provided in this section address the questions important to the safety of the CP4 EPSPS protein in ASR368, including its potential allergenicity and toxicity. To summarize, the CP4 EPSPS protein from ASR368 was determined and shown to be equivalent to *E. coli*-produced CP4 EPSPS and Roundup Ready soybean (event 40-3-2). An assessment of the allergenic potential of the protein supports the

conclusion that the CP4 EPSPS protein does not pose an allergenic risk to humans or animals. The donor organisms for the CP4 EPSPS coding sequence, *Agrobacterium* sp. strain CP4, is ubiquitous in the environment and not commonly known for human or animal pathogenicity or allergenicity. The CP4 EPSPS protein lacks structural similarity to allergens, toxins or other proteins known to have adverse effects on mammals. The CP4 EPSPS protein is rapidly digested in simulated digestive fluid and demonstrate no oral toxicity in mice at the level tested. Based on the above information, the consumption of the CP4 EPSPS protein from ASR368 or its progeny is considered safe for humans and animals. Furthermore, as humans do not consume creeping bentgrass and animal exposure would be negligible since Scotts and Monsanto have no intention to commercialize ASR368, exposure of humans and animals to CP4 EPSPS from ASR368 is negligible. Given the protein safety data presented herein, the identical nature of the CP4 EPSPS protein in ASR368 to CP4 EPSPS contained in other products that have been deregulated by USDA-APHIS, as well as previous safety assessments, CP4 EPSPS contained in ASR368 is also considered as safe for the environment as conventional creeping bentgrass.

VII. COMPOSITIONAL ASSESSMENT OF ASR368

Safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius 2009) in which the composition of raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. Compositional equivalence between biotechnology-derived and conventional crops supports an “equal or increased assurance of the safety of foods [or feed] derived from genetically modified plants” (OECD 2002).

Although humans do not consume creeping bentgrass, the straw and screenings that remain after the seed is cleaned are used minimally as animal feed. Comparative compositional analyses were conducted on leaf forage samples from ASR368, the non-transformed parent, B99061R and three conventional varieties produced in replicated plots established at sites in Marion County, OR, Union County, OH, Ottawa County, MI and Clinton County, IL and collected from replicated field sites during the 2000-2001 growing season. Single samples of four additional conventional varieties were also included to establish commercial ranges and 99% tolerance intervals to provide additional information on the range of natural variability for each component.

McCrimmon (1994), Youngberg and Vough (1977) and the Atlas of Nutritional Data on United States and Canadian Feeds (1972) were consulted to determine the appropriate compositional analytes and their range in creeping bentgrass straw. Comparative analyses of proximates (protein, fat, ash and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber, minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium and zinc) and carbohydrates by calculation were performed. In all, 17 different components were analyzed to assess the composition of event ASR368.

In a combined-site analysis in which the data were pooled among the sites, there were no statistically significant differences observed between ASR368 and the control B99061R for any of the analytical components (Table VII-1). In an individual-site analysis of the data, four statistically significant differences were observed between ASR368 and B99061R among three different analytical components (Table VII-2). Statistically significant differences were detected for the content of moisture (1 site), phosphorus (1 site), and NDF (2 sites). Of the four comparisons observed to be statistically different between ASR368 and B99061R, all values of ASR368 were within the range and 99% tolerance interval of the conventional, commerical varieties. The significant differences were only observed at one or two sites, not in the combination of all the field sites, and were not considered to be biologically meaningful from a food and feed safety or nutritional perspective. Therefore, it is concluded that event ASR368 is compositionally equivalent to and as safe and nutritious as the forage produced from other conventional creeping bentgrass varieties.

A food/feed safety and nutritional assessment summary document, that included the compositional analysis data, was submitted to the FDA in September 2002, and the FDA consultation, identified under BNF No. 000079, was completed on September 23, 2003[6].

[6] http://www.fda.gov/food/foodscienceresearch/geplants/submissions/ucm155781.htm

Table VII-1. Statistical summary of combined sites creeping bentgrass forage proximate, fiber and mineral content of forage for glyphosate tolerant creeping bentgrass event ASR368, non-transformed parental control line (B99061R) and several commercial cultivars.

Analytical Component	ASR368[1] Mean ± S.E. (Range)	B99061R Mean ± S.E. (Range)	Difference (Test Event ASR368 minus Control Line B99061R) Mean ± S.E. (Range)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Proximate						
Ash (% DW)	10.17 ± 2.02 (6.19 - 16.81)	10.35 ± 2.02 (6.33 - 17.34)	-0.18 ± 1.06 (-3.24 - 3.17)	-2.48,2.13	0.869	(6.11 - 21.43) [0,19.83]
Carbohydrates (% DW)	68.51 ± 2.68 (62.39 - 75.00)	68.34 ± 2.68 (60.97 - 73.78)	0.17 ± 1.47 (-3.00 - 3.59)	-3.04,3.38	0.908	(59.16 - 78.12) [56.74,87.86]
Moisture (% FW)	71.05 ± 1.84 (65.80 - 76.30)	72.08 ± 1.84 (68.80 - 78.40)	-1.03 ± 0.80 (-4.80 - 1.20)	-2.77,0.72	0.225	(65.70 - 78.00) [64.30,79.89]
Protein (% DW)	17.43 ± 1.52 (14.31 - 21.54)	17.74 ± 1.52 (13.79 - 22.28)	-0.31 ± 0.93 (-2.67 - 1.91)	-2.34,1.71	0.741	(10.70 - 22.81) [9.57,21.25]
Total Fat (% DW)	3.89 ± 0.28 (2.44 - 5.65)	3.57 ± 0.28 (2.24 - 4.93)	0.32 ± 0.34 (-2.49 - 2.47)	-0.35,0.99	0.346	(2.36 - 6.37) [0.52,6.69]
Fiber						
Acid Detergent Fiber (% DW)	24.34 ± 1.27 (19.47 - 29.53)	24.07 ± 1.27 (21.48 - 26.06)	0.27 ± 0.84 (-2.89 - 4.30)	-1.57,2.11	0.756	(21.73 - 32.84) [14.45,37.01]
Crude Fiber (% DW)	18.07 ± 0.83 (16.70 - 20.49)	17.43 ± 0.83 (14.65 - 19.78)	0.64 ± 0.72 (-0.80 - 2.69)	-0.92,2.20	0.389	(15.45 - 23.48) [11.79,27.27]
Neutral Detergent Fiber (% DW)	48.07 ± 1.35 (42.34 - 54.27)	45.43 ± 1.35 (41.64 - 50.90)	2.64 ± 1.50 (-4.21 - 8.95)	-0.62,5.90	0.102	(41.60 - 53.52) [40.90,55.81]
Mineral						
Calcium (100g/kg DW)	0.052 ± 0.0075 (0.036 - 0.076)	0.055 ± 0.0075 (0.037 - 0.10)	-0.0026 ± 0.0044 (-0.026 - 0.0033)	-0.011,0.0062	0.559	(0.029 - 0.096) [0.030,0.081]

[1] Number represents average across the four sites and the range of values observed.
[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII-2. Summary of statistically significant results for the comparison of component levels for event ASR368 vs. non-transformed parental control line (B99061R) and several commercial cultivars.

VI]

Analytical Component	Units	Mean ASR368	Mean B99061R	Mean Diff.(ASR368 minus B99061R) % of B99061R	Mean Diff.(ASR368 minus B99061R) Signif. (p-Value)	ASR368 (Range)	Commercial (Range)	Commercial (Tolerance Int.)[1]
Site OR								
Fiber								
Neutral Detergent Fiber	% DW	48.33	42.77	13.00	0.020	(45.48 - 50.00)	(41.60 - 53.52)	[40.90,55.81]
Site IL								
Fiber								
Neutral Detergent Fiber	% DW	52.76	45.68	15.50	<0.001	(51.38 - 54.27)	(41.60 - 53.52)	[40.90,55.81]
Proximate								
Moisture	% FW	67.93	71.83	-5.43	0.026	(65.80 - 70.70)	(65.70 - 78.00)	[64.30,79.89]
Mineral								
Phosphorus	100g/kg DW	0.035	0.040	-13.46	0.046	(0.032 - 0.038)	(0.020 - 0.040)	[0,0.055]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

VIII. PHENOTYPIC, AGRONOMIC, AND ENVIRONMENTAL INTERACTIONS ASSESSMENT

This section provides a comparative assessment of the phenotypic, agronomic, and environmental interaction characteristics, establishment and persistence in managed and unmanaged environments of ASR368 compared to the conventional control. The data support a conclusion that ASR368 is not meaningfully different from the conventional control with the exception of the glyphosate tolerance trait, and therefore, is not expected to pose a plant pest risk compared to conventional creeping bentgrass. These conclusions are based on the results of multiple evaluations from laboratory, greenhouse, and field assessments.

Phenotypic, agronomic, and environmental interaction characteristics of ASR368 were evaluated in a comparative manner to assess plant pest potential. These assessments included evaluation of seed germination characteristics, plant growth and development characteristics, observations of plant responses to abiotic stress, plant-disease and plant-arthropod interactions, pollen characteristics, and volunteer potential, and persistence outside of cultivation characteristics. Results from these assessments demonstrate that ASR368 does not possess a) increased weediness characteristics; b) increased susceptibility or tolerance to specific abiotic stresses, diseases, or insect pests; or c) characteristics that would confer a plant pest risk compared to the conventional control.

In the phenotypic, agronomic, and environmental interactions assessment of ASR368, data were collected to evaluate altered plant pest potential. A detailed description of the regulated article phenotype is requested as part of the petition for determination of nonregulated status in 7 CFR §340.6 including differences from the unmodified recipient organism that would "substantiate that the regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived". As part of the characterization of ASR368, data were collected to provide a detailed description of the phenotypic, agronomic, and environmental interaction characteristics of ASR368.

The plant characterization of ASR368 encompassed eight general data categories: 1) seed establishment; 2) vegetative establishment; 3) relative growth; 4) flowering; 5) pollen characteristics; 6) fecundity; 7) seed physiology, and 8) botanical structures.

The phenotypic, agronomic, and environmental interactions data were evaluated from a basis of familiarity (OECD, 1993) and were comprised of a combination of field, greenhouse, and laboratory studies conducted by scientists who are familiar with the production and evaluation of creeping bentgrass. In each of these assessments, ASR368 was compared to an appropriate conventional control but did not possess glyphosate tolerance trait. In addition, other cultivars developed through conventional breeding and selection were included to provide a range of comparative values for each characteristic that are representative of the variability in existing conventional creeping bentgrass cultivars. Data collected for the various characteristics from the conventional reference cultivars provide context for interpreting experimental results.

As discussed in Section II of this petition, *A. stolonifera* has been studied for decades and much is known of its biology, life history and pest potential. This body of knowledge establishes familiarity with the species and serves as a baseline for the variability common to creeping bentgrasses for a particular plant characteristic. This baseline can also be used to help compare the plant pest potential of glyphosate tolerant creeping bentgrass ASR368 with other conventional creeping bentgrass cultivars (Hokanson et al., 1999).

To help establish greater familiarity with ASR368 and to better understand its plant pest or weed potential, more than 90 experiments were performed between 1999 and 2003 at 65 locations representing the northern or cool, southern or warm and transition climate zones of turfgrass adaptation. These experiments describe the biology, morphology and life history of plants derived from ASR368. The results of these experiments are presented in Section VIII, organized to represent a different aspect of the creeping bentgrass biology and life history. The stages of the life cycle and the plant characteristics evaluated at each stage that could contribute to ASR368 posing a plant pest risk are provided in Figure VIII-1. These include:

1) Establishment via seed.

2) Establishment via vegetative stolons.

3) Relative growth in several environments under competitive and non-competitive environments representing cool season, warm season and transition zone turfgrass growing areas.

4) Flowering period: initiation of flowering, beginning of anthesis and anthesis duration.

5) Pollen biology: size and viability/longevity.

6) Fecundity: duration of seed set and components of seed yield.

7) Seed longevity, dormancy, germination energy and seedling vigor.

8) Quantitative and/ or qualitative assessments of a number of botanical structures or characteristics such as: flag leaf, panicle, floret, ligule, stolons, nodes, bud leaf vernation and leaf venation.

Experimental Comparators

ASR368 was chosen from among more than four hundred transformation events because of its commercially acceptable agronomic and phenotypic characteristics and tolerance to glyphosate herbicide. Using a forward breeding strategy, clones of the ASR368 R0 generation were crossed with a number of Elite Parent Plants to develop the R1 and F1 progeny populations (Figure V-17). As a result of this unique breeding strategy, each

individual plant of an ASR368 seedling population is genotypically and phenotypically distinct yet representative of an *A. stolonifera* population.

A number of different comparators were employed for ASR368 in the experiments presented in this section. Due to the forward breeding process and the potential for somaclonal variation among plants regenerated from tissue culture (Evans et al., 1984; Fluminhan et al., 1996; Muller et al., 1990), near isogenic or tissue culture lines were considered of limited value as comparators for ASR368. Therefore, the following were also employed as comparators: (1) conventional cultivars that represent the range of *A. stolonifera* agronomic and phenotypic characteristics, (2) the Elite Parent Plants (EPPs), which were selected from conventional *A. stolonifera* cultivars developed before 1994 and were crossed with ASR368 R0 generation plants to produce the R1, F1 and F2 progeny populations and/or, (3) null segregant or "Glyphosate Susceptible" plants from which non-transgenic populations were developed. The commercial cultivars, EPPs and null segregants were considered more appropriate non-transgenic organism comparators for assessing the plant pest risk of ASR368 than either near-isogenic or tissue culture lines.

The ASR368 treatments and comparators employed in each experiment presented in this section are depicted in Table VIII-1 and include:

R0: Initial generation ASR368 plants derived directly from transformation, subsequently regenerated in tissue culture and further maintained by vegetative propagation. Identified as ASR368 R0 in the experiments presented in this section and considered the first generation.

R1: Second progeny population of ASR368 plants resulting from the hybridization of an ASR368 R0 mother plant and pollen from a population of conventional Elite Parent Plants. Identified as ASR368 R1 in the experiments presented in this section.

F1: Second progeny population of ASR368 plants resulting from the hybridization of a conventional Elite Parent Plant mother plant and pollen from R0 ASR368. Identified as ASR368 F1 in the experiments presented in this section.

F2: Third progeny population of ASR368 plants resulting from the hybridization of conventional Elite Parent Plants with pollen from a population of F1 ASR368 progeny. Identified as ASR368 F2 in the experiments presented in this section.

GT: A glyphosate tolerant plant that has inherited the *cp4 epsps* gene.

GS: A glyphosate susceptible plant identified among the segregating ASR368 F1 or R1 progeny population. These "GS" plants or "null segregants" did not inherit the *cp4 epsps* gene. GS has also been used to refer to other non-transgenic conventional plants used as comparators in experiments.

B99061R/990028 (non-transgenic tissue culture line): Developed from randomly selected non-transgenic embryogenic callus regenerated in tissue culture and further maintained by vegetative propagation. This tissue culture line was included as an additional comparator in many of the experiments in which ASR368 R0 was evaluated. B99061R/990028 is neither an isogenic nor near-isogenic line to ASR368. Identified as B99061R in the experiments presented in this section.

Elite Parent Plants (EPPs): The EPPs are individual plants selected based on progeny turf trials from the traditionally bred and commercially available creeping bentgrass cultivars Penneagle, Pennlinks, Providence, Putter and Southshore. These varieties, which were developed prior to 1994, were chosen for intercrossing with ASR368 because they possess numerous characteristics desired by the golf course industry.

Backspin: Backspin was developed by Texas A&M University through the intercrossing of selections from Penncross and three other non-transgenic collections of creeping bentgrass obtained from golf courses in the United States and France. The variety was commercially introduced in 1999. B99061R and ASR368 were developed from callus tissue derived from separate seeds selected from Backspin.

Penncross: Penncross was released nearly 50 years ago and is the most widely planted creeping bentgrass cultivar in the United States. The cultivar was developed by breeders at Pennsylvania State University from existing creeping bentgrass turf strains. It is a parent of many currently commercial cultivars and is used as a standard cultivar for variety comparison in the National Turf Evaluation Program[7].

Penn A-4: Penn A-4 was developed by Pennsylvania State University turfgrass breeders and released in 1995. The parents for this cultivar were selected from existing greens likely planted to the creeping bentgrass cultivars Penncross, Penneagle and Pennlinks. Penn A-4 exhibits the highest shoot density of existing cultivars and has been in the National Turf Evaluation Program for more than five years[7].

Crenshaw: Crenshaw was developed by Texas A&M University in the 1990s and was commercially released in 1993. The variety was selected for heat tolerance, is highly susceptible to dollarspot disease and is widely planted in the Southeast United States. Crenshaw has been in the National Turf Evaluation Program trials for more than 10 years[7].

Other bentgrass cultivars: In a number of experiments several other commercial bentgrass cultivars served as comparators for ASR368, e.g. SR1020, Seaside, and Penneagle (*A. stolonifera*), Highland and Trust dryland bentgrass (*A. castellana*), Bardot and SR7100 colonial bentgrass (*A. capillaris*) and Streaker and Reton redtop bentgrass (*A. gigantea*).

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[7] http://www.ntep.org/

This collection of non-transgenic genotypes is generally representative of the genotypic variability of *A. stolonifera* and are appropriate comparators to assess whether glyphosate tolerant creeping bentgrass ASR368 has been altered in a biologically meaningful manner. With these comparators the weed or plant pest potential of ASR368 can be assessed through agronomic and phenotypic evaluations.

The experiments presented in Section VIII were conducted under field conditions and/or in a greenhouse or poly-house. The controlled conditions possible in a greenhouse enable cultivars to be developed and evaluated throughout the year in environments representative of that naturally inhabited by *A. stolonifera* or in which it is agriculturally produced. Greenhouses also enabled the flowering characteristics of ASR368 to be studied without the isolation required under field conditions. In addition, if one or more characteristics of plant growth or development were changed as a result of the plant transformation process, the change(s) would likely be expressed in a consistent manner across multiple environments, including those maintained in a greenhouse or poly-house.

Finally, in order to simplify the text of Section VIII, unless otherwise noted, "genotype" is used to represent a population of individual genotypes that comprise a conventional creeping bentgrass cultivar or a population of ASR368 R1, F1 or F2 progeny as well as single genotypes such as the ASR368 R0 or B99061R.

Figure VIII-1. Summary of creeping bentgrass life cycle, organization of Section VII and studies performed to assess the agronomic characteristics of ASR368

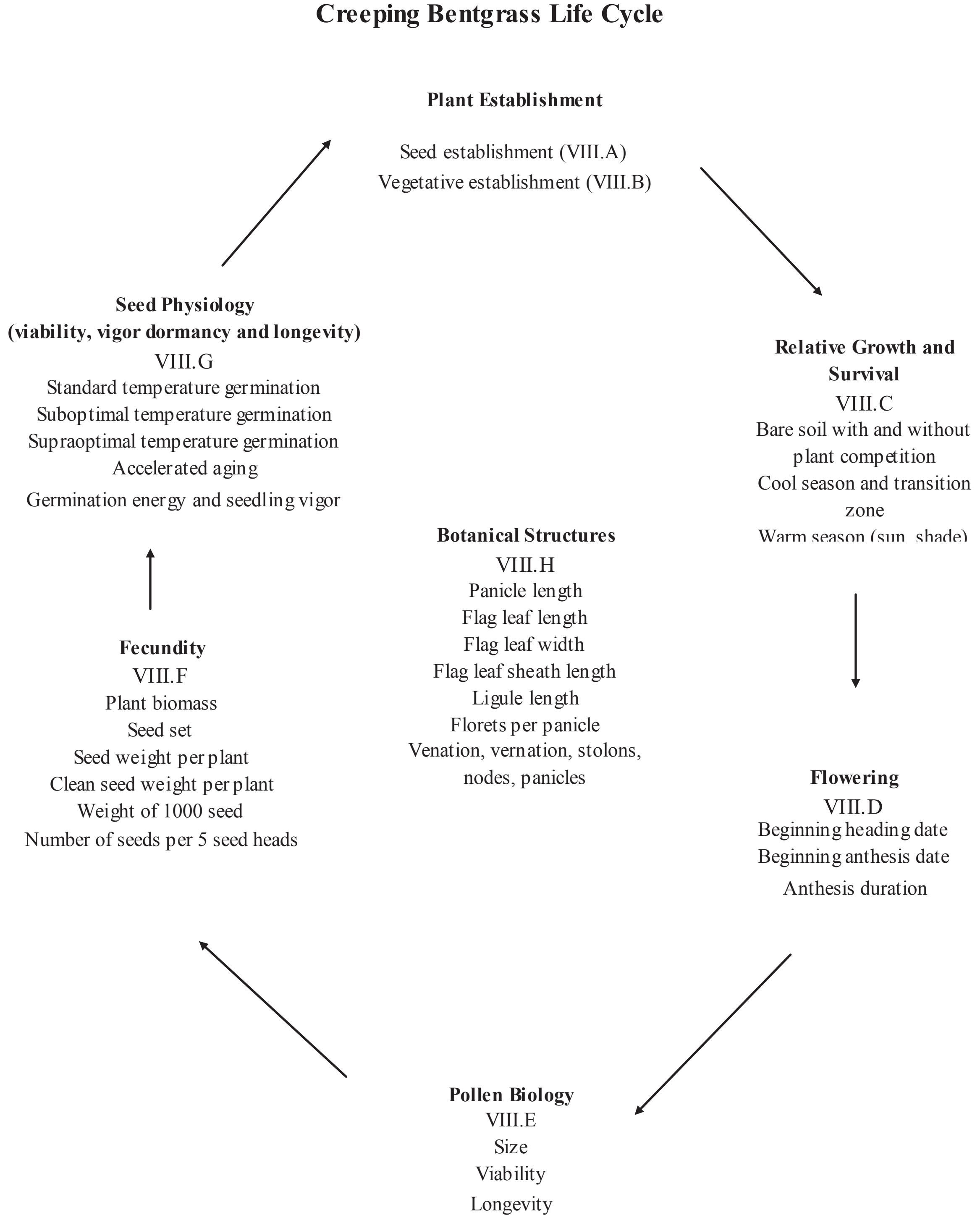

Table VIII-1. Studies performed from 2000 – 2003 to assess the agronomic and phenotypic characteristics of ASR368

Study	Gen.[1]	Controls[2]	Location[3]
Plant Establishment			
Bare soil - seed	R1	A-4, BS, CR, PC	MA, OR
Competition - seed	R1	A-4, BS, CR, PC	MA, OR
Vegetative – stolons	R1	A-4, CR, PC, SR1020, NS	KY (GH)
Vegetative – stolons	R1	BS, PE	OR (PH)
Vegetative – stolons (field/GH)	F1, F2	PC, A-4, CR, EPP	KY, OH, OR, AL
Relative Growth			
Bare soil	R0	A-4, B99, CR, PC	IL, MI, OH, OR
Competition, cool season (R0)	R0	A-4, B99, CR, PC	NJ, OH, OR
Competition, cool season (F1)	F1	A-4, B99, BS, CR, PC, HL, SR7, ST	OH
Competition, warm season (shade)	F1	A-4, B99, BA, BS, CR, PC, HL, SR7, ST	AL
Competition, warm season (sun)	F1	A-4, B99, BA, BS, CR, PC, HL, SR7, ST	AL
Bare soil, reduced irradiance	F1	A-4, B99, BS, CR, PC, HL, SR7, ST	MI
Flowering[4]			
Flowering - greenhouse	R0, F1	A-4, B99, CR, PC	IA (GH)
Flowering - greenhouse	R0, F1, F2	A-4, BS, PC	IA (GH)
Flowering - field	R1	NS	WA (2)
Flowering – field	F1, F2	A-4, BS, CR	OR

Table VIII-1. Studies performed from 2000 – 2003 to assess the agronomic and phenotypic characteristics of ASR368 (continued)

Pollen Biology			
Pollen size, viability and long.	R0, F1	A-4, B99, CR, PC	IA (GH)
Pollen size, viability and long.	F2	A-4, BS, CR	IA (GH)
Fecundity			
Seed set, yield and veg. prod.	R0, F1	A-4, B99, CR, PC	IA (GH)
Seed set, yield and veg. prod.	R1	NS, EPP	WA (2)
Seed set, yield and veg. prod.	F1	A-4, BS, CR	OR
Seed set, yield and veg. prod.	F2	A-4, BS, CR	OR
Seed Physiology[5]			
SGT, SUB, SuOP, AAT, vigor	R1	NS, SR1020, HL	OR (GC, GH)
Comparative Botanical Characteristics[6, 7]			
Botanical characteristics	F1	EPP	OR (1), WA (1)
Botanical characteristics	F2	EPP	OR
Flower morphology	R0, F1,	A-4, B99, CR, PC	IA (GH)
Flower morphology	F2	A-4, BS, PC	IA (GH)

[1] R0 = Initial generation ASR368 plants derived directly from transformation, R1 = seed harvested from ASR368 plants (maternal carried trait), F1 = seed harvested from EPP (ASR368 as pollen donor), F2 = seed harvested from EPP (ASR368 F1 or R1 as pollen donor)
[2] Key to controls: A-4 = Penn A-4, BA = Bardot (colonial bentgrass), BS = Backspin, B99 = B99061R, CR = Crenshaw, NS = Null segregant, PC = Penncross, PE = Penneagle, HL = Highland bentgrass, SR7 = SR7100 (colonial bentgrass) ST = Streaker (redtop bentgrass), EPP = Elite parent plants (either individual plants of Penneagle, Pennlinks, Providence, Putter, Southshore or as a synthetic population of these creeping bentgrass cultivars)
[3] Locations: AL = Alabama, IL = Illinois, , IA = Iowa, KY = Kentucky, MA = Massachusetts, MI = Michigan, NJ = New Jersey, OH = Ohio, OR = Oregon, WA = Washington, GH = greenhouse, GC = growth chamber, PH = poly-house
[4] Flowering characteristics include: beginning head date, beginning anthesis date and anthesis duration
[5] SGT = standard germination test, SUB = suboptimal germination test, SuOP = supraoptimal germination test and AAT = accelerated aging germination test, vigor = high and low germination and seedling vigor
[6] Botanical characteristics include: panicle length, flag leaf length, flag leaf width, flag leaf sheath length, ligule length, florets per panicle and florets per spikelet, leaf venation, leaf vernation, anthers, stolons with nodes
[7] Flower morphology: panicle length and florets/spikelet

VIII.A. Seed Establishment

The rate and breadth of environmental conditions and the diversity of mechanisms by which a plant species is able to establish influences that species' potential to persist in the environment. As described in Section II.E.2, creeping bentgrass colonizes and prospers in environments with the potential for direct and firm contact of a seed or node with soil and little environmental stress, i.e. good mineral nutrition, water availability and abundant light (Hunt et al., 1987). Seed establishment of ASR368 was asessed in experiments depicted in this section. The establishment of vegetative (detached stolon) and whole plants will be reported in Sections VIII.B and VIII.C, respectively.

Studies were conducted to determine the relative ability of seed from ASR368 R1 and four conventional creeping bentgrass cultivars (Backspin, Crenshaw, Penn A-4 and Penncross) to establish and persist in bare soil (non-competitive) and mature turf (competitive) environments. These studies were conducted in Marion County, Oregon during 2000, 2001 and 2002, and Franklin County, Massachusetts during 2000 - 2001 under USDA notification numbers 00-224-01n and 01-228-02n.

VIII.A.1. Marion County, Oregon and Franklin County, Massachusetts (2000 – 2001)

VIII.A.1.1. Experimental methods

Plant Material

Five genotypes of creeping bentgrass seed material were used in this study. ASR368 R1 seed (expected to segregate 1:1 for glyphosate tolerant and glyphosate sensitive phenotypes) and four conventional cultivars: Penncross, Penn A-4, Crenshaw, and Backspin. Prior to planting, the percent germination of each seed lot was determined using the standard AOSA seed germination test (AOSA, 1998). The germination percentages are presented in Table VIII-2 and the number of potential seedlings per plot are presented in Table VIII-3. The number of potential seedlings per plot of the 25 seed planted per genotype was calculated using the corresponding germination data.

Table VIII-2. Creeping bentgrasses and germination percentages for the 2000 – 2001 seed establishment studies in Marion County, Oregon and Franklin County, Massachusetts.

Genotype	% Germination[1]
ASR368 GT	88
Penncross	94
Penn A-4	96
Crenshaw	93
Backspin	96

[1] Mean % germination determined by AOSA standard methods on 4 replications of 100 seed subsamples each from the same seed lots.

Table VIII-3. Creeping bentgrasses and number of potential seedlings per plot in the 2000 – 2001 seed establishment studies in Marion County, Oregon and Franklin County, Massachusetts.

Genotype	Number of Potential Seedlings Per Plot[1]
ASR368 GT	22
Penncross	24
Penn A-4	24
Crenshaw	23
Backspin	24

[1] Number of potential seedlings was calculated using the formula: (% germination × 25 seed planted = number of potential seedlings per plot).

Field Plantings (2000 – 2001)

Two plantings were made at the Marion County, Oregon location. A fall planting was made on October 27, 2000 at the initiation of the rainy season and a spring planting was made on March 21, 2001 prior to the end of the rainy season. In Franklin County, Massachusetts, one fall planting was made on September 20, 2000.

Experimental design

Two levels of competition were used during establishment at both the Oregon and Massachusetts locations.

The Oregon study included: (1) a bare soil test plot void of vegetation and (2) a vegetative plot consisting of a mature plant canopy (4-6 inches) of ‘Brigade’ hard fescue. The bare soil plot was fumigated, tilled and irrigated prior to study initiation on October 27, 2000. The competitive plots were established on the same dates as the bare soil plots. No additional tillage or mowing occurred once the studies were initiated. Winter and spring rains in Oregon provided sufficient soil moisture for germination of both the fall and the spring planting dates.

The Massachusetts location also included two contrasting levels of competition for establishment: (1) a bare soil test plot site stripped of the existing vegetation with a sod cutter, and (2) a vegetative site covered with a mature 10-year old Kentucky bluegrass, fine fescue and perennial ryegrass turf stand with a plant canopy height of three to five inches under low maintenance. This experiment was established on September 20, 2000 as an irrigated and non-irrigated fall planting within each competition level. However, no irrigation events were needed after the initial irrigation as the fall season received ample amounts of natural precipitation. A frost occurred within 10 days of initiation at this site. Dormancy of the surrounding turfgrass was observed by mid-October and the test site was covered with snow and ice by the end of October, which subsequently killed all seedlings.

The plots at both study sites were arranged in a completely randomized design with three replications within each of the four combinations of competition level (bare soil or vegetated) and season (spring or fall in Oregon) or irrigation (+ or – in Massachusetts) regimes. Each plot was 1 m × 1 m square. A 30 cm × 30 cm square was centered within each plot as the test area. A total of 25 seeds were added to each plot on a uniform 6 cm spacing using a grid pattern as a guide in the 30 cm × 30 cm square. The seed was dropped onto the surface of the bare soil or the fescue-covered site without incorporation or pressing into the soil to simulate natural seed dissemination conditions.

Data Collection

Monthly seedling and plant counts were taken throughout the duration of the experiments in Oregon. The total number of potential seedlings per plot was calculated by multiplying the germination percentage determined for each seed line by the number of seed sown (Table VIII-2). Survivability was then calculated as the number of seedlings established divided by the total number of potential seedlings (Table VIII-3).

In Massachusetts, seedling and plant counts were made on October 5 and 12, 2000. Observations were not recorded again until the following spring as plant dormancy was observed by mid-October and persistent low temperatures coupled with snow and ice followed for the remainder of the winter. Data collection resumed in March 2001 and all plots were observed monthly through September.

Statistical Methods

Fisher's Exact Test ($\alpha = 0.05$) was used to compare the survivability of ASR368 versus each commercial cultivar in Oregon. A Least Significant Difference analysis ($\alpha = 0.05$) was used to examine the potential for significant differences between means in the Massachusetts data.

VIII.A.1.2. Results of 2000 – 2001 seed establishment studies

VIII.A.1.2.1. Bare soil seedling establishment - Oregon

VIII.A.1.2.2. Fall Planting

Seedling survival rates for seed of ASR368 R1 and the four conventional cultivars, Backspin, Crenshaw, Penn A-4 and Penncross from October 2000 through September 2001 are presented in Table VIII-4. No seedlings were observed in any plot from October through February. Survival rates fluctuated over time. Fluctuation in seedling number suggests that: (1) individual seed germination occurred over time, (2) seedlings were difficult to locate at earlier stages of development and/or (3) seedlings may have germinated and subsequently perished. A maximum of 30% field survival (Crenshaw on the 9/7/01 observation date; Table VIII-4) was noted for any creeping bentgrass genotype planted in non-competitive bare soil plots during the fall. Throughout the duration of the study, ASR368 R1 seed or seedling establishment was significantly lower than Crenshaw on all but one date. In comparison to the other conventional cultivars ASR368 R1 establishment tended to be not significantly less ($\alpha = 0.05$) or within the range of the

conventional creeping bentgrass cultivars evaluated. These differences would not be expected to increase the plant pest potential of ASR368.

VIII.A.1.2.3. Spring Planting

Table VIII-5 depicts the spring 2001 seedling survival rates in Oregon for ASR368 R1 and the four conventional cultivars: Backspin, Crenshaw, Penn A-4 and Penncross. Survival rates fluctuated over time similar to what was observed for the fall sowing dates. The fluctuation in plant numbers again suggests: 1) that individual seed germination occurred over time, (2) seedlings were difficult to locate at earlier stages of development and/or (3) seedlings may have germinated, established and subsequently perished. Throughout the duration of the study, ASR368 R1 seed or seedling establishment was not significantly different from the commercial creeping bentgrass cultivars evaluated ($\alpha = 0.05$). Less than 9% maximum field survival from spring sowing was observed among any creeping bentgrass genotype in non-competitive environments.

In several of the following tables (VIII-4 through VIII-9), the term "survivability" in the context of this petition section is used as a relative measure of the plant establishment from seed versus the potential for establishment from the pure live seed count planted. Pure live seed counts are based on standard germination tests of the seed lots in the trial multiplied by the number of seed units planted in each plot. Survivability is calculated based on the number of plants actually found within each plot on any given date. In order to avoid undue disruption of plots, no attempts were made to track individual plants to determine if a seed that germinated and was counted on one date actually survived until the next observation date. Consequently zero survivability was recorded on some dates.

Seed planting dates used in Oregon during 2000 and 2001 coincide with the approximate start of the rainy winter season in the Pacific Northwest. The objective of the experiments was to optimize germination and establishment under natural conditions so earlier seeding dates were not used. Earlier seeding dates would coincide with dry weather patterns typical of September (ET_{50} moisture deficit) that are unlikely to result in optimum seedling germination or establishment (see tables VIII-18 vs. VIII-22). If seed were planted earlier and an unlikely and infrequent precipitation event occurred, it is likely that germination would initiate, but would result in higher seedling mortality since consistent moisture is not expected until October or November. Bentgrass seed growers in the Willamette Valley usually provide supplementary irrigation to expedite germination and maintain rapid establishment of new seed production fields.

Bentgrass seedlings can establish and survive under optimum environmental conditions. However, bentgrass is unlikely to germinate and establish under unmanaged conditions (Jonsdottir, 1991; Howe and Snaydon, 1986) and does have difficulty even under highly managed systems (Kendrick and Danneberger, 2002). For example, a comparison of seedling establishment under irrigated versus non-irrigated conditions initiated during September of 2001 in Oregon provides evidence of the importance of irrigation in the Willamette Valley for improved fall seedling establishment. Germination and establishment under optimized environmental (temperature and moisture) conditions is observed in trials reported in Section VIII.G. Field studies provide additional perspective on whether germination and establishment is more or less likely to occur than traditional

species or at what frequency germination and establishment of any bentgrass is likely to occur, if at all, in more naturalized conditions.

Table VIII-4. Fisher's Exact Test comparison of survivability of ASR368 R1 seed and seedlings with several conventional creeping bentgrass cultivars planted on bare soil during fall 2000 in Marion County, Oregon

Obs. Date	Genotype	Survivability	Difference[1]	95% Conf. Int.[2]		P - value
3/12/01	ASR368	0.015				
	Backspin	0.043	-0.028	-0.084	0.028	0.6645
	Crenshaw	0.153	-0.138	-0.226	-0.049	*0.0071
	Penn A-4	0.069	-0.054	-0.120	0.011	0.2513
	Penncross	0.057	-0.042	-0.104	0.020	0.4035
04/01/01	ASR368	0.091				
	Backspin	0.086	0.005	-0.090	0.101	1.0000
	Crenshaw	0.264	-0.173	-0.296	-0.050	*0.0141
	Penn A-4	0.222	-0.131	-0.250	-0.013	0.0586
	Penncross	0.157	-0.066	-0.176	0.044	0.3646
05/12/01	ASR368	0.076				
	Backspin	0.129	-0.053	-0.154	0.048	0.4669
	Crenshaw	0.278	-0.202	-0.324	-0.080	*0.0034
	Penn A-4	0.222	-0.146	-0.262	-0.031	*0.0286
	Penncross	0.229	-0.153	-0.270	-0.036	*0.0237
06/07/01	ASR368	0.167				
	Backspin	0.128	0.039	-0.092	0.170	0.7689
	Crenshaw	0.250	-0.083	-0.218	0.051	0.3217
	Penn A-4	0.236	-0.069	-0.203	0.064	0.4237
	Penncross	0.186	-0.019	-0.147	0.109	0.9485
07/04/01	ASR368	0.076				
	Backspin	0.157	-0.081	-0.188	0.025	0.2268
	Crenshaw	0.236	-0.160	-0.277	-0.043	*0.0173
	Penn A-4	0.208	-0.133	-0.246	-0.019	*0.0462
	Penncross	0.171	-0.096	-0.205	0.013	0.1516
08/07/01	ASR368	0.068				
	Backspin	0.157	-0.089	-0.202	0.024	0.2623
	Crenshaw	0.236	-0.168	-0.291	-0.045	*0.0319
	Penn A-4	0.229	-0.161	-0.301	-0.021	0.0594
	Penncross	0.213	-0.145	-0.283	-0.006	0.0913
09/07/01	ASR368	0.045				
	Backspin	0.143	-0.097	-0.200	0.005	0.1742
	Crenshaw	0.292	-0.246	-0.368	-0.125	*0.0014
	Penn A-4	0.229	-0.184	-0.318	-0.050	*0.0221
	Penncross	0.143	-0.097	-0.200	0.005	0.1742

[1] Difference between ASR368 survival and conventional cultivar survival

[2] 95% Confidence Interval of difference

* Significant difference detected between specified commercial cultivar and ASR368 R1 seed based on Fishers exact test (α =0.05)

Table VIII-5. Fisher's Exact Test comparison for survivability of ASR368 R1 seed and seedlings with several conventional creeping bentgrass cultivars planted on bare soil during spring 2001 in Marion County, Oregon

Obs. Date	Genotype	Survivability	Difference[1]	95% Conf. Int.[2]		P-value
04/01/01	ASR368	0.000				
	Backspin	0.000	0.000	0.000	0.000	1.0000
	Crenshaw	0.000	0.000	0.000	0.000	1.0000
	Penn A-4	0.014	-0.014	-0.041	0.013	1.0000
	Penncross	0.000	0.000	0.000	0.000	1.0000
05/12/01	ASR368	0.000				
	Backspin	0.000	0.000	0.000	0.000	1.0000
	Crenshaw	0.000	0.000	0.000	0.000	1.0000
	Penn A-4	0.042	-0.042	-0.088	0.004	0.2783
	Penncross	0.057	-0.057	-0.112	-0.003	0.1345
06/07/01	ASR368	0.000				
	Backspin	0.000	0.000	0.000	0.000	1.0000
	Crenshaw	0.042	-0.042	-0.098	0.015	0.3503
	Penn A-4	0.056	-0.056	-0.108	-0.003	0.1423
	Penncross	0.071	-0.071	-0.132	-0.011	0.0673
07/04/01	ASR368	0.045				
	Backspin	0.086	-0.040	-0.130	0.050	0.6768
	Crenshaw	0.028	0.018	-0.055	0.090	0.9797
	Penn A-4	0.056	-0.010	-0.091	0.071	1.0000
	Penncross	0.043	0.003	-0.075	0.080	1.0000
08/07/01	ASR368	0.023				
	Backspin	0.043	-0.020	-0.085	0.045	0.9967
	Crenshaw	0.042	-0.019	-0.091	0.053	1.0000
	Penn A-4	0.014	0.009	-0.043	0.061	1.0000
	Penncross	0.057	-0.034	-0.104	0.036	0.7145
09/07/01	ASR368	0.045				
	Backspin	0.043	0.003	-0.075	0.080	1.0000
	Crenshaw	0.000	0.045	-0.016	0.107	0.4520
	Penn A-4	0.014	0.032	-0.036	0.099	0.6420
	Penncross	0.014	0.031	-0.036	0.099	0.6609

[1] Difference between ASR368 survival and conventional cultivar survival

[2] 95% Confidence Interval of difference

VIII.A.1.2.4. Bare soil seedling establishment - Massachusetts

No significant difference among genotypes in the number of germinated seedlings was observed on either the October 5 or 12, 2000 observation dates (LSD, $\alpha = 0.05$; Table VIII-6). Although the study was continued past October 12, the winter of 2000/2001 was considered one of the worst with regard to overall cold injury of bentgrass at virtually all of the golf courses in the New England region (McCabe, 2001). Ice formation occurred in late December and remained until early April 2001. Germination and establishment ratings were resumed at this time but due to the severity of the weather, none of the

seedlings established in the fall had survived. Germination and establishment ratings continued through September 2001 but no latent germination was noted for any genotype treatment. These results demonstrate that ASR368 R1 seed are no more likely to germinate, establish or persist under severe climatic conditions than the conventional cultivars.

Table VIII-6. The average number of germinated seedlings at the first and second leaf stage on two October 2000 dates following the planting of ASR368 R0 and conventional cultivars on bare soil during September 2000 in Franklin County, Massachusetts[1]

Seed Line	# of Germinated Plants		# in First Leaf		# in Second Leaf	
	10/5	10/12	10/5	10/12	10/5	10/12
ASR368	12.0	16.5	1.7	12.2	0.0	0.0
Penncross	11.3	13.3	1.2	10.0	0.0	0.7
Penn A-4	11.8	14.0	1.7	11.2	0.0	0.2
Crenshaw	10.5	12.3	0.8	9.2	0.0	0.0
Backspin	13.8	14.8	1.2	10.0	0.0	0.0
LSD ($\alpha = 0.05$)	6.51	6.79	1.88	6.15	0.17	0.89

[1] Number of seedlings of 25 seed planted

VIII.A.1.2.5. Seed establishment in a competitive environment

Despite adequate rainfall, no seed of ASR368 R1 or the conventional cultivars were observed to establish in any competitive plot throughout the 12-month duration of the studies in Marion County, Oregon or Franklin County, Massachusetts as illustrated in Tables VIII-7 through VIII-9, respectively. These results reflect the general inability of creeping bentgrass plants to fully establish from seed and persist in competitive environments regardless if adequate moisture is present, as discussed in Section II.E.2 of this petition.

Table VIII-7. Number of surviving ASR368 R1 and conventional cultivar plants observed in 2001 when sown from seed into a mature unmanaged sward of hard fescue during fall 2000 in Marion County, Oregon

	Number of surviving seedlings							
	Date							
Seed Line	**10/1/00 - 2/2/01**	**3/12/01**	**4/1/01**	**5/12/01**	**6/7/01**	**7/4/01**	**8/7/01**	**9/7/01**
ASR368	0	0	0	0	0	0	0	0
Backspin	0	0	0	0	0	0	0	0
Crenshaw	0	0	0	0	0	0	0	0
Penn A-4	0	0	0	0	0	0	0	0
Penncross	0	0	0	0	0	0	0	0

Table VIII-8. Number of surviving ASR368 R1 and conventional cultivar plants observed in 2001 when sown from seed into a mature unmanaged sward of hard fescue during spring 2001 in Marion County, Oregon

	Number of surviving seedlings					
	Date					
Seed Line	**4/1/01**	**5/12/01**	**6/7/01**	**7/4/01**	**8/7/01**	**9/7/01**
ASR368	0	0	0	0	0	0
Backspin	0	0	0	0	0	0
Crenshaw	0	0	0	0	0	0
Penn A-4	0	0	0	0	0	0
Penncross	0	0	0	0	0	0

Table VIII-9. Number of surviving ASR368 R1 and conventional cultivar plants observed in 2001 when sown from seed into a mature sward of Kentucky bluegrass and fine fescue during fall 2000 in Franklin County, Massachusetts*

	Number of surviving seedlings						
	Date						
Seed Line	**10/1/00 – 2/2/01**	**4/5/01**	**5/12/01**	**6/10/01**	**7/6/01**	**8/16/01**	**9/15/01**
ASR368	0	0	0	0	0	0	0
Backspin	0	0	0	0	0	0	0
Crenshaw	0	0	0	0	0	0	0
Penn A-4	0	0	0	0	0	0	0
Penncross	0	0	0	0	0	0	0

* Plots inaccessible for observation during November, 2000 through March 2001 due to snow and ice cover.

VIII.A.2. Marion County, Oregon (2001 – 2002)

VIII.A.2.1. Experimental methods

VIII.A.2.1.1. Plant Material

Seed of the same five creeping bentgrass genotypes i.e., ASR368 R1, Backspin, Crenshaw, Penncross, and Penn A-4 were used in the establishment of studies conducted in Marion County, Oregon in 2001 - 2002. The percentage germination of each seed lot was determined using the standard AOSA seed germination test (AOSA 1998). The germination percentage for each seed lot is provided in Table VIII-10.

VIII.A.2.1.2. Field Plantings

Two plantings were made in Marion County, Oregon. A fall planting was made on September 27, 2001 and a spring planting was made on April 12, 2002. These plantings were chosen to evaluate seed establishment under four distinct environments i.e., irrigated and non-irrigated conditions in both competitive and non-competitive environments. The irrigated plots received at least one inch of water, total, either through supplemental irrigation and/or rainfall each week for eight weeks following planting. The non-irrigated plots received only natural rainfall.

VIII.A.2.1.3. Experimental design

Four different environments were employed: (1) an irrigated, non-competitive bare soil test plot void of vegetation, (2) non-irrigated, non-competitive bare soil test plot void of vegetation, (3) an irrigated vegetated and competitive plot in an open, but mature plant canopy (4-6 inches tall) of ‘Brigade’ hard fescue, (4) a non-irrigated vegetated and competitive plot in an open, but mature plant canopy (4-6 inches tall) of ‘Brigade’ hard fescue. Plots were prepared prior to planting and managed post-planting as previously described for the 2000 – 2001 studies in Section VIII.A.1.1.

The studies were arranged in a randomized complete block design with three replications. Each plot was 1 m × 1 m square. A 30 cm × 30 cm square was centered within each plot as the test area. This 30 cm × 30 cm square subsequently received the seed drop. A seed count of 100/plot was used for all genotypes. A total of 100 seed were added to each plot in a random pattern within the 30 cm × 30 cm square. The seed was dropped onto the surface of the plots without incorporation or pressing into the soil to simulate natural seed dissemination conditions.

Data collection

Seedling plant counts per plot were recorded monthly throughout the duration of the study. A rank of plant size was based on estimated tiller counts for the fall planting for the months of May, June, July, September and October. All seedlings and plants surviving the summer season were tested with a non-destructive immuno-assay strip test (Strategic Diagnostics Inc, Newark, DE) to determine the ratio of GT:GS on September 26, 2002. Plant diameter data was collected from all plots on September 27, 2002.

The total number of potential seedlings per plot was calculated by multiplying the germination percentage determined for each seed line by the number of seed dropped (Table VIII-10). Survivability was then calculated as the number of seedlings established divided by the total number of potential seedlings. Data on the number of plants that established and persisted were recorded.

Table VIII-10. GT to GS ratio, percent germination and expected seedlings per plot of transgenic and conventional creeping bentgrass cultivars used in the establishment and persistence study in Marion County, Oregon

Genotype	GT : GS	Percent Germination	Expected Seedlings Per Plot[1]	
			GT+GS	GT
ASR 368 R1	49:51	88	88	43
Backspin	0:100	96	96	0
Penncross	0:100	94	94	0
Penn A-4	0:100	96	96	0
Crenshaw	0:100	93	93	0

[1] Calculated by: percent germination × 100 seed added to plot.

VIII.A.2.2. Results of 2001 – 2002 seed establishment studies

VIII.A.2.2.1. Bare soil seedling establishment – Oregon

VIII.A.2.2.2. Fall Planting 2001

Seed establishment in the fall 2001 irrigated study ranged from ca. 1% to a maximum of 8% (Penn A-4) over the twelve months in which observations were made. The rate of ASR368 R1 seed establishment tended to be less than but not significantly different from the conventional cultivars other than that of Penn A-4, which consistently established at a significantly greater rate (Table VIII-11). Maximum survivability of any genotype at the end of the study in October 2002 was < 7% under these optimized conditions.

Percent establishment in the non-irrigated study ranged from less than 0.1% to ca. 10% for the genotypes tested (Table VIII-12). ASR368 R1 seed establishment was not significantly different from Backspin throughout the entire study and to the other commercial cultivars for the first five months in which observations were taken (November 2001 to March 2002). Seed establishment of ASR368 R1 was significantly greater than Crenshaw in April through October, Penn A-4 in June through September and Penncross in July (Table VIII-12). Despite these transient differences, seed establishment was variable and low for each of the genotypes evaluated.

VIII.A.2.2.3. Spring Planting 2002

Seed establishment for the Spring 2002 irrigated and non-irrigated plantings was essentially zero (< 0.005%) for each of the genotypes tested, which precluded the performance of a statistical analysis. These results help to demonstrate the difficulty

creeping bentgrass seed has establishing in bare soil regardless of whether adequate moisture is present[8].

VIII.A.2.2.4. Competitive establishment - Oregon

No seed of any genotype established in the fall or spring, irrigated or non-irrigated competitive plantings. These results are consistent with those of the 2000/2001 studies and the published scientific literature. Therefore, it is concluded that the ability of ASR368 seed to establish in an existing turf sward is not different from conventional creeping bentgrass cultivars representative of *A. stolonifera*.

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[8] Supplementary irrigation was only provided during the initial eight-week establishment period when up to 1" of water was provided from a combination of natural precipitation and supplementary irrigation (except for a soaking irrigation event near the end of the study to revive plants for final counts). The objective of the early maintenance was to provide a more optimized condition for bentgrass emergence in spring before the typical dry summer season started. Germination and establishment of bentgrass is relatively slow compared to other grass species and bentgrass seedlings are inconspicuous and may not be distinguishable from other grassy weeds until about 8-10 weeks after emergence. The natural conditions of the Willamette Valley during summer were not conducive to further germination and survival of bentgrass seed.

Table VIII-11. Fisher's Exact Test comparison for survivability of ASR368 R1 seed and seedlings with several conventional creeping bentgrass cultivars planted on bare soil and irrigated during fall 2001 in Marion County, Oregon

Obs. Date	Genotype	Survivability	Difference[1]	95% Conf. Int.[2]		P-value
11/07/01	ASR368	0.015				
	Backspin	0.031	-0.016	-0.041	0.009	0.267
	Crenshaw	0.065	-0.049	-0.082	-0.017	0.004*
	Penn A-4	0.142	-0.127	-0.170	-0.084	<0.0001*
	Penncross	0.050	-0.034	-0.064	-0.005	0.030*
11/20/01	ASR368	0.072				
	Backspin	0.104	-0.032	-0.079	0.015	0.231
	Crenshaw	0.082	-0.010	-0.055	0.034	0.748
	Penn A-4	0.240	-0.168	-0.226	-0.109	<0.0001*
	Penncross	0.057	0.015	-0.026	0.056	0.489
11/26/01	ASR368	0.114				
	Backspin	0.153	-0.039	-0.096	0.017	0.211
	Crenshaw	0.125	-0.012	-0.066	0.043	0.694
	Penn A-4	0.243	-0.129	-0.192	-0.067	<0.0001*
	Penncross	0.121	-0.007	-0.061	0.047	0.894
12/17/01	ASR368	0.152				
	Backspin	0.167	-0.015	-0.076	0.046	0.643
	Crenshaw	0.143	0.008	-0.052	0.068	0.810
	Penn A-4	0.274	-0.123	-0.190	-0.056	0.001*
	Penncross	0.142	0.010	-0.050	0.069	0.809
12/27/01	ASR368	0.140				
	Backspin	0.181	-0.040	-0.101	0.021	0.205
	Crenshaw	0.140	0.000	-0.058	0.059	1.0000
	Penn A-4	0.264	-0.124	-0.190	-0.058	0.0003*
	Penncross	0.145	-0.005	-0.064	0.053	0.903
01/09/02	ASR368	0.167				
	Backspin	0.201	-0.035	-0.099	0.030	0.324
	Crenshaw	0.154	0.013	-0.049	0.074	0.726
	Penn A-4	0.278	-0.111	-0.180	-0.043	0.002*
	Penncross	0.152	0.014	-0.047	0.076	0.726
02/27/02	ASR368	0.155				
	Backspin	0.080	0.075	0.022	0.129	0.007*
	Crenshaw	0.108	0.048	-0.009	0.105	0.126
	Penn A-4	0.253	-0.098	-0.165	-0.032	0.004*
	Penncross	0.138	0.017	-0.042	0.076	0.629

[1] Difference between ASR368 survival and conventional cultivar survival

[2] 95% Confidence Interval of difference

* Significant difference detected between survivability of commercial cultivar and ASR368 R1 seed based on Fishers exact test (α =0.05)

Table VIII-11. Fisher's Exact Test comparison for survivability of ASR368 R1 seed and seedlings with several conventional creeping bentgrass cultivars planted on bare soil and irrigated during fall 2001 in Marion County, Oregon (continued)

Obs. Date	Genotype	Survivability	Difference[1]	95% Conf. Int.[2]		P-value
03/28/02	ASR368	0.155				
	Backspin	0.076	0.079	0.026	0.132	0.005*
	Crenshaw	0.104	0.051	-0.005	0.108	0.095
	Penn A-4	0.253	-0.098	-0.165	-0.032	0.004*
	Penncross	0.135	0.021	-0.039	0.080	0.543
04/24/02	ASR368	0.155				
	Backspin	0.083	0.072	0.018	0.126	0.012
	Crenshaw	0.108	0.048	-0.009	0.105	0.126
	Penn A-4	0.250	-0.095	-0.161	-0.028	0.998
	Penncross	0.131	0.024	-0.035	0.083	0.464
05/24/02	ASR368	0.144				
	Backspin	0.080	0.064	0.011	0.117	0.020*
	Crenshaw	0.108	0.036	-0.019	0.092	0.2430
	Penn A-4	0.247	-0.103	-0.168	-0.037	0.003*
	Penncross	0.124	0.020	-0.037	0.077	0.531
06/24/02	ASR368	0.140				
	Backspin	0.094	0.046	-0.007	0.100	0.110
	Crenshaw	0.104	0.036	-0.019	0.091	0.237
	Penn A-4	0.247	-0.106	-0.171	-0.041	0.002*
	Penncross	0.121	0.020	-0.037	0.076	0.526
07/25/02	ASR368	0.140				
	Backspin	0.087	0.053	0.000	0.106	0.058
	Crenshaw	0.097	0.043	-0.011	0.098	0.143
	Penn A-4	0.243	-0.103	-0.168	-0.038	0.002*
	Penncross	0.128	0.012	-0.045	0.070	0.707
09/04/02	ASR368	0.061				
	Backspin	0.073	-0.012	-0.054	0.029	0.612
	Crenshaw	0.057	0.003	-0.036	0.043	1.0000
	Penn A-4	0.108	-0.047	-0.093	-0.001	0.066*
	Penncross	0.078	-0.017	-0.060	0.025	0.502
10/11/02	ASR368	0.068				
	Backspin	0.069	-0.001	-0.044	0.041	1.0000
	Crenshaw	0.039	0.029	-0.009	0.067	0.051
	Penn A-4	0.069	-0.001	-0.044	0.041	0.133
	Penncross	0.060	0.008	-0.033	0.049	0.730

[1] Difference between ASR368 survival and conventional cultivar survival

[2] 95% Confidence Interval of difference

* Significant difference detected between survivability of commercial cultivar and ASR368 R1 seed based on Fishers exact test (α =0.05)

Table VIII-12. Fisher's Exact Test comparison for survivability of ASR368 R1 seed and seedlings with several conventional creeping bentgrass cultivars planted on bare soil and non-irrigated during fall 2001 in Marion County, Oregon

Obs. Date	Genotype	Survivability	Difference[1]	95% Conf. Int.[2]		P-value
11/07/01	ASR368	0.008				
	Backspin	0.003	0.004	-0.008	0.017	0.609
	Crenshaw	0.004	0.004	-0.009	0.017	0.614
	Penn A-4	0.010	-0.003	-0.019	0.013	1.000
	Penncross	0.014	-0.007	-0.024	0.011	0.687
11/20/01	ASR368	0.019				
	Backspin	0.014	0.005	-0.016	0.026	0.743
	Crenshaw	0.025	-0.006	-0.031	0.018	0.773
	Penn A-4	0.042	-0.023	-0.051	0.006	0.144
	Penncross	0.028	-0.009	-0.035	0.016	0.579
11/26/01	ASR368	0.080				
	Backspin	0.052	0.027	-0.014	0.069	0.228
	Crenshaw	0.068	0.011	-0.033	0.055	0.626
	Penn A-4	0.083	-0.004	-0.049	0.042	0.878
	Penncross	0.067	0.012	-0.032	0.056	0.625
12/17/01	ASR368	0.080				
	Backspin	0.056	0.024	-0.018	0.066	0.308
	Crenshaw	0.065	0.015	-0.029	0.059	0.511
	Penn A-4	0.063	0.017	-0.026	0.060	0.507
	Penncross	0.074	0.005	-0.040	0.050	0.873
12/27/01	ASR368	0.095				
	Backspin	0.052	0.043	-0.001	0.086	0.070
	Crenshaw	0.061	0.034	-0.011	0.079	0.949
	Penn A-4	0.052	0.043	-0.001	0.086	0.982
	Penncross	0.060	0.034	-0.011	0.079	0.953
01/09/02	ASR368	0.087				
	Backspin	0.063	0.025	-0.019	0.069	0.330
	Crenshaw	0.072	0.015	-0.030	0.061	0.529
	Penn A-4	0.059	0.028	-0.015	0.072	0.250
	Penncross	0.064	0.023	-0.021	0.068	0.332
02/27/02	ASR368	0.061				
	Backspin	0.049	0.012	-0.026	0.050	0.577
	Crenshaw	0.029	0.032	-0.003	0.067	0.094
	Penn A-4	0.056	0.005	-0.034	0.044	0.856
	Penncross	0.053	0.007	-0.032	0.046	0.716

[1] Difference between ASR368 survival and conventional cultivar survival

[2] 95% Confidence Interval of difference

Table VIII-12. Fisher's Exact Test comparison for survivability of ASR368 R1 seed and seedlings with several conventional creeping bentgrass cultivars planted on bare soil and non-irrigated during fall 2001 in Marion County, Oregon (continued)

Obs. Date	Genotype	Survivability	Difference[1]	95% Conf. Int.[2]		P-value
03/28/02	ASR368	0.061				
	Backspin	0.049	0.012	-0.026	0.050	0.577
	Crenshaw	0.025	0.036	0.001	0.070	0.054
	Penn A-4	0.052	0.009	-0.030	0.047	0.714
	Penncross	0.050	0.011	-0.027	0.049	0.580
04/24/02	ASR368	0.072				
	Backspin	0.066	0.006	-0.036	0.048	0.867
	Crenshaw	0.025	0.047	0.011	0.083	0.015*
	Penn A-4	0.052	0.020	-0.020	0.060	0.378
	Penncross	0.039	0.033	-0.006	0.071	0.132
05/24/02	ASR368	0.083				
	Backspin	0.056	0.028	-0.015	0.070	0.239
	Crenshaw	0.025	0.058	0.020	0.096	0.004*
	Penn A-4	0.045	0.038	-0.003	0.079	0.080
	Penncross	0.046	0.037	-0.004	0.079	0.083
06/24/02	ASR368	0.091				
	Backspin	0.069	0.021	-0.024	0.067	0.432
	Crenshaw	0.018	0.073	0.035	0.111	0.0002*
	Penn A-4	0.042	0.049	0.008	0.091	0.024*
	Penncross	0.043	0.048	0.006	0.090	0.025
07/25/02	ASR368	0.098				
	Backspin	0.059	0.039	-0.006	0.085	0.111
	Crenshaw	0.025	0.073	0.033	0.114	0.0005*
	Penn A-4	0.024	0.074	0.034	0.114	0.0002*
	Penncross	0.043	0.056	0.013	0.099	0.012*
09/04/02	ASR368	0.057				
	Backspin	0.035	0.022	-0.013	0.057	0.226
	Crenshaw	0.004	0.053	0.024	0.082	0.0002*
	Penn A-4	0.021	0.036	0.004	0.068	0.043*
	Penncross	0.028	0.028	-0.006	0.062	0.135
10/11/02	ASR368	0.049				
	Backspin	0.042	0.008	-0.027	0.042	0.688
	Crenshaw	0.004	0.046	0.019	0.073	0.001*
	Penn A-4	0.028	0.021	-0.011	0.054	0.265
	Penncross	0.032	0.017	-0.016	0.051	0.385

[1] Difference between ASR368 survival and conventional cultivar survival

[2] 95% Confidence Interval of difference

* Significant difference detected between survivability of commercial cultivar and ASR368 R1 seed based on Fishers exact test (α =0.05)

VIII.A.3. Conclusion for seed establishment studies

The percent germination for each of the seed lots used in these experiments ranged from 88% for the ASR368 R1 to 96% for the Penn A-4 and Backspin conventional varieties. These germination rates exceed the minimal germination threshold of 85% for Certified quality bentgrass seed production (Oregon State University, 2001). Despite this expected germination rate, establishment and field survival of all the creeping bentgrass cultivars seeded in either bare soil or existing vegetative stands was generally low, never exceeding 30%. ASR368 R1 seed establishment was significantly less than Penn A-4 in both the Marion County, Oregon bare soil fall 2000 study and fall 2001 irrigated study. In comparison to the other conventional cultivars, ASR368 R1 seed establishment tended to fall within the range of the other conventional cultivars in both the Oregon and Massachusetts bare soil studies. None of the genotypes were able to establish when seeded into existing turf.

As discussed in Section II.E.2, successful seedling establishment can be limited in existing turf swards due to either insufficient disturbance or excessive competition from the existing turfgrass population. Howe and Snaydon (1986); Jonsdottir (1991) and Bullock et al. (1994) noted that seedling recruitment into natural stands and pastures, respectively, was unsuccessful in augmenting *A. stolonifera* populations. Sweeney and Danneberger (1998) and Kendrick and Danneberger (2002) were also unable to demonstrate with molecular markers the success from direct inter-seeding *A. stolonifera* into an existing *A. stolonifera* stand utilized for golf putting green turf. The difficulty in establishment of *Agrostis* seedlings may be a consequence of the extremely small seed (0.07 mg seed^{-1}) size since small seed require a long duration of disturbance (reduced competition) and low stress for establishment and survival after germination (Cattani and Struik, 2001). Small seed may also preclude direct contact with soil in an existing vegetative stand.

The main conclusion from these seed establishment studies is that ASR368 is similar to conventional cultivars. However, the results provide further evidence that creeping bentgrass requires more optimum moisture conditions than what were provided artificially in order to establish and persist in the Willamette Valley. Bentgrass can be established in the Willamette Valley on golf courses and seed fields; however, it is unlikely to be very successful without adequate moisture for an extended timeframe. Golf courses may irrigate new greens five or more times each day in order to optimize germination and establishment. Seed testing provides for optimum moisture and temperature conditions in order to determine maximum germination potential of a seed lot. Natural conditions in unmanaged ecosystems infrequently provide optimum conditions for the extended timeframe necessary for *Agrostis stolonifera* establishment and persistence.

Consequently, given the results from these experiments, which further confirm reports in the scientific literature, seed of ASR368 would not be expected to germinate, establish or persist in unmanaged competitive and non-competitive ecosystems differently from conventional creeping bentgrasses. The results also support an overall conclusion that ASR368 is not different than conventional creeping bentgrass in terms of plant pest or weediness characteristics and is no more likely to pose a plant pest risk than conventional creeping bentgrass.

VIII.B. Vegetative Establishment

Creeping bentgrass can reproduce vegetatively through the production of stolons. Stolons are true lateral stems that contain nodes and internodes. The meristematic tissue present at the nodes can initiate new independent plants when they are disseminated into favorable conditions, which include intimate contact with soils, available moisture and low competition for resources. An enhanced ability to vegetatively establish could increase the persistence and plant pest potential of ASR368. This section compares the establishment ability of detached stolon sections containing nodes from plants of ASR368 R1, F1 and/or F2 progeny, ASR368 glyphosate susceptible segregants (GS) and several commercial cultivars in several field environments and under controlled greenhouse or poly-house conditions.

Two concurrent studies were conducted in 2001 - 2002 to compare vegetative stolon propagation of ASR368 F1 and F2 plants with that of several different creeping bentgrass conventional cultivars. Experiment I was conducted in a growth chamber in Fayette County, Kentucky and Experiment II was conducted in a poly-house in Marion County, Oregon. Experiment II was not a "follow-up experiment" to Experiment I. Experiments I and II (Tables VIII-13 and VIII-14) in this section were initiated as concurrent experiments. Repetitions of Experiment I were initiated on December 12, 19 and 28, 2001 and repetitions of Experiment II were initiated on December 11, 2001, January 3 and February 7, 2002. As these experiments were run independently of each other, Experiment II was not influenced in any way by the results of Experiment I.

Additional studies were performed during 2002 - 2003 to assess the ability of ASR368 F1 and F2 progeny and conventional cultivars to vegetatively establish under field conditions in Marion County, Oregon; Union County, Ohio; Baldwin County, Alabama and in the greenhouse in Fayette County, Kentucky. At each site the study was performed under irrigated and non-irrigated conditions. These trials were performed under USDA Notification Number 02-193-04n.

VIII.B.1. Growth chamber and poly-house vegetative establishment studies (2001 - 2002)

VIII.B.1.1. Experimental methods

VIII.B.1.1.1. Experiment I – Kentucky Growth Chamber

Six genotypes were evaluated in this study: ASR368 R1 (GT) and ASR368 (GS) and the conventional cultivars Penn A-4, Penncross, Crenshaw, and SR-1020. Six plants of each genotype were grown in pots in a poly-house in Marion County, Oregon and then shipped to Fayette County, Kentucky where they were acclimated in a greenhouse prior to stolon harvest for use in the study. Viable stolons were harvested from each pot based on a visual assessment of good health and the presence of two nodes within a length of 2.5 cm. Only nodes that had not yet initiated tiller production were chosen, and each stolon fragment contained exactly two nodes. Four stolon fragments (eight nodes) were clipped from each pot and trimmed to the correct length (approximately 2.5 cm) with scissors. All extraneous tissues were removed. Fragments were placed in Petri dishes (100 × 15 mm), which had been filled with approximately 50 g of soil. Stolons were pressed lightly

by hand to ensure good stolon/soil contact. Dishes were then irrigated to field capacity using a wash bottle to gently apply water and not disturb stolon placement. The experiment was initiated on December 12, 19, and 28, 2001, constituting three complete runs of the experiment, each consisting of seven days.

Dishes with stolons were placed uncovered in a plant growth chamber (Conviron Model E7, Controlled Environments, Ltd., Winnipeg, Manitoba, Canada). The environment within the chamber simulated spring/fall conditions in the transitional climatic zone. Total day length was 14 hours. Daytime high temperatures were 23°C and nighttime low temperatures were 10°C. Relative humidity was maintained as high as possible (always > 95%) to retard drying of soil and stolons in dishes. Dishes were irrigated daily to field capacity with a wash bottle. Dishes remained in the chamber for seven days during each run. At the end of the seventh day, each individual node was evaluated for initiation of new tillers.

The experimental design was completely randomized with six replications. Each Petri dish represented one replication. The stolons within a single dish were harvested from one pot or plant. Tiller counts per dish were recorded (maximum = 8, minimum = 0) at the completion of each seven-day run per planting date.

VIII.B.1.1.2. Experiment II: Oregon poly-house

Four genotypes were evaluated in this study: two independent ASR368 F2 progeny populations, ASR368a[9] and ASR368b, and the conventional cultivars Penneagle and Backspin. Each ASR368 F2 population had a different conventional maternal parent that had been pollinated by a random population of ASR368 F1 (Figure V-17). ASR368 progeny were each hemizygous for the *cp4 epsps* transgene. Each population or cultivar was represented by 50 plants.

Plants were maintained in a poly-house environment in Marion County, Oregon prior to stolon harvest. Stolons were selected and harvested at random from the population of respective plants on December 11, 2001, January 3 and February 7, 2002. The harvested stolons contained the terminal bud through the second node. The terminal bud with the first node subtending the apex was removed with a scissors from each stolon fragment and discarded. Each stolon fragment was trimmed with a scissors to 0.65 cm above and

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[9] ASR368a and ASR368b stolons were randomly harvested from 50 progeny plant populations (50 different genotypes), each derived from seed harvested from a topcross of ASR368 Fl progeny to two different maternal Elite Parent Plants, EPPs. The two EPP plants are believed to trace from two different conventional cultivars. EPPs VI5-2-5 and V4-1-6 are the conventional maternal parent plants for populations A and B, respectively. EPP 15-2-5 traces to a single plant segregant from cv. Pennlinks. EPP V4-1-6 traces to a single plant segregant from cv. Penneagle. EPP plants are likely to be highly heterozygous plants that segregate for multiple characteristics when crossed to other bentgrass genotypes. EPP lines were selected for crosses with ASR368 based upon good turf performance in progeny turf evaluations during 1998 - 2000. EPP V15-2-5 and V4-1-6 progeny (F2 ASR368) were harvested after being pollinated by a population of Fl ASR368 progeny (heterogeneous and heterozygous population hemizygous for the *cp4 epsps* gene derived from crosses of ASR368 RO with four EPPs). GT progeny used as a source of stolon nodes were initially harvested as seed from the EPP plants and then selected for glyphosate tolerance by spraying the segregating seedling populations twice with Roundup herbicide to identify and confirm plants derived from event ASR368 (GT).

below the second node. The stolon containing the second node was then placed directly into 14 cm diameter pots containing a washed sand media that had been leveled, firmed and placed at a 10 cm depth. Each stolon fragment was planted in a vertical/upright position with the node just below the sand surface.

The experimental design was a randomized complete block with three replications at each planting date. Each experimental plot consisted of three pots containing 20 nodes each for a total of 60 nodes. All nodes were planted in sequence one replication at a time. All containers were placed on four thermostatically controlled heating mats in an enclosed poly-house. The media temperature was maintained at 16°C and monitored with soil thermometers. Each container received 20 to 25 mm of water daily via a gentle water curtain to prevent moisture stress. Day length was natural. Each of the three replications of the study was evaluated at seven days after planting for number of nodes producing tillers (maximum = 60, minimum = 0).

VIII.B.1.1.3. Statistical analysis

Data from both experiments were transformed to percent of nodes producing at least one tiller at the end of the growth periods. Production of multiple tillers from single nodes was rare and not evaluated. Statistical analyses were conducted using PROC GLM of SAS (SAS Institute, Cary, NC). Means were separated by F-protected LSD tests (α = 0.05) after determining replication × genotype interactions did not exist. Orthogonal comparisons were used to compare ASR368 populations with non-transgenic populations in each experiment.

VIII.B.1.2. Results for experiments I and II

Nodes from ASR368 R1 GT progeny produced significantly more tillers than ASR368 GS progeny within seven days in Experiment I. However, the percentage of nodes producing tillers among ASR368 GT progeny was not significantly different from three of the four conventional creeping bentgrass cultivars including Penn A-4, Penncross and SR1020. The statistical comparisons for all three experimental runs combined are presented in Table VIII-13.

The statistical comparisons for all three combined experimental runs of Experiment II are presented in Table VIII-14. No significant differences (α = 0.05) for the percentage of nodes producing tillers were noted between the ASR368 genotypes or commercial cultivars tested.

ASR368 establishment was not significantly different from the commercial cultivars Penn A-4, Penncross and SR1020 in Experiment I and is, therefore, unlikely to demonstrate any additional competitive ability compared to conventional cultivars. The results of Experiment I were confirmed in Experiment II during 2001 — 2002, although with different comparators representative of the conventional *Agrostis stolonifera.*

Table VIII-13. Comparison of the mean percent of nodes producing tillers of ASR368 GT and GS genotypes and four creeping bentgrass conventional cultivars after a seven-day growth period during Experiment I

Genotype	Mean % of nodes producing tillers[1]
ASR368 (GT)	57.6a
Penn A-4	51.4ab
Penncross	44.4abc
SR-1020	41.7abc
ASR368 (GS)	37.5bc
Crenshaw	31.9c
LSD (α =0.05)	16.3

[1] Means followed by the same letter are not significantly different according to LSD (α = 0.05).

Table VIII-14. Comparison of the mean percent of nodes producing tillers of two F2 ASR368 GT populations and two creeping bentgrass conventional cultivars after a seven-day growth period during Experiment II

Genotype	Mean % of nodes producing tillers[1]
Backspin	55.7a
ASR368-A	54.3a
Penneagle	50.9a
ASR368-B	48.1a
LSD (α =0.05)	21.3

[1] Means followed by the same letter are not significantly different according to LSD (α = 0.05).

VIII.B.2. Vegetative establishment studies (2002 - 2003)

Field studies were established in the fall of 2002 in Marion County, Oregon; Union County, Ohio and Baldwin County, Alabama. An additional greenhouse study was also performed in Fayette County, Kentucky. The objectives of these studies were to: (1) determine if there was a difference in the ability of ASR368 to establish vegetatively compared to an elite parent plant (EPP) population and conventional creeping bentgrass cultivars under irrigated and non-irrigated field conditions and (2) supplement the results of the Kentucky greenhouse study performed in 2001, i.e. Experiment I. ASR368 F1 and F2 progeny populations and an Elite Parent Plant breeding line population plus three conventional creeping bentgrass cultivars were evaluated at each of the four locations as follows:

Backspin Alabama and Oregon
Crenshaw Alabama, Kentucky, Ohio and Oregon
Penn A-4 Alabama, Kentucky, Ohio and Oregon
Penncross Kentucky and Ohio

VIII.B.2.1. Experimental methods

Plant propagation and establishment

In the fall of 2001 plant material of each of the creeping bentgrass genotypes was potted and maintained in a poly-house in Marion County, Oregon. Prior to planting the Ohio and Kentucky trials in 2002, potted plants of each genotype population were sent to the Union County, Ohio location where they were maintained in a greenhouse for subsequent harvest and planting of both the Ohio and Kentucky sites.

The Oregon site maintained plant material for both the Oregon and Alabama sites. Excised stolon nodes were harvested late in the afternoon from each of the populations in Oregon for shipping via overnight early morning parcel to the Baldwin County, Alabama location for immediate planting upon receipt. Harvested stolon nodes were placed on saturated blotter paper within a Petri dish within another sealed bag to prevent desiccation prior to shipping and planting. The second node subtending the stolon apex with ½ cm of internode on either side was cut from the plants of each population for planting at each site.

Field and greenhouse planting

Prior to planting at the Alabama, Ohio and Oregon sites, on October 11, 7 and 8, 2002, respectively, the vegetation from a 45 cm × 45 cm square in an established turf area was removed. The soil was loosened to a ½" depth to simulate a seedbed. Within this area a 30 cm × 30 cm plot was delineated into which 30 stolon nodes were introduced. The nodes were spread so that they did not overlap and were pressed uniformly into the soil surface with a board for good soil contact. The plots were arranged in a randomized complete block design with three replications per genotype per irrigation regime.

The experiment was established in the Fayette County, Kentucky greenhouse on December 10, 2002 in a similar manner but using 6" pots as individual plots.

Irrigation

Every plot was irrigated with 1 cm of water on the day of planting. Thereafter the 'irrigated' plots were irrigated daily with 0.5 cm of water. Irrigated treatments were designed to mimic a golf course where irrigation might be applied each day or where consistent precipitation events are provided each day. The 'non-irrigated' plots received only natural precipitation or 0.5 cm per week total, which was intended to mimic the conditions found in unmanaged ecosystems at each location.

Data collected

The number of nodes with viable shoots in each plot was counted a minimum of two times each week for up to five weeks per location. Based on the four locations and two irrigation regimes provided at each location, potential for vegetative establishment of transgenic ASR368 (two generations) and conventional creeping bentgrasses were compared in eight different environments during this study.

Tables VIII-15 through VIII-22 all present data in the same format. The variation in the number of nodes establishing among sites is due to the differences in actual establishment as impacted by the use of irrigation to supplement natural precipitation versus trial locations with only natural precipitation and no supplemental irrigation.

Statistical analysis

Data from each location was analyzed using PROC GLM of SAS (SAS Institute, Cary, NC). Means were separated by F-protected LSD tests ($\alpha = 0.05$).

VIII.B.2.2. Results

Irrigated trials

The number of ASR368 F1 and F2 (GT) nodes producing viable tillers under irrigated conditions was not significantly different from the conventional cultivars on any observation date in either Baldwin County, Alabama or Fayette County, Kentucky (Tables VIII-15 and VIII-16).

Table VIII-15. Number of nodes out of 30 producing viable tillers under irrigated conditions among six genotype populations planted in Baldwin County, Alabama in October 2002

Genotype population		Observation date (2002)									
	N	10/14	10/16	10/18	10/21	10/23	10/25	10/28	10/30	11/1	11/4
		Number of nodes									
Backspin	3	11.0a	19.0a	18.7a	20.0a	19.0a	20.7a	21.7a	23.3a	23.0a	21.3a
Crenshaw	3	11.7a	16.7a	16.0a	16.7a	16.0a	18.0a	22.3a	22.0a	21.3a	20.0a
EPP	3	10.7a	15.0a	15.7a	17.0a	19.0a	20.3a	22.7a	24.0a	21.7a	20.7a
Penn A-4	3	12.7a	17.3a	17.7a	17.3a	20.0a	22.0a	25.3a	25.7a	24.3a	23.7a
ASR368 F1	3	10.0a	16.0a	15.7a	17.3a	17.0a	17.7a	20.3a	20.7a	20.3a	19.7a
ASR368 F2	3	7.0a	13.7a	13.3a	15.3a	20.3a	20.3a	21.7a	23.3a	23.0a	23.0a
LSD ($\alpha = 0.05$)[1]		7.70	7.12	6.78	6.53	6.67	4.82	5.45	4.66	4.59	5.29
Pr > F		0.681	0.653	0.613	0.741	0.668	0.380	0.497	0.325	0.478	0.497

[1] Means with the same letter during each observation date are not significantly different, $\alpha = 0.05$.

Table VIII-16. Number of nodes out of 30 producing viable tillers under irrigated conditions among six genotype populations planted in Fayette County, Kentucky in December 2002

Genotype Population	N	Observation date (2002)						2003
		12/16	12/18	12/20	12/23	12/25	12/27	1/3
		Number of nodes						
Penncross	3	20.7a	24.7a	26.0a	26.7a	27.7a	25.3a	26.3a
Crenshaw	3	20.3a	26.3a	29.0a	29.0a	29.3a	28.0a	32.7a
EPP	3	20.3a	21.3a	24.7a	25.7a	25.7a	28.3a	28.0a
Penn A-4	3	20.0a	24.0a	27.0a	27.7a	27.7a	26.3a	27.7a
ASR368 F1	3	18.3a	21.3a	26.3a	26.7a	28.3a	26.0a	30.7a
ASR368 F2	3	17.0a	23.3a	27.0a	27.3a	27.7a	26.0a	31.3a
LSD ($\alpha = 0.05$)[1]		8.57	7.47	4.96	5.07	4.17	4.71	7.71
Pr > F		0.919	0.668	0.578	0.790	0.579	0.682	0.474

[1] Means with the same letter during each observation date are not significantly different, $\alpha = 0.05$.

At the Union County, Ohio location, the ASR368 F2 produced significantly fewer tillers than Penn A-4 and EPP populations by October 15, 2002 but was not significantly different from any conventional populations on the remaining observation dates. The ASR368 F1 had significantly more stolon nodes than Crenshaw on October 18, 2002 but was not significantly different from the other conventional cultivars or the EPP population on that and all other observation dates in Union County, Ohio (Table VIII-17).

Table VIII-17. Number of nodes out of 30 producing viable tillers under irrigated conditions among six genotype populations planted in Union County, Ohio in October 2002

Genotype Population	N	Observation date (2002)					
		10/15	10/18	10/22	10/25	10/30	11/5
		Number of nodes					
Penncross	3	7.7bcd	9.7ab	10.7b	10.7b	12.7ab	12.0ab
Crenshaw	3	7.0cd	7.7b	9.0b	9.3b	9.7b	9.7b
EPP	3	11.0a	12.7a	14.7a	14.7a	14.7a	14.7a
Penn A-4	3	10.0ab	11.7a	12.7ab	12.7ab	13.0ab	13.0ab
ASR368 F1	3	9.3abc	11.3a	11.3ab	11.3ab	12.0ab	12.0ab
ASR368 F2	3	5.3d	9.7ab	11.7ab	12.0ab	13.0ab	12.7ab
LSD ($\alpha = 0.05$)[1]		2.77	3.63	3.68	3.45	4.10	4.42
Pr > F		0.010	0.109	0.088	0.080	0.247	0.319

[1] Means with the same letter during each observation date are not significantly different, $\alpha = 0.05$.

In Marion County, Oregon, Backspin had significantly more nodes than the other genotype populations including, ASR368 F1 and F2, from October 21 through October 31, 2002. ASR368 F1 had significantly more nodes than the EPPs on October 21 and 25,

2002 and ASR368 F2 had significantly fewer nodes than the EPPs on October 14, 2002 (Table VIII-18). However, these differences were not consistent and the ASR368 F1 and F2 fell within the range of the conventional cultivars on all dates.

Table VIII-18. Number of nodes out of 30 producing viable tillers under irrigated conditions among six genotype populations planted in Marion County, Oregon in October 2002

Genotype population	Observation date (2002)							
	N	10/11	10/14	10/18	10/21	10/25	10/28	10/31
	Number of nodes							
Backspin	3	0.0a	5.3a	16.7a	22.7a	22.3a	22.7a	22.7a
Crenshaw	3	0.0a	1.7c	8.0b	13.3cb	13.3bc	13.3b	13.3b
EPP	3	0.0a	4.7a	9.3b	11.3c	11.3c	11.3b	11.3b
Penn A-4	3	0.0a	2.3bc	9.0b	12.3bc	13.0bc	12.7b	12.7b
ASR368 F1	3	0.0a	3.7ab	12.3ab	15.7b	16.0b	15.7b	15.7b
ASR368 F2	3	0.0a	2.0bc	8.0b	13.3bc	13.3bc	12.7b	12.7b
LSD ($\alpha = 0.05$)[1]		0.00	1.96	4.79	4.24	3.90	4.55	4.55
Pr > F		na	0.008	0.015	0.002	0.001	0.003	0.003

[1] Means with the same letter during each observation date are not significantly different, $\alpha = 0.05$.

na not available, due to 0.0 counts neither a Pr > F or LSD value could be calculated

Non-irrigated trials

The number of ASR368 F1 and F2 nodes producing viable tillers under non-irrigated conditions was not significantly different from the conventional creeping bentgrass populations on all evaluation dates at all locations other than two instances (Tables VIII-20 – VIII-22). On October 18, 2002, in Baldwin County, Alabama ASR368 F1 had significantly more nodes than the EPPs and on October 30, 2002 ASR368 F2 had significantly fewer nodes than Crenshaw. It is clear from the results at the Kentucky, Ohio and Oregon locations that irrigation is necessary to supplement precipitation for the successful establishment of creeping bentgrass stolon nodes at these locations.

Table VIII-19. Number of nodes out of 30 producing viable tillers under non-irrigated conditions among six genotype populations planted in Baldwin County, Alabama in October 2002

Genotype Population	N	Observation date (2002)									
		10/14	10/16	10/18	10/21	10/23	10/25	10/28	10/30	11/1	11/4
		Number of nodes									
Backspin	3	9.0a	17.3a	17.0a	13.0a	14.3a	12.3a	14.7a	13.3a	12.7a	11.7a
Crenshaw	3	6.3ab	16.7a	16.7ab	13.0a	13.0a	13.3a	15.0a	14.3a	14.0a	14.3a
EPP	3	5.3b	11.3b	10.7c	12.3a	12.7a	12.0a	15.7a	17.0a	16.0a	15.7a
Penn A-4	3	6.3ab	14.0ab	12.7bc	10.0a	9.3a	9.3a	12.7a	12.0a	11.7a	10.7a
ASR368 F1	3	6.0ab	14.3ab	16.3ab	15.0a	12.0a	12.3a	13.0a	13.0a	12.3a	13.7a
ASR368 F2	3	8.3ab	14.3ab	14.7abc	13.0a	13.3a	12.7a	14.0a	12.7a	12.0a	12.0a
LSD (α = 0.05)[1]		3.61	4.96	4.03	6.76	8.66	8.72	11.58	11.94	11.5	11.27
Pr > F		0.253	0.191	0.031	0.726	0.8472	0.931	0.990	0.945	0.956	0.917

[1] Means with the same letter during each observation date are not significantly different, α = 0.05.

Table VIII-20. Number of nodes out of 30 producing viable tillers under non-irrigated conditions among six genotype populations planted in Fayette County, Kentucky in December 2002

Genotype population	N	Observation date (2002)						2003
		12/16	12/18	12/20	12/23	12/25	12/27	1/3
		Number of nodes						
Penncross	3	11.0a	5.0a	1.7a	0.7a	0.0a	0.0a	0.0a
Crenshaw	3	14.0a	10.0a	4.0a	2.0a	1.0a	0.0a	0.0a
EPP	3	13.0a	12.3a	7.7a	4.3a	2.7a	0.0a	0.0a
Penn A-4	3	7.0a	5.0a	1.0a	0.0a	0.0a	0.0a	0.0a
ASR368 F1	3	17.0a	11.0a	3.0a	2.0a	1.7a	0.0a	0.0a
ASR368 F2	3	11.0a	6.0a	2.0a	1.3a	0.7a	0.0a	0.0a
LSD (α = 0.05)[1]		11.80	11.65	9.32	4.93	3.08	0.00	0.00
Pr > F		0.586	0.603	0.674	0.525	0.425	na	na

[1] Means with the same letter during each observation date are not significantly different, α = 0.05.

Table VIII-21. Number of nodes out of 30 producing viable tillers under non-irrigated conditions among six genotype populations planted in Union County, Ohio in October 2002

Genotype Population	N	Observation date (2002)					
		10/15	10/18	10/22	10/25	10/30	11/5
		Number of nodes					
Penncross	3	0.0a	0.0a	0.0a	0.0a	0.0b	0.0a
Crenshaw	3	1.0a	1.3a	1.3a	1.3a	1.0a	0.0a
EPP	3	0.0a	0.0a	0.0a	0.0a	0.3ab	0.3a
Penn A-4	3	0.3a	0.3a	0.7a	0.7a	0.3ab	0.3a
ASR368 F1	3	1.0a	0.7a	0.7a	0.7a	0.3ab	0.3a
ASR368 F2	3	0.0a	0.3a	0.3a	0.3a	0.0b	0.0a
LSD ($\alpha = 0.05$)[1]		1.65	1.47	1.49	1.49	1.00	0.81
Pr > F		0.529	0.395	0.396	0.396	0.326	0.770

[1] Means with the same letter during each observation date are not significantly different, $\alpha = 0.05$.

Table VIII-22. Number of nodes out of 30 producing viable tillers under non-irrigated conditions among six genotype populations planted in Marion County, Oregon in October 2002

Genotype population	N	Observation date (2002)						
		10/11	10/14	10/18	10/21	10/25	10/28	10/31
		Number of nodes						
Backspin	3	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
Crenshaw	3	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
EPP	3	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
Penn A-4	3	0.0a	0.0a	0.0a	0.3a	0.3a	0.0a	0.0a
ASR368 F1	3	0.0a	0.0a	0.3a	0.0a	0.0a	0.0a	0.0a
ASR368 F2	3	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
LSD ($\alpha = 0.05$)[1]		0.00	0.00	0.43	0.43	0.43	0.00	0.00
Pr > F		na	na	0.465	0.465	0.465	na	na

[1] Means with the same letter during each observation date are not significantly different, $\alpha = 0.05$.

VIII.B.3. Conclusion for vegetative establishment studies

The results of the vegetative establishment experiments described above demonstrate that glyphosate tolerance imparted by plant transformation had neither a positive or negative effect on the production of tillers from the stolon nodes of two progeny populations of ASR368. It is important to note that even though the environmental and edaphic conditions in Experiments I and II (SectionVIII.B.1.1) were different, the maximum mean percentage of nodes producing tillers was essentially the same (maximum mean percentage; Exp. I = 57.6%, Exp. II = 55.7%). Significant differences between ASR368

GT and both GS segregants and cv. Crenshaw were detected for vegetative establishment in Experiment I[10]. However, ASR368 GT was not significantly different from the three other conventional cultivars (Penn A-4, Penncross and SR-1020) and Crenshaw was not significantly different from Penncross, SR-1020 and ASR368 GS.

The vegetative establishment experiments conducted during 2002 - 2003 in eight different environments (four irrigated and four non-irrigated) (Tables VIII-15 through VIII-22) further confirm that the potential for vegetative establishment of plants derived from ASR368 (two different generations of progeny) is not significantly different from that of several accepted conventional cultivars and a population consisting of EPPs used in crosses with ASR368 to generate Fl and F2 generation progeny. Furthermore, the 2002 - 2003 experiments provide additional evidence that an extended optimum moisture regime is critical for the vegetative establishment and persistence of *Agrostis stolonijera.*

The results of the 2002 field and greenhouse studies lend further support to the conclusion that ASR368 plants are not different from conventional creeping bentgrass cultivars in their ability to produce new tillers from viable stolon nodes. The results also support an overall conclusion that ASR368 is not different than conventional creeping bentgrass in terms of plant pest or weediness characteristics and is no more likely to pose a plant pest risk than conventional creeping bentgrass.

VIII.C. Relative Growth

Turfgrasses are perennial plants not because individual shoots survive indefinitely, but because the plant community is dynamic (Turgeon, 2002). During vegetative growth and development, there is no basic alteration in the structural design of the turfgrass plant. Organs originate in a repetitious well-defined manner following a typical sigmoidal curve. The size of the plant and the number of plant organs, e.g., roots, tillers, leaves, etc. typically does not change (Turgeon, 2002).

Tillers, the basic unit of the plant, arise from axillary buds. They are most active in the late summer to early autumn and may function vegetatively or reproductively according to environmental stimuli (Turgeon, 2002). Tillers enable the in-place expansion of the plant and usually live for not more than one year and frequently less (Turgeon, 1985). Jonsdottir (1991) reported that creeping bentgrass tillers had a very short life span with a half-life of two to seven months. Mortality of a tiller typically occurs after flowering, which is usually followed by replacement with a new tiller. The number of leaves per tiller remains fairly constant and the rate of new leaf emergence is approximately the same as the rate of senescence. A turfgrass plant is considered mature when tillers are receptive to floral induction (Turgeon, 1985). However, grasses maintained as turfgrasses may not go through a reproductive phase because the mowing height and regularity of

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[10] Although no GS segregant progeny were included in Experiment II due to lack of availability of similar age plants maintained in similar fashion to the ASR368 A, ASR368 B, Penneagle and Backspin populations, conventional cultivars are considered to be GS phenotypes. The heterozygous nature of *Agrostis stolonifera* provides for a range of phenotypes that must be considered as representative of the conventional *Agrostis stolonifera,* when determining relative performance.

mowing, particularly on golf courses, precludes flowering (Lush 1988; Johnson and Riordan, 1999).

Stolons lead to the production of independently rooted daughter plants at stolon nodes (Cattani and Struik, 2001). Attached stolons generally develop as competition within the plant necessitates better light for growth (Cattani and Struik, 2001). A. stolonifera can exhibit substantial stolon growth (Eriksson, 1989; Watschke, 1995) under favorable environmental conditions, i.e., good mineral nutrition and water availability (Hunt et al., 1987).

Golf courses manage creeping bentgrass with frequent close mowing, aggressive cultivation and sufficient agronomic inputs to achieve the highest vertical tiller density (shoots per unit area) and shortest internode length possible. Large numbers of individual plants that contribute to visual and physical uniformity across the entire sward are favored over small numbers of large clonal (stolon generated) colonies under these conditions. Seed producers prefer individual plants to enhance the potential for reproductive tillering. Consequently, seed is planted in rows and the top-growth is removed after harvest to encourage lateral stolon growth.

In this section, we examine the relative growth of ASR368 compared to conventional creeping bentgrass. Increased growth, either when established in bare soil or in competition with other turfgrasses, could increase a plant speices' weediness. Studies were performed across multiple locations representing the northern or cool, southern or warm and transition climate zones of turfgrass adaptation in 2000, 2001, 2002 and 2003 (USDA # 99-203-04n, 00-159-02n, 00-224-01n, 00-201-03n, 01-151-02n, 01-177-02n and 02-193-04n). Seasonal variation over the duration of the studies contributed to additional stresses within each study location. The studies performed, identity of ASR368 test material, control plants and cultivars, locations and study duration are provided in Tables VIII-23 and VIII-24.

Table VIII-23. ASR368, B99061R, and conventional bentgrass cultivars evaluated for relative growth and competitive ability in 2000 – 2003

Locations County, State	Climate Zone	Planting Date (m/d/y)	Competitive Turf[1]	ASR368 Generation	Bentgrass Cultivars Evaluated[2]	Study Duration (mos)[3]
Clinton, IL	Transition	7/26/00	Bare soil	R0	B99061R, CR, PE, P4	13
Ottawa, MI	Cool	7/27/00	Bare soil	R0	B99061R, CR, PE, P4	13
Union, OH	Cool	6/23/00	Bare soil	R0	B99061R, CR, PE, P4	15
Marion, OR	Cool	6/28/00	Bare soil	R0	B99061R, CR, PE, P4	14
Middlesex, NJ	Transition	7/14/00	KB	R0	B99061R, CR, PE, P4	25
Union, OH	Cool	6/23/00	KB	R0	B99061R, CR, PE, P4	14
Franklin, OH	Cool	10/31/00	PR	F1	B99061R, BS, CR, PE, P4, HB, ST, SR	24
Marion, OR	Cool	6/20/00	KB/PR	R0	B99061R, CR, PE, P4	32
Baldwin, AL[4]	Warm	11/2/00	BG	F1	B99061R, BS, CR, PE, P4, HB, ST, SR	9
Baldwin, AL[5]	Warm	11/2/00	SA	F1	B99061R, BS, CR, PE, P4, HB, ST, SR	9
Baldwin, AL[4]	Warm	11/2/01	BG	F1	B99061R, BA, CR, PE, P4, HB, ST,SEA	9
Baldwin, AL[5]	Warm	11/2/01	SA	F1	B99061R, BA, CR, PE, P4, HB, ST,SEA	9
Ingham, MI	Cool	3/31/01	Bare soil	F1	B99061R, BS, CR, PE, P4, HB, ST, SR	6
Ingham, MI	Cool	2/2/02	Bare soil	F1	B99061R, BS, CR, PE, P4, HB, ST, SR	4

[1]Mature turf varieties into which ASR368 and other test genotypes were planted: KB = Kentucky bluegrass, PR = perennial ryegrass, BG = bermudagrass, SA = St. Augustinegrass, KB/PR = a uniform mixture of Kentucky bluegrass and perennial ryegrass

[2] Bentgrass cultivars tested: BA = Bardot colonial bentgrass (*Agrostis capillaris*), BS = Backspin, CR = Crenshaw, PE = Penncross, P4 = Penn A-4, HB = Highland dryland bentgrass (*Agrostis castellana*), ST = Streaker redtop (*Agrostis gigantea*) and SR = SR7100 colonial bentgrass (*Agrostis capillaris*)

[3] Months post-planting

[4] ASR368, B99061R and bentgrass cultivars were planted into direct sun

5 ASR368, B99061R and bentgrass cultivars were planted in the shade to assess shade tolerance

Table VIII-24. Months in which observations were made of relative plant growth and competitive ability at each location during 2000 to 2003

Location	2000						2001												2002												2003	
	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F
Clinton, IL		*	*	*	*					*	*	*	*	*																		
Ottawa, MI		*	*	*	*			*	*	*	*	*	*	*																		
Union, OH				*							*	*	*	*	*																	
Marion, OR		*	*	*				*	*	*	*	*	*	*																		
Franklin, OH										*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*				
Union, OH	*	*	*		*				*	*	*	*	*	*																		
Middlesex, NJ		*	*	*							*	*	*													*						
Marion, OR		*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*				*
Baldwin, AL (sun)									*	*	*	*	*	*																		
Baldwin, AL (shade)									*	*	*	*	*	*																		
Baldwin, AL (sun)																						*	*	*	*	*						
Baldwin, AL (shade)																						*	*	*	*	*						
Ingham, MI (shade)												*	*		*																	
Ingham, MI (shade)																				*	*	*	*									

VIII.C.1. Experiment I - relative growth in bare soil without mowing

Studies were conducted during 2000 – 2001 to compare the relative growth rates of un-mowed ASR368 R0 generation plants, B99061R and three conventional creeping bentgrass cultivars (Crenshaw, Penncross, and Penn A-4) in non-competitive, bare soil environments.

The experiment was conducted at four locations representing a range of cool season and transition zone environments where creeping bentgrass is grown: Clinton County, Illinois; Marion County, Oregon; Union County, Ohio and Ottawa County, Michigan. These field releases were conducted under USDA # 00-159-02n and 01-151-02n.

VIII.C.1.1. VIII.C.1.a. Experimental methods

Site establishment and maintenance

The starting material for each creeping bentgrass genotype consisted of stolon nodes grown in Jiffy® Pellets (42 mm) in a poly-house in Marion County, Oregon during spring 2000. Each field location was established with three replicated 3’ × 5’ plots per treatment

arranged in a randomized complete block design. Each plot was populated with three plants of a single genotype. Vegetative plugs of each entry were planted at a depth of two to four inches and were separated from the adjoining plot by a buffer of at least five feet.

The plants at each location were managed with standard agronomic practices to maintain plant growth. Irrigation and insecticide and fungicide applications were applied as needed to maintain the integrity of the plots and fertilizer was applied to avoid nutrient deficiency symptoms. Although the plants were not mowed, individual seed heads were removed manually as necessary to preclude pollen development.

Data collection

Data was collected on a near monthly basis between the summer of 2000 and the fall of 2001 (Table VIII-24). Early season evaluations consisted of measuring the minimum and maximum stolon length for each of three plants within each plot. As the plants matured, treatment evaluations were made by measuring the percentage ground cover within each 3 × 5' plot and shoot density. A 1 to 9 rating scale, where 9 was the greatest number of shoots per unit area, was used to assess shoot density. This change in data collection facilitated a more rapid and direct assessment of development since the plants within each plot had grown into one another and precluded additional stolon length measurements.

Statistical analysis

Analysis of variance was used to determine differences in growth among the bentgrass lines. Means were compared using Fisher's LSD ($\alpha = 0.05$). Fisher's LSD was chosen because it has the highest power of the pair-wise separation procedures.

VIII.C.1.2. Results

Ottawa County, Michigan

The Ottawa County, Michigan location was planted on July 27, 2000 and represents a cool season climate. Early season observations of stolon length were taken from August 27, 2000 through March 28, 2001. Each mean represents the average of two observations each on nine plants (i.e., three plots, three plants per plot). Observations taken after March 28, 2001 reflect the percent ground cover in the plots and relative shoot density (shoots / unit area). Values for shoot density were recorded on a 1 to 9 scale with 1 being low and 9 being high.

Stolon Length:

There were no differences in stolon length, percentage plot cover or shoot density observed between ASR368, B99061R and the conventional creeping bentgrass cultivars according to Fisher's LSD ($\alpha = 0.05$) over the duration of the trial. Therefore, with respect to stolon growth, percentage ground cover and shoot density, ASR368 was not different from B99061R or the conventional creeping bentgrass cultivars evaluated at this location between August 2000 and July 2001 (Tables VIII-25 and VIII-26).

Table VIII-25. Plant growth as measured by stolon length (cm) of ASR368 R0, B99061R and three conventional cultivars in Ottawa County, Michigan from August, 2000 to March, 2001

	2000												2001					
	August			September			October			November			February			March		
	centimeters																	
	Mx[1]	X[2]	Mn[3]	Mx	X	Mn	Mx	X	Mn	Mx	X	Mn	Mx	X	Mn	Mx	X	Mn
Crenshaw	70	42.3	28	89	53.0	33	93	59.0	37	106	61.3	41	90	57.0	35	102	61.3	37
Penn A-4	55	34.7	17	64	51.3	28	94	63.7	40	96	65.0	38	88	58.7	36	92	63.3	38
Penncross	77	46.7	35	98	61.0	45	102	65.0	43	108	66.3	40	96	63.0	40	98	67.0	37
B99061R	56	40.3	25	84	54.7	40	81	63.3	41	98	64.3	38	97	57.3	38	90	62.3	47
ASR368 R0	64	42.7	28	75	57.7	36	93	67.0	43	97	69.0	44	90	64.3	43	90	67.7	40
LSD (0.05)		NS[4]			NS			NS			NS			NS			NS	
Trt. Prob		0.17			0.33			0.60			0.56			0.64			0.69	
CV		12.6			10.3			9.4			8.3			12			10.1	

[1] Mx = maximum.
[2] Means (X) were calculated from the maximum (Mx) and minimum (Mn) values from each of three plants per replicate (n=9).
[3] Mn = minimum.
[4] NS = not significant according to Fisher's LSD ($\alpha = 0.05$).

Table VIII-26. Plant growth as measured by percentage ground cover and shoot density[1] of ASR368 R0, B99061R and three conventional cultivars in Ottawa County, Michigan from April, 2001 to July, 2001

	2001							
Genotype	**April**		**May**		**June**		**July**	
	% Cover	Density	% Cover	Density	% Cover	Density	% Cover	Density
Crenshaw	48.3	8.0	58.3	8.7	66.7	8.0	78.3	9.0
Penn A-4	51.7	7.7	65.0	8.3	75.0	8.7	85.0	9.0
Penncross	66.7	8.0	78.3	8.3	83.3	7.7	95.0	8.3
B99061R	45.0	6.3	50.0	7.7	60.0	8.0	78.3	8.7
ASR368 R0	50.0	7.3	66.7	8.3	76.7	8.3	85.7	8.7
LSD (0.05)	NS[2]	NS	NS	NS	NS	NS	NS	NS
Trtmnt Prob (F)	0.204	0.217	0.125	0.265	0.281	0.211	0.395	0.232
CV	20.1	11.9	18.1	6	17.6	5.9	13	4.2

1 Shoot density (shoots per unit area) was recorded on a 1-9 scale with 1 being low and 9 being high

[2] NS = not significant according to Fisher's LSD ($\alpha = 0.05$).

Clinton County, Illinois

The Clinton County, Illinois location represents a transition zone climate for cool and warm season grass adaptation. The location was planted on July 26, 2000. Early season observations of stolon length were taken from August 30, 2000 through April 15, 2001. Observations taken after April 15, 2001 reflect percent ground cover and relative shoot density (shoots / unit area). Values for percentage ground cover and shoot density were recorded in the same manner as described previously for Ottawa County, Michigan.

Throughout two seasons of growth in Clinton County, Illinois, the stolon length, percentage ground cover and shoot density of ASR368 R0 plants were not significantly different from either B99061R or Crenshaw. ASR368 R0 was significantly larger than Penn A-4 in September 2000, and April and June 2001 and significantly smaller than Penncross in July and August 2001 (Table VIII-27 and Table VIII-28). No significant differences between ASR368 and the conventional creeping bentgrasses were detected on any other dates.

Table VIII-27. Comparative growth as measured by stolon length (cm) of ASR368 R0, B99061R and three conventional cultivars in Clinton County, Illinois from August 2000 to April 2001

	2000												2001		
Genotype	August			September			October			November			April		
	centimeters														
	Mx[1]	Mean[2]	Mn[3]	Mx	Mean[5]	Mn	Mx	Mean	Mn	Mx	Mean	Mn	Mx	Mean[5]	Mn
Crenshaw	56	29.7	16	74	39.0a	19	76	39.7	25	80	43	30	68	35.3a	18
Penn A-4	46	24.7	12	41	22.3b	7	46	30.3	15	60	39.7	20	39	24.7b	13
Penncross	52	33.7	22	62	42.0a	29	60	44	30	70	50	25	57	42.0a	30
B99061R	58	30	16	53	38.0a	19	50	36.7	20	55	37.7	25	49	35.0a	24
ASR368	49	30.3	18	51	35.0a	20	50	35.3	20	55	37.3	15	52	33.7a	26
LSD (0.05)		NS[4]			10.9			NS			NS			8.6	
Trt Prob (F)		0.215			0.023			0.235			0.41			0.019	
CV		13.9			16.4			17.9			20.6			13.3	

[1] Mx = maximum.
[2] Means were calculated from the maximum (Mx) and minimum (Mn) values from each of three plants per replicate (n=9).
[3] Mn = minimum.
[4] NS = not significant according to Fisher's LSD (α = 0.05).
[5] Means not followed by the same letter are significantly different according to Fisher's LSD (α = 0.05).

Table VIII-28. Comparative growth as measured by percentage ground cover and shoot density[1] of ASR368 R0, B99061R and three conventional cultivars in Clinton County, Illinois from May, 2001 to August, 2001

	2001							
Genotype	**May**		**June**		**July**		**August**	
	% Cover	Density	% Cover[3]	Density	% Cover[3]	Density	% Cover[3]	Density
Crenshaw	58.3	7.0	71.7a	6.5	58.3ab	8.0	66.7ab	7.7
Penn A-4	35.0	5.8	31.7b	6.7	40.0c	7.0	43.3c	6.8
Penncross	71.7	8.0	76.7a	6.0	66.7a	8.3	75.0a	7.7
B99061R	51.7	7.8	65.0a	8.3	60.0ab	8.3	63.3ab	7.8
ASR368 R0	53.3	6.7	55.0a	7.7	45.0bc	7.7	55.0bc	7.2
LSD (0.05)	NS[2]	NS	22.0	NS	18.4	NS	19.1	NS
Trtmnt Prob (F)	0.09	0.46	0.01	0.09	0.049	0.24	0.04	0.42
CV	25.2	21.8	19.4	13.6	18.1	9.4	16.8	9.3

Shoot density (shoots per unit area) was recorded on a 1-9 scale with 1 being low and 9 being high.

[2] NS = not significant according to Fisher's LSD ($\alpha = 0.05$).

[3] Means not followed by the same letter are significantly different according to Fisher's LSD ($\alpha = 0.05$).

Marion County, Oregon

The Marion County, Oregon location was planted on June 28, 2000. Oregon's cool season and mild climate provided for additional growth of individual plants relative to other sites. The rapid increase in plant growth permitted a measurement of stolon size only in August and November 2000. Percentage ground cover was recorded on the observation dates from September through April 2001, and from May to August 2001 both percent ground cover and shoot density were recorded. Values for shoot density were recorded as described above.

Throughout two seasons of growth in Marion County, Oregon the growth of the ASR368 R0 plants was not significantly different from B99061R and the three conventional cultivars (Tables VIII-29 – VIII-31).

Table VIII-29. Comparative growth as measured by stolon length (cm) of ASR368 R0, B99061R and three conventional cultivars in Marion County, Oregon in August, 2000 and November 2000

Genotype	2000					
	August			November		
	centimeters					
	Max[1]	Mean[2]	Min[3]	Max	Mean	Min
Crenshaw	52	39.7	24	129	103.0	52
Penn A-4	61	40.7	23	149	101.7	66
Penncross	57	49.7	38	136	111.3	84
B99061R	54	42.7	33	128	106.3	80
ASR368 R0	52	41	29	127	99.3	80
LSD (0.05)		NS[4]			NS	
Trtmnt Prob (F)		0.08			0.83	
CV		9.2			12.8	

[1] Max = maximum.
[2] Means were calculated from the maximum (Max) and minimum (Min) values from each of three plants per replicate (n=9).
[3] Min = minimum.
[4] NS = not significant according to Fisher's LSD ($\alpha = 0.05$).

Table VIII-30. Comparative growth as measured by percentage ground cover of ASR368 R0, B99061R and three conventional cultivars in Marion County, Oregon from September 2000 to March, 2001

Genotype	2000		2001	
	September	October	February	March
	% Cover	% Cover	% Cover	% Cover
Crenshaw	61.0	80.0	84.3	78.3
Penn A-4	63.3	76.7	88.7	82.7
Penncross	74.3	94.3	92.3	90.7
B99061R	54.3	76.7	87.0	88.3
ASR368 R0	51.7	75.0	83.7	80.0
LSD (0.05)	NS[1]	NS	NS	NS
Treatment Prob (F)	0.32	0.52	0.72	0.33
CV	21.4	18.3	9.6	9.4

[1] Shoot density observations were recorded on a 1-9 scale with 1 being low and 9 being high.
[2] NS = not significant according to Fisher's LSD ($\alpha = 0.05$).

Table VIII-31. Comparative growth as measured by percentage ground cover and shoot density[1] of ASR368 R0, B99061R and three conventional cultivars in Marion County, Oregon from May, 2001 to August, 2001

	2001							
Genotype	May		June		July		August	
	% Cover	Density	% Cover	Density	% Cover	Density	% Cover	Density
Crenshaw	96.7	6.3	96.7	7	100	6.0bc[3]	100	6.3
Penn A-4	75.0	7	78.3	6.3	90	8.0a	91.7	6.7
Penncross	100	6.3	100	6	100	6.7bc	100	6.3
B99061R	99.3	7	99.3	7.3	100	7.7a	100	8.0
ASR368 R0	99.3	7	98.3	7.3	100	7.3ab	100	6.7
LSD (0.05)	NS[2]	NS	NS	NS	NS	0.9	NS	NS
Treatment Prob (F)	0.44	0.77	0.44	0.06	0.46	0.01	0.46	0.18
CV	19.3	14.1	16.4	8.1	7.9	6.8	6.6	10.9

[1] Shoot density observations were recorded on a 1-9 scale with 1 being low and 9 being high.
[2] NS = not significant according to Fisher's LSD ($\alpha = 0.05$).
[3] Means followed by the same letter on date are not significantly different according to Fisher's LSD ($\alpha = 0.05$).

Union County, Ohio

The Union County, Ohio location was planted on June 23, 2000 and represents a cool season climate. An observation of stolon length was taken on October 3, 2000. Due to winter conditions no observations were taken again until May 4, 2001. From this date forward observations of percent ground cover and shoot density were taken.

Throughout two seasons of growth in Union County, Ohio, the stolon length, percentage ground cover and/or shoot density of ASR368 R0 were not significantly different from that of B99061R and Penn A-4. ASR368 R0 had a significantly lower percent cover in June through September 2001 and significantly lower density in August and September 2002 than Crenshaw. ASR368 R0 also had a lower percent cover and density in August and less coverage in September 2002 than Penncross (Table VIII-32 and Table VIII-33).

Table VIII-32. Comparative growth as measured by stolon length (cm) of ASR368 R0, B99061R and three conventional cultivars in Union County, Ohio in October, 2000

	October 2000		
	centimeters		
Genotype	**Max[1]**	**Mean[2]**	**Min[3]**
Crenshaw	99	45.7	21
Penn A-4	63	38.3	14
Penncross	65	41.7	26
B99061R	65	40	19
ASR368 R0	81	43.7	19
LSD (0.05)		NS[4]	
Treatment Prob(F)		0.69	
CV		15.9	

Max = maximum.

[2] Means were calculated from the maximum (Max) and minimum (Min) values from each of three plants per replicate (n=9).

[3] Min = minimum.

[4] NS = not significant according to Fisher's LSD (α = 0.05).

Table VIII-33. Comparative growth as measured by percentage ground cover and shoot[1] density of ASR368 R0, B99061R and three conventional cultivars in Union County, Ohio from May, 2001 to September, 2001

	2001									
Genotype	**May**		**June**		**July**		**August**		**September**	
	%	Densit	%	Densit	%	Densit	%	Densit	%	Densit
Crenshaw	36.7a[3]	5.3	43.3a	6.3	53.3a	6.7	36.7a	5.0a	58.3a	5.7a
Penn A-4	17.7b	3.7	13.3c	5.7	23.3bc	5.0	13.3b	1.7c	18.3b	3.3b
Penncross	37.7a	5.3	33.3ab	6.3	33.3b	6.7	36.7a	4.0ab	46.7a	4.3ab
B99061R	27.0ab	4.7	11.7c	7.0	13.3c	4.3	15.0b	2.7bc	26.7b	3.3b
ASR368 R0	26.7ab	5.0	20.0bc	7.0	23.3bc	5.3	15.0b	2.0c	26.7b	3.3b
LSD (0.05)	13.2	NS[2]	15.7	NS	19.7	NS	19.7	1.8	16	1.6
Treatment Prob (F)	0.04	0.09	0.01	0.13	0.01	0.15	0.04	0.01	0.002	0.05
CV	24.2	14.5	34.2	9.6	35.7	21.4	44.9	30.9	24.1	21.3

[1] Shoot density was recorded on a 1-9 scale with 1 being low and 9 being high.

[2] NS, not significant according to Fisher's LSD (α = 0.05).

[3] Means followed by the same letter on date are not significantly different according to Fishers LSD (α = 0.05)

VIII.C.1.3. Conclusion from bare soil relative growth studies

Throughout the 13 to 15 months that these studies were performed during 2000 and 2001, ASR368 R0 plants were not significantly different from B99061R and significantly different from the conventional cultivars on only a few dates. These results support the conclusion that ASR368 plants will not differ in establishment and growth rate from conventional creeping bentgrass genotypes when planted into bare soil under non-competitive conditions. Furthermore, ASR368 plants show no additional growth ability related to shoot density or ability to colonize open ground compared to conventional creeping bentgrass, which further supports a conclusion of no contribution to increased weed potential based on these growth characteristics.

VIII.C.2. Experiment II - Relative growth in competitive and managed turfgrass stands

A number of experiments were performed in 2000, 2001 and 2002 to evaluate the relative growth of ASR368 compared to a number of conventional cultivars in competitive managed turfgrass stands. These experiments were conducted at six locations representing several distinct environments: (1) cool season, (2) transitional climate, (3) warm season full sun, (4) warm season shade and (5) reduced irradiance (Table VIII-23). Each of the cool and transitional climate zone studies was conducted for a minimum of two growing seasons, including the year of establishment as presented in Table VIII-24. The trials in Alabama and Michigan were repeated in 2001 and 2002 and 2000 and 2002, respectively.

VIII.C.2.1. Relative growth of ASR368 R0 generation plants in competitive and managed cool season and transition zone turfgrass regions

VIII.C.2.1.1. Experimental methods

Field studies were initiated on June 23, 2000 in Union County, Ohio; October 31, 2000 in Franklin County, Ohio; July 14, 2000 in Middlesex County, New Jersey, and June 20, 2000 in Marion County, Oregon. The turf area (perennial ryegrass, and/or Kentucky bluegrass) to be inter-planted was maintained for uniform turf coverage and surface drainage (Table VIII-23). Soil cores (35 mm in diameter and 60 mm deep) were removed from the original turf area on 60 – 90 cm centers. Bentgrass plugs were transplanted directly into the core holes so that the crowns of the plant were at or slightly below the soil surface to ensure firm contact between the Jiffy pot media was maintained with the field soil. The turf area was maintained under a regime appropriate for the original dominant species following an initial establishment phase to acclimate the bentgrass transplants.

The plots were irrigated as needed to prevent stress during a six-week establishment phase, and then irrigated to maintain the existing turf. Plots were mown regularly at a clipping height of 1.25 cm in Oregon, 3.75 cm in New Jersey, and 5 cm or less at both Ohio locations. Nitrogen and other nutrients were applied as needed to compensate for visual deficiency symptoms. Insecticides and fungicides were applied as needed to control the integrity of plots following standard agronomic practices.

Bentgrass plant diameter was measured in centimeters each month of the growing season when possible. Mean plant diameter was calculated as an average of two measurements made on each tiller plot.

Statistical analysis

The study was conducted as a completely randomized design with three replications at each of four locations. Analysis of variance was used to determine differences in growth among the bentgrass lines. Means were compared using Fisher's LSD ($\alpha = 0.05$).

The data were analyzed over all locations with lines nested within plant type (control, reference, or ASR368). However, the number of reporting locations varied by month as listed in Table VIII-24. Data collection at the Union County, Ohio and Middlesex County, New Jersey sites was restricted during several winter months (November to April) by snow cover and weather conditions. Variation from site to site necessitated a separate analysis of monthly data from each location.

VIII.C.2.1.2. Results

Middlesex County, New Jersey (transition zone climate)

Mean plant diameter measurements were made from August through October 2000 and May through July 2001 (Table VIII-34). Adverse weather conditions and plant dormancy precluded sampling in the intervening months. An additional observation was made in August 2002 to further assess the long-term growth differences of the test genotypes.

The mean plant diameter of ASR368 R0 plants and the three conventional creeping bentgrass cultivars was consistently greater than B99061R on all sampling dates (Table VIII-34). The relatively poor plant vigor of B99061R in comparison to ASR368 and the conventional cultivars suggests that the vegetative plugs planted were not particularly vigorous in this experiment. The mean plant diameter of ASR368 R0 fell within the range of values for the conventional cultivars for every rating over the 25 months or three growing seasons encompassed by the study. ASR368 R0 was not significantly different from the conventional cultivars throughout the duration of the study other than one instance where it was significantly smaller than Penncross during the final measurement in August 2002. Therefore, we would not expect this event to have any greater fitness or competitive advantage in New Jersey or transition zone turf situations than conventional available creeping bentgrass cultivars.

Table VIII-34. Mean plant diameter (cm) of ASR368 R0, B99061R and three conventional cultivars, from August 2000 to August 2002 in Middlesex County, NJ

	2000						2001						2002	
Genotype	August		September		October		May		June		July		August	
	centimeters													
	Mea	SD[2]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Penn A-4	6.5	2.6	6.6	3.4	7.8	5.7	4.2	3.8	8.5	7.1	10.2	7.4	4.6	10.1
Crenshaw	6.3	1.8	7.3	3.1	12.8	3.9	14.7	7.7	19.0	8.4	18.6	5.8	6.3	19
Penncross	7.6	2.7	8.3	3.4	12.2	3.4	14.2	7.5	18.6	4.5	21.2	4.0	32.8*	15.2
B99061R	4.7	2.6	4.0*	1.8	6.5	3.5	0.0*[1]	0.0	7.0	7.5	5.2*	5.3	1.0	1.7
ASR368 R0	7.8	0.6	8.8	1.3	11.3	2.5	10.5	5.1	12.5	0.5	13.8	3.5	1.8	3.2
LSD	3.3		3.9		8.3		7.4		5.5		7.8		16.3	

* Mean diameter is significantly different than ASR368 according to Fisher's LSD ($\alpha = 0.05$).

[1] B99061R plants were still dormant during May 2001, which resulted in a 0.0 reading for this observation. The plants resumed vegetative growth prior to the next observation, which enabled calculation of the mean plant diameter in both June and July 2001.

[2] SD = Standard deviation

Union County, Ohio (cool season climate)

The mean plant diameter of ASR368 R0 plants was not significantly different from B99061R and the conventional cultivars throughout the 15 months of the study (Table VIII-35, Fisher's LSD, $\alpha = 0.05$). The density of the Kentucky bluegrass stand as well as competition from broadleaf weeds, such as dandelion and white clover, resulted in more variation among replications of the same cultivar than were observed between cultivars. This variation contributed to the considerably greater mean plant diameter and standard deviation for ASR368 R0 in May 2001. The individual plant diameter for each replication on this date was 5, 8 and 25 cm. The unusually large 25 cm diameter rating for the third replicate is believed to result from inadvertently including the measurement of bentgrass volunteers not related to the ASR368 R0 plants originally transplanted. This measuring error did not occur on either the preceding or subsequent sampling dates.

Table VIII-35. Mean plant diameter (cm) of ASR368 R0[4], B99061R and three conventional cultivars during 2000 and 2001 in Union County, OH

	2000								2001											
Genotype	**Jul**		**Aug**		**Sep**		**Nov**		**Mar**		**Apr**		**May**		**Jun**		**Jul**		**Aug**	
	centimeters																			
	X[1]	SD[3]	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD
Penn A-4	5.5	1.6	8.8	6.2	7.2	3.2	5.9	1.8	4.7	2	2.9	3.1	5.1	8.2	8.3	8.1	6.9	4.5	8	8.4
Crenshaw	5.5	0.8	5.8	1.6	6.4	1.6	5.8	1.3	5.1	1.9	3.9	2.5	4.6	4.1	3.9	5	5.3	2.2	4.1	4.2
Penncross	5.2	1.1	5.2	1.2	5.8	2.6	5.1	1.3	4.3	1.8	3.8	2.2	4.1	4.9	5.8	8	6.7	3.4	6.5	6.8
B99061R	6.0	1.3	4.8	1.6	5.0	2.6	3.3	1.8	3.3	1.9	1.7	1.4	2.2	2.3	3.3	2.9	3.7	3.8	3.6	3.7
ASR368 R0	5.7	0.2	6.3	1.9	5.0	0.5	5.0	1.8	4.3	2.5	3.2	2.8	12.8	10.7	4	3.5	4.7	4.2	5.8	5.9
LSD	NS[2]		NS		NS		3.7		NS		4.4		NS		NS		NS		NS	

[1] X = Mean

[2] NS = not significantly different from ASR368 according to Fisher's LSD ($\alpha = 0.05$).

[3] SD = Standard deviation

[4] ASR368 R0 is the only GT genotype in this dataset

Franklin County, Ohio (cool climate)

The creeping bentgrass conventional cultivars in this study were: Backspin, Crenshaw, Penn A-4 and Penncross. Three other bentgrass species were also evaluated: 'Highland' dryland bentgrass (*A. castellana*), 'SR7100' colonial bentgrass (*A. capillaris*) and 'Streaker' redtop bentgrass (*A. gigantea*) to provide a comparison to other commonly planted bentgrasses.

The mean plant diameter of ASR368 F1 progeny was within the range of B99061R and the three creeping bentgrass conventional cultivars on all sampling dates throughout the 23 months encompassed by this study (Table VIII-36 – VIII-38). Crenshaw was significantly larger than ASR368 F1 progeny during June 2001, but was not significantly different in all other months. Penncross was significantly larger than ASR368 at the end of the study during August and September 2002.

The other bentgrass reference species were more variable in growth in comparison to ASR368 F1 and the other creeping bentgrass cultivars, which may be attributed to species differences. Colonial and dryland bentgrasses were among the slower growing of all of the bentgrasses in the trial.

Table VIII-36. Mean plant diameter (cm) of ASR368 F1[6], B99061R and conventional bentgrass cultivars, during April to September 2001 in Franklin County, OH

Cultivar	2001 April		May		June		July		August		September	
	centimeters											
	Mean	SD[5]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Backspin	5.5	1.8	6.8	1.4	4.7	0.8	4.9	1.4	7.7	2.4	7.6	1.8
Crenshaw	5.2	1.2	6.6	1.1	5.7*	1.5	6.0	1.8	7.6	1.7	7.7	2.4
Penncross	5.6	1.1	6.2	0.9	4.3	0.4	5.2	1.7	8.6	3.8	7.6	2.1
Penn A-4	4.9	0.9	6.2	1.0	5.0	0.8	4.6	1.5	6.2	3.6	6.2	3.8
Highland[1]	6.1	3.0	7.5*	1.1	4.0	0.5	3.8	0.3	4.1*	0.7	4.4	1.6
SR7100[2]	3.7	0.8	5.0	0.9	3.9	0.5	3.3	0.4	4.4*	1.3	6.2	1.6
Streaker[3]	6.8*	1.0	8.8*	1.7	4.8	0.7	4.6	2.4	6.1	2.9	6.3	3.1
B99061R	5.0	1.2	6.3	0.5	4.8	0.6	5.3	3.2	7.2	1.0	6.8	3.1
ASR368 F1	5.0	1.2	5.9	1.4	4.3	0.9	5.4	2.1	8.1	1.8	6.9	2.6
LSD	1.6		1.3		1.0		NS[4]		2.6		NS	

* Mean diameter is significantly different from ASR368 on date according to Fisher's LSD ($\alpha = 0.05$).

1 Highland dryland bentgrass (*Agrostis castellana*)

2 SR7100 colonial bentgrass (*Agrostis capillaris*)

3 Streaker redtop bentgrass (*Agrostis gigantea*)

4 NS = not significantly different than ASR368 according to Fisher's LSD ($\alpha = 0.05$).

5 SD = standard deviation

6 ASR368 F1 is the only GT genotype in this dataset

Table VIII-37. Mean plant diameter (cm) of ASR368 F1[6], B99061R and conventional cultivars, during October 2001 through May 2002 in Franklin County, OH

	2001				2002							
Cultivar	Oct		Dec		Jan		Mar		Apr		May	
	centimeters											
	Mean	SD[5]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Backspin	8.1	2.8	7.9	2.7	8.1	2.7	6.0	1.9	6.6	1.7	8.9	2.4
Crenshaw	9.2	2.8	8.4	2.5	7.6	2.4	6.4	1.3	7.6	1.8	10.8	4.1
Penncross	9.5	2.7	8.9	2.6	7.9	2.8	6.9	2.2	7.7	2.4	10.2	3.6
Penn A-4	7.5	3.8	7.7	3.9	7.3	3.5	5.7	3.0	6.8	3.2	7.0	3.4
Highland[1]	5.6	3.0	5.7	3.4	4.8	3.3	4.6	2.5	3.7*	1.5	2.7*	1.4
SR7100[2]	6.3	2.0	6.6	2.1	5.9	2.0	5.0	1.6	4.7	2.3	4.1*	2.4
Streaker[3]	11.4	3.1	10.4	4.3	9.2	4.2	11.6*	3.6	12.9*	4.5	13.9*	6.2
B99061R	7.3	2.3	7.3	2.1	6.8	2.9	5.2	2.5	6.3	0.8	8.0	2.2
ASR368 F1	7.9	3.0	8.2	3.0	6.7	2.2	5.9	2.3	6.9	2.1	8.6	2.5
LSD	3.0		NS[4]		NS		2.5		2.7		3.6	

* Mean diameter is significantly different from ASR368 on date according to Fisher's LSD ($\alpha = 0.05$).
[1] Highland dryland bentgrass (*Agrostis castellana*)
[2] SR7100 colonial bentgrass (*Agrostis capillaris*)
[3] Streaker redtop bentgrass (*Agrostis gigantea*)
[4] NS = not significantly different than ASR368 according to Fisher's LSD ($p = 0.05$).
[5] SD = standard deviation
[6] ASR368 F1 is the only GT genotype in this dataset

Table VIII-38. Mean plant diameter (cm) of ASR368 F1[5], B99061R and conventional bentgrass cultivars, during June 2002 to September 2002 in Franklin County, OH

	2002							
Cultivar	June		July		August		September	
	centimeters							
	Mean	SD[4]	Mean	SD	Mean	SD	Mean	SD
Backspin	7.9	3.7	7.7	3.6	8.2	1.9	9.5	3.5
Crenshaw	8.9	3.2	8.2	3.7	7.4	3.8	10.3	6.1
Penncross	11.1	2.9	10.4	2.3	11.1*	5.9	13.8*	7.6
Penn A-4	7.0	1.8	8.4	4.0	8.6	4.1	6.2	4.4
Highland[1]	1.3*	1.1	1.4*	1.2	1.8*	0.4	2.2*	0.9
SR7100[2]	3.1*	2.7	3.7*	2.3	3.6	1.9	4.8	2.9
Streaker[3]	13.7*	5.7	11.9	5.9	6.2	2.8	14.4*	5.2
B99061R	10.0	2.3	11.8	2.1	9.7	2.0	9.5	2.2
ASR368 F1	9.7	4.0	7.9	4.2	7.0	3.8	7.8	3.3
LSD	3.3		4.1		3.7		4.9	

* Mean diameter is significantly different from ASR368 on date according to Fisher's LSD ($p = 0.05$).
[1] Highland dryland bentgrass (*Agrostis castellana*)
[2] SR7100 colonial bentgrass (*Agrostis capillaris*)
[3] Streaker redtop bentgrass (*Agrostis gigantea*)
[4] SD = standard deviation
[5] ASR368 F1 is the only GT genotype in this dataset

Marion County, Oregon (cool season)

Observations were taken at the Marion County, Oregon site on 27 dates that spanned 32 months beginning August 2000 and ending February 2003. The mean plant diameter of ASR368 R0 generation plants was greater than B99061R on nearly all measurement dates but differences were not always significant. The three conventional creeping bentgrass cultivars were also consistently larger than B99061R. In comparison to the three commercial cultivars, the mean plant diameter of ASR368 R0 was not significantly different from at least one or more of these cultivars on every measurement date during the 32-month study (Tables VIII-39 – VIII-41, Fisher's LSD, $\alpha = 0.05$).

Table VIII-39. Mean plant diameter (cm) of ASR368 R0, B99061R and three conventional creeping bentgrass cultivars during 2000 in Marion County, Oregon

	2000							
Genotype	August		September		November		December	
	centimeters							
	Mean	SD[1]	Mean	SD	Mean	SD	Mean	SD
Penn A-4	6.6	1.6	4.6	1.4	5.1	1.4	5.6	1.7
Crenshaw	6.7	1.5	4.9	1.0	6.8	1.0	6.7	1.1
Penncross	7.3	0.9	5.5	0.7	5.6	1.7	6.2	0.9
B99061R	6.2*	2.9	4.0	0.5	4.3*	1.4	4.0	0.0
ASR368 R0	9.0	1.3	5.3	0.6	6.7	1.3	6.0	0.9
LSD	2.2		1.5		2.2		2.0	

* Mean diameter is significantly different than ASR368 according to Fisher's LSD ($\alpha = 0.05$). [1] SD = Standard deviation

Table VIII-40. Mean plant diameter (cm) of ASR368 R0, B99061R and three conventional creeping bentgrass cultivars during 2001 in Marion County, Oregon

	2001																							
Genotype	Jan		Feb		Mar		Apr		May		Jun		Jul		Aug		Sep		Oct		Nov		Dec	
	centimeters																							
	X[1]	SD[2]	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD
Penn A-4	9.6*	2.4	8.8*	2.4	9.7*	1.3	11.6	2.0	9.9	3.4	11.9	3.4	12.2	2.9	14.1	5.7	12.9	5.4	14.2	5.8	14.9	6.6	18.9	7.6
Crenshaw	10.6	1.8	9.5*	2.1	11.6	3.2	15.0	6.3	14.6	2.0	17.6	3.7	17.1	4.0	17.8	5.6	15.6	3.9	18.1	4.4	15.9	3.4	23.0	3.8
Penncross	10.2*	1.8	11.2	1.8	11.6	1.8	16.6	2.6	17.6	5.7	16.1	3.8	15.3	3.0	19.6	5.6	17.7	3.8	19.7	5.1	17.2	4.6	21.9	6.1
B99061R	7.0*	0.9	7.7*	1.2	6.7*	1.6	9.0	3.8	8.5	1.7	10.0*	1.8	10.7	1.6	12.0	3.9	8.7	2.6	11.2	5.0	10.7	2.1	8.2*	4.6
ASR368	13.7	2.8	13.5	2.0	13.5	2.5	14.3	0.8	13.2	1.4	15.7	3.3	14.5	0.9	18.2	5.3	14.7	2.8	13.8	2.1	13.0	3.5	17.5	4.0
LSD	3.4		3.2		3.5		5.6		6.5		5.2		5.0		7.4		6.0		6.9		6.8		8.9	

[1] X = Mean plant diameter
[2] SD = Standard deviation
* Mean diameter is significantly different from ASR368 according to Fisher's LSD ($\alpha = 0.05$).

Table VIII-41. Mean plant diameter (cm) of ASR368 R0, B99061R and three conventional creeping bentgrass cultivars during 2002 and 2003 in Marion County, Oregon

	2002																				2003	
Genotype	Jan		Feb		Mar		Apr		May		Jun		Jul		Aug		Sep		Oct		Feb	
	X[1]	SD[2]	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD
	cm		Cm		Cm		Cm		Cm		Cm		Cm		Cm		Cm		Cm		cm	
Penn A-4	22.8	9.7	17.3	8.7	13.1	8.0	12.9	7.8	14.7	8.7	16.8	9.7	23.0	13.0	24.5	12.2	26.9	14.0	26.1	14.4	24.7*	13.2
Crenshaw	28.3	6.4	19.4	4.2	14.3	5.0	15.7	7.7	13.7	7.0	15.7	6.7	24.2	9.3	28.1	8.8	27.6	10.9	26.8	12.5	25.6*	12.1
Penncross	19.4	6.3	20.9	7.4	18.9	7.4	19.7	7.8	16.6	7.1	19.9	6.2	28.5	9.2	32.2	8.5	31.3	9.2	31.0	11.6	31.3*	9.2
B99061R	7.8	5.3	7.5	4.4	5.8	3.6	6.3	2.0	6.8	2.4	7.0	0.0	10.7	0.8	14.8	6.0	16.7	3.8	14.2	6.0	18.0	10.3
ASR368	18.2	10.3	12.7	2.5	13.5	4.6	13.7	2.5	11.0	1.3	13.2	3.8	19.7	6.8	20.3	4.3	22.5	5.3	21.2	9.0	10.8	2.3
LSD	10.6		8.4		8.2		8.8		8.9		9.2		12.9		13.1		13.9		15.1		13.7	

[1] X = Mean plant diameter
[2] SD = Standard deviation
* Mean diameter is significantly different from ASR368 according to Fisher's LSD ($\alpha = 0.05$).

VIII.C.2.1.3. Conclusion of competitive cool season and transition zone relative growth studies

The relative growth and competitive ability of ASR368 as measured by plant diameter was examined in three managed cool season environments and a transition zone turfgrass environment. ASR368 R0 plants were evaluated at three locations and ASR368 F1 plants at one location (Franklin County, Ohio). At three of the four locations the mean plant diameter of ASR368 plants was consistently larger than B99061R. On several dates this difference was statistically significant according to Fisher's LSD ($\alpha = 0.05$). However, the mean plant diameter of ASR368 was not significantly different from at least one of the conventional cultivars on sampling dates spanning 12 to 32 months.

Event ASR368 was selected from among more than 400 transformation events because of its commercially acceptable agronomic, genotypic and phenotypic characteristics. The ASR368 R0 was then crossed with a number of plants derived from conventional cultivars (Elite Parent Plants) to produce the ASR368 F1 progeny population. The same forward breeding process is used to develop the conventional bentgrass cultivars used as controls in these experiments. B99061R was randomly selected amongst other callus tissue derived from other explant genotypes and regenerated for use as an experimental control. It was not possible to go through the same selection and forward breeding process to ensure it was identical and as commercially acceptable as ASR368 or the other conventional bentgrasses except for the expression of the *cp4 epsps* gene. Somaclonal variation among plants regenerated from tissue culture in terms of morphology, growth habit, etc. is well documented in the scientific literature (Evans et al. 1984; Fluminhan et al. 1996; Muller et al. 1990). Consequently, the difference in growth between plants of B99061R and ASR368 may more likely be attributed to somaclonal variation, the event selection criteria and/or the forward breeding process rather than to the insertion of the *cp4 epsps* gene.

In summary, the mean plant diameter of ASR368 tended to fall within the range of the conventional cultivars at each of the four locations. It is not expected that plants of

ASR368 would possess any greater ability to persist than conventional creeping bentgrass cultivars in competitive cool season or transition zone turfgrass environments. Therefore, it is not expected that ASR368 would pose additional weediness or plant pest risk in managed environments as compared to conventional creeping bentgrass.

VIII.C.2.2. Relative growth of ASR368 plants in competitive and managed warm season turfgrass stands

VIII.C.2.2.1. Experimental methods

The plant growth rate of ASR368 F1 was compared to B99061R and Backspin (2001 only), Crenshaw, Penn A-4, Penncross and Seaside (2002 only) creeping bentgrasses in a managed, competitive, warm season turfgrass environment in both 2001 and 2002. Three other bentgrass species were also evaluated: 'Highland' dryland bentgrass, 'SR7100' (2001) and 'Bardot' (2002) colonial bentgrasses and 'Streaker' redtop bentgrass, to provide a comparison to other commonly planted bentgrasses (Table VIII-23). Vegetative plants of the bentgrass cultivars (nine per cultivar per environment) originating from Marion County, Oregon (as described above in Section VIII.C.2.2), were transplanted into a mature stand of St. Augustinegrass (*Stenotaphrum secondatum*) in the shade and bermudagrass (*Cynodon dactylon*) in full sun in Baldwin County, Alabama on November 2, 2000 and again November 2, 2001. The plots were watered as needed to prevent moisture stress on the transplanted material. The competitive ability of the creeping bentgrass events and conventional cultivars was recorded monthly by measuring the average diameter of each creeping bentgrass patch.

Statistical analysis

In both years the study was conducted as a completely randomized design in three replicates as described above in Section VIII.C.2.1.1. Analysis of variance was used to determine differences in growth among the bentgrass lines. Means were compared using Fisher's LSD ($\alpha = 0.05$).

VIII.C.2.2.2. Results

St. Augustinegrass, shade

The mean plant diameter of ASR368 F1 was within the range of B99061R and the commercial bentgrass cultivars on all observation dates in both 2001 and 2002. In 2001, ASR368 F1 was consistently larger than B99061R from June to August 2001 (Table VIII-42). Penncross was significantly larger than ASR368 F1 during all but the March 2001 measurement date. In 2002, ASR368 F1 was significantly smaller than Penncross on all sampling dates and significantly larger than B99061R on all but the first observation date (Table VIII-43). As discussed in the previous section, the difference between ASR368 F1 and B99061R may be attributed to somaclonal variation, the event selection criteria and/or the forward breeding process rather than the insertion of the *cp4 epsps* gene.

Table VIII-42. Mean plant diameter (cm) of *Agrostis* reference species, B99061R and ASR368 F1 during March 2001 to August 2001 in the shade in Baldwin County, Alabama

	2001											
Cultivar	March		April		May		June		July		August	
	centimeters											
	Mean	SD[1]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Backspin	22.1	3.3	22.9	3.1	22.6*	3.3	23.7	4.3	23.1	4.4	23*	4.8
Crenshaw	19.4	1.9	21.7	1.7	21.1	1.8	22.1	2	21.1	2.8	20.1	3.1
Highland	21.0	2.4	20.7	3.1	20.0	3.3	21.9	4.2	20.1	3.2	20.7	3.4
Penncross	22.1	2.7	24.6*	3.7	23.7*	2.9	26.8*	3.4	25.8*	2.7	24.3*	4.9
Penn A-4	18.3	2.6	20.8	2.1	19.8	2.4	20.5	2.5	19.6	3.9	19.9	3.6
SR7100	18.9	2.4	20.7	2.5	21.1	2.4	23.1	3.2	22.6	2.8	22.3*	3.7
Streaker	24.8*	4.8	24.8*	5	21.0	3.6	21.4	3	19.8	3.6	19.4	3.1
B99061R	18.3	2.4	18.4	3.9	18.3	1.3	18.2	1.5	17.5	1.4	11.9	10
ASR368 F1	19.1	3.8	20.5	2.2	19.3	1.8	20.7	2.1	19.4	2.1	16.8	6.7
LSD	3.2		3.5		3.2		3.5		3.7		5.0	

* Mean diameter is significantly different from ASR368 according to Fisher's LSD ($\alpha = 0.05$). [1] SD = Standard deviation

Table VIII-43. Mean diameter (cm) of creeping bentgrass plants of ASR368 F1, B99061R and conventional cultivars in the shade from April 2002 to August 2002 in Baldwin County, Alabama

	2002									
Genotype	April		May		June		July		August	
	centimeters									
	Mean	SD[1]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Bardot	20.6*	3.5	19.8	3.9	21.1	3.9	22.0	3.8	19.9	4.1
Crenshaw	19.4	2.5	18.9	2.7	22.1	3.0	22.1	2.2	21.0	3.0
Highland	18.3	4.3	16.0	2.8	18.2	4.4	18.9	5.1	19.6	4.1
Penn A-4	18.5	2.3	18.9	2.6	21.4	3.0	22.5	3.4	20.4	3.5
Penncross	20.6*	2.1	20.4*	2.2	23.5*	3.2	24.7*	3.4	23.1*	3.6
Seaside	19.5	1.9	19.4	3.2	21.5	3.3	22.5	3.8	21.3	4.5
Streaker	20.0*	5.5	18.5	7.1	19.5	7.6	19.1	6.1	17.0	5.6
B99061R	15.2	1.9	13.6*	2.2	15.3*	1.3	15.4*	1.8	15.3*	2.0
ASR368 F1	17.1	1.1	16.9	2.2	19.2	2.5	20.4	3.0	19.5	3.4
LSD (0.05)	2.8		3.2		3.5		3.5		3.5	

* Mean diameter is significantly different from ASR368 according to Fisher's LSD ($\alpha = 0.05$) [1] SD = Standard deviation

Bermudagrass, full sun

The mean plant diameter of ASR368 F1 plants fell within the range of the mean diameter of B99061R or the conventional bentgrass cultivars and species growing in competition with bermudagrass in full sun in both 2001 and 2002 in Baldwin County, Alabama. In 2001, ASR368 F1 was not significantly different from the commercial creeping bentgrass cultivars on most dates except for two when it was significantly smaller than Crenshaw (March) and Penncross (June) (Table VIII-44). ASR368 F1 was significantly smaller than

Streaker from March through June 2001 but not by the end of the study. ASR368 F1 was also significantly smaller than SR7100 in June and August 2001. In 2002, ASR368 F1 was not significantly different from the conventional creeping bentgrass cultivars on most dates except for two when it was significantly smaller than Crenshaw (April; and June) and Seaside (June) (Table VIII-45). ASR368 F1 was significantly larger than B99061R on all dates other than for the final observation (Table VIII-45).

Table VIII-44. Mean plant diameter (cm) of ASR368 F1, B99061R and conventional bentgrass cultivars during March 2001 to August 2001 in full sun in Baldwin County, Alabama

	2001											
	March		**April**		**May**		**June**		**July**		**August**	
	centimeters											
Genotype	Mean	SD[2]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Backspin	17.1	2.6	15.8	2.6	15.6	2.0	15.1	1.6	13.4	1.2	11.7	1.0
Crenshaw	18.8*	2.3	17.2	2.8	16.5	1.1	15.8	1.7	13.9	1.1	12.9	1.4
Highland	16.5	4.4	16.2	4.0	15.7	3.9	15.5	3.7	12.8	1.4	12.7	2.3
Penncross	17.0	2.2	16.4	1.7	16.8	1.6	16.9*	1.7	14.0	1.8	12.8	1.2
Penn A-4	13.5	2.5	14.5	3.6	14.3	1.8	13.5	2.5	12.6	1.6	12.3	1.2
SR7100	17.0	2.3	16.2	2.2	16.1	2.1	16.3*	1.4	14.8	2.2	13.7*	0.6
Streaker	18.6*	3.4	20.2*	2.2	18.6*	3.2	16.9*	3.0	14.1	1.5	11.6	0.9
B99061R	15.7	1.9	16.0	2.1	14.9	0.8	14.3	1.6	11.7	0.6	11.8	0.3
ASR368 F1	15.2	1.7	13.8	3.3	14.6	1.8	13.6	1.4	13.1	2.5	12.3	1.1
LSD (0.05)	3.1		3.7		2.7		2.4		NS[1]		1.4	

* Mean diameters are significantly different from ASR368 according to Fisher's LSD ($\alpha = 0.05$).
[1] NS = Mean diameter is not significantly different from ASR368 according to Fisher's LSD ($\alpha = 0.05$).
[2] SD = Standard deviation

Table VIII-45. Mean plant diameter (cm) of ASR368 F1, B99061R and conventional bentgrass cultivars in full sun from April 2002 to August 2002 in Baldwin County, Alabama

	2002									
Genotype	**April**		**May**		**June**		**July**		**August**	
	centimeters									
	Mean	SD[1]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Bardot	15.6	1.5	14.4	1.6	13.6	0.9	13.3	1.4	12.6	1.5
Crenshaw	16.4*	2.3	13.7	2.6	14.4*	1.8	14.0	1.6	12.1	1.3
Highland	15.3	2.8	13.1	3.1	12.7	1.6	12.8	2.3	10.7	1.3
Penn A-4	12.3	2.7	11.4	1.8	11.8	2.1	11.8	2.4	10.3	1.8
Penncross	13.8	1.4	13.1	2.5	13.6	2.1	12.9	2.0	11.5	1.5
Seaside	15.4	2.5	14.2	2.6	14.5*	2.1	13.5	2.0	11.2	1.1
Streaker'	15.7	3.2	13.7	3.4	13.1	2.7	12.5	1.6	10.3	1.5
B99061R	9.9*	2.8	8.6*	2.5	9.3*	1.6	9.8*	1.8	10.5	1.9
ASR368 F1	14.1	2.0	12.3	1.6	12.3	1.6	12.7	2.4	11.5	1.5
LSD (0.05)	2.2		2.3		1.8		1.8		1.5	

* Mean diameters are significantly different from ASR368 according to Fisher's LSD ($\alpha = 0.05$). [1] SD = Standard deviation

VIII.C.2.2.3. Conclusion for competitive and managed warm season relative growth studies

No observations were made in either the sun or shade relative growth studies conducted in 2001 and 2002 to indicate that ASR368 F1 plants are any more fit or aggressive than the traditional *Agrostis* cultivars in competition with St. Augustinegrass or bermudagrass in shade or sun, respectively. ASR368 F1 was consistently larger than B99061R in these studies in both 2001 and 2002. However, this difference may be attributed to somaclonal variation, the event selection criteria and/or the forward breeding process rather than the insertion of the *cp4 epsps* gene.

VIII.C.2.3. Relative growth under reduced irradiance (shade)

Shade or reduced light quality is a limiting factor in the establishment and persistence of creeping bentgrass in managed and unmanaged ecosystems. Studies were initiated in 2001 and 2002 under low light conditions in Ingham County, Michigan to compare the growth and competitive ability of ASR368 FI with B99061R and several commercial bentgrass cultivars. Conventional cultivars included Backspin, Penn A-4, Crenshaw and Penncross creeping bentgrasses and related species *A. castellana*, 'Highland' dryland bentgrass, *A. capillaris*, 'SR7100' colonial bentgrass and *A. gigantea*, 'Streaker' redtop bentgrass.

VIII.C.2.3.1. Experimental methods

Plant propagation and establishment

The creeping bentgrass cultivars or populations were established from stolon nodes or seed and grown in a greenhouse in Oregon prior to shipment to Michigan. Immediately

after receipt at the Michigan location, the plants were clipped to 1 inch or less and then transplanted in the indoor shade research facility in Ingham County. The facility is covered with a translucent material that provides 17% to 20% of normal irradiant sunshine in a uniform environment.

Plantings occurred on May 31, 2001 and February 2, 2002 and similar methods were used each time. The plants were set in the soil so that the root and shoot interface (crown) was at or slightly below the soil surface and firm contact between the potting media was maintained with the soil. The trial area was maintained under a nutrient regime appropriate for good plant health and was irrigated every day in the first two weeks of establishment. Thereafter supplemental irrigation was applied three times each week to prevent drought stress for the duration of the trial.

Data collected

The trial was conducted in a randomized complete block design in three replications. The competitive ability of each genotype was evaluated by measuring the ground cover of each creeping bentgrass patch. Ground cover was measured by centering a grid with 121 points on the plant, counting the number of points corresponding with green tissue and then calculating the mean number of points with green tissue.

Statistical analysis

An analysis of variance and least significant difference (LSD) test at the $\alpha = 0.05$ level were used to make multiple comparisons of the mean ground cover of the different genotypes in both 2001 and 2002.

VIII.C.2.3.2. Results

ASR368 F1 was intermediate to the creeping bentgrass cultivars, Backspin, Penn A-4, Crenshaw and Penncross and the tissue culture line B99061R, during all measurement dates in 2001 (Table VIII-46). ASR368 F1 progeny and the conventional creeping bentgrass cultivars were significantly larger than Highland dryland bentgrass, Streaker redtop bentgrass and SR 7100 colonial bentgrass on all dates.

In the 2002 trial, ASR368 F1 progeny were not significantly different from and within the range of B99061R and the four conventional creeping bentgrass cultivars, Backspin, Crenshaw, Penn A-4 and Penncross, during all measurement dates (Table VIII-47). Also similar to 2001 trial, the Streaker, Highland and SR7100 were consistently among those cultivars with the lowest ground coverage/plant.

VIII.C.2.3.3. Conclusions of reduced irradiance relative growth studies

ASR368 F1 progeny population plants were generally intermediate in ground cover compared to those of B99061R or the other conventional creeping bentgrass cultivars in both the 2001 and 2002 evaluations. Therefore, it can be concluded that the vegetative growth of ASR368 progeny in either managed or unmanaged ecosystems under reduced light conditions is not expected to be different from conventional creeping bentgrass.

Table VIII-46. Comparative growth as measured by mean ground cover of ASR368 F1 progeny, B99061R and *Agrostis* reference genotypes under reduced irradiance conditions in 2001 in Ingham County, Michigan in 2001

Genotype	Mean Ground Cover[1]		
	2001		
	June 28	July 12	September 3
Streaker	18.7d*	17.7e	17.7bc
Highland	24.3d	15.7e	14.7c
SR 7100	31.0d	25.0de	3.3c
B99061R	44.3c	31.0cd	41.0ab
ASR368 F1	47.7c	45.0ab	49.3a
Penn A-4	50.3bc	36.3bcd	45.0a
Backspin	50.7bc	43.3abc	45.0a
Penncross	61.3ab	47.3ab	58.7a
Crenshaw	64.0a	53.3a	53.0a
LSD (α= 0.05)	12.47	13.33	25.22
SD[2]	7.2	7.7	14.57
CV[3]	16.52	22.03	40.02

[1] Mean ground cover is based on total points out of a maximum value of 121 points/ft^2
[2] SD = Standard Deviation
[3] CV = Coefficient of Variation
* Means followed by same letter within dates are not significantly different (α = 0.05, Student-Newman-Keuls)

Table VIII-47. Comparative growth as measured by ground cover of ASR368 F1 progeny, B99061R and *Agrostis* reference genotypes under reduced irradiance conditions in Ingham County, Michigan in 2002

Genotype	Mean Ground Cover[1]				
	2002				
	February 22	March 14	April 4	April 17	May 17
ASR368 F1	1.3a*	4.4ab	15.8ab	19.1a	36.7ab
B99061R	1.6a	4ab	17.7ab	22.2a	29.8bc
Streaker	1.0a	2.6b	7.2b	8b	14.2cd
Penn A-4	1.7a	4.8ab	18.8a	22.8a	48.4ab
Highland	1.0a	2.4b	7.7b	8.2b	8.7d
Backspin	1.6a	4.4ab	17.2ab	21.9a	47ab
SR7100	1.4a	4.1ab	15.3ab	15.2ab	19.2cd
Penncross	1.2a	4.2ab	20.1a	20.1a	47.6ab
Crenshaw	1.2a	5.0a	20.1a	21.8a	54.3a
LSD ($\alpha = 0.05$)	0.44	1.49	7.27	7.2	13.95
SD	0.25	0.86	4.2	4.16	8.06
CV	18.87	21.45	27.03	23.48	23.71

[1] Mean ground cover is based on total points out of a maximum value of 121 points/ft^2

[2] SD = Standard Deviation

[3] CV = Coefficient of Variation

* Means followed by same letter within dates are not significantly different ($\alpha = 0.05$, Student-Newman-Keuls)

VIII.C.3. Conclusion for relative growth studies

ASR368 and its progeny displayed no increase in vegetative growth, aggressiveness, invasiveness or relative fitness compared to conventional creeping bentgrass cultivars when established in bare soil with no competition or with competition from other turfgrasses in cool, warm and transition climate zones. ASR368 also demonstrated no competitive advantage in direct sun or shade or reduced light (Ingham County, MI and Baldwin County, AL).

There are a number of reports in the scientific literature confirming that the variability in creeping bentgrass growth observed in these studies was less than that typically observed due to differences in climate and cultural practices, such as mowing, irrigation, and fertilization (Turgeon, 1985; Beard, 1973; Holt and Payne, 1951). Therefore, given the results from these experiments conducted in twelve diverse environments over three years, which further confirm reports in the scientific literature, ASR368 and its progeny would not be expected to grow in a different manner in either managed or unmanaged ecosystems than conventional creeping bentgrass. Furthermore, these findings also support an overall conclusion that ASR368 is not different than conventional creeping bentgrass in terms of plant pest or weediness characteristics and is no more likely to pose a plant pest risk than conventional creeping bentgrass.

VIII.D. Flowering

Changes in the reproductive processes of flowering or inflorescence formation may be an unintended effect of the plant transformation process. Several flowering characteristics could enhance the relative fitness of a given species. For example, a creeping bentgrass variety that sets flowers earlier in the season or has a longer flowering period may have enhanced reproductive potential. Characteristics such as heading date, anthesis initiation date, anthesis duration and maturity date of ASR368 relative to other creeping bentgrasses were evaluated in various environments. This section reviews the relative floral development of ASR368 in greenhouse studies conducted at Iowa State University in 2001 and 2002 and field studies conducted in Franklin County, Washington during 2000 and 2001, and Jefferson County, Oregon in 2002 (USDA # 00-220-02n, 01-01714n, 01-177-01n and 00-177-02n).

VIII.D.1. Greenhouse studies

VIII.D.1.1. Experimental methods

Genotypes

In 2001, the time to first inflorescence emergence (days to heading, heading date), anthesis initiation and anthesis duration of the ASR368 R0 and F1 progeny were compared to that of B99061R, and plants of the conventional cultivars Penncross, Penn A4 and Crenshaw. In 2002, ASR368 R0 plants and F1 and F2 progeny populations were compared to plants of B99061R, Penncross, Penn A-4 and Crenshaw.

Plant Propagation

2001 Study: Plants of R0 generation ASR368, B99061R and the conventional cultivars were established in 6" pots from vegetative stolon nodes during September 2000. ASR368 F1 progeny were derived from seed harvested from maternal Elite Parent Plants (EPP) pollinated by ASR368 R0 in a crossing block during summer, 2000. The ASR368 F1 plants were established from seed and maintained similar to the other plant material during September 2000. All R0 and F1 ASR368 plants were hemizygous and dominant for the *cp4 epsps* gene. All plants were propagated and grown to maintain similar plant age across genotypes within each experiment. All plants were maintained in a cold frame poly-house in Marion County, Oregon for development, vernalization and floral induction prior to overnight shipment to Iowa.

Approximately each week beginning in March 2001 and ending in May 2001, one set (batch) of plants was shipped from vernalization and floral induction conditions (natural day length, 0 to 10^0C) in Oregon to floral initiation conditions (16 hr light, 20 to 25^0C) in the greenhouse in Iowa. Plants were irrigated as needed to prevent plant moisture stress. Once moved into the greenhouse, irrigation was performed twice per week and greenhouse temperature was set at 20 to 21^0C.

Plants were fertilized to maintain good plant vigor and health and to avoid nutrient deficiency symptoms. Insecticides and fungicides were applied as needed following standard agronomic practices to control pests.

Plants shipped with each batch from Oregon to Iowa in 2001 are listed below:

- three clones of ASR368 R0;
- three random ASR368 F1 progeny plants;
- three clones of B99061R; and
- five random plants each of Penncross, Crenshaw and Penn A-4.

2002 Study: Plants of ASR368 R0, B99061R and the conventional cultivars were established from vegetative stolon nodes during September 2001. ASR368 F1 plants were derived from seed harvested from maternal EPPs pollinated by ASR368 R0 in a crossing block during summer, 2001. Similarly, ASR368 F2 plants were derived from seed harvested from maternal EPPs pollinated by ASR368 F1 plants in a crossing block during summer, 2001. Both F1 and F2 progeny plants were established from random seeds. All ASR368 R0, F1 and F2 plants are hemizygous dominant for the *cp4 epsps* transgene. All plants were propagated and grown to maintain similar plant age across genotypes within each experiment. All plants were maintained in a cold frame poly-house in Marion County, Oregon for development, vernalization and floral induction prior to overnight shipment to Iowa.

Approximately each week beginning in March 2002 and ending in June 2002, one set (batch) of plants was shipped from vernalization and floral induction conditions (natural day length, 0 to 10^0C) in Oregon to floral initiation conditions (16 hr light, 20 to 25^0C) in the greenhouse in Iowa. Plants were maintained as in 2001.

Plants shipped with each batch from Oregon to Iowa in 2002 are listed below:

- three clones of the ASR368 R0 plant;
- five random ASR368 F1 progeny plants;
- five random ASR368 F2 progeny plants;
- three clones of B99061R; and
- five random plants each of three conventional cultivars: Penncross, Crenshaw and Penn A-4.

Data Collected (2001 and 2002)

Inflorescence initiation (first head date) for a plant was determined as the number of days required from the initial placement in floral initiation conditions in the Iowa greenhouse to the emergence of the first three panicles on a plant.

Anthesis date was determined as the number of days required from the initial placement in floral initiation conditions in the greenhouse to the date when the first three panicles on a plant had anthers exterted from the floret.

The duration of anthesis was defined as the number of days from the beginning of anthesis to the end of anthesis for each genotype. End of anthesis was identifed as the day when no additional panicles were observed shedding pollen.

Statistical analysis

The data analysis for both the 2001 and 2002 studies was performed using SAS (version 8.1, the SAS Institute, Cary, NC). The effect of genotype was fixed and the effect of block

(different batches) was random in these experiments. Genotype means were calculated taking into consideration block effects and a mixed model was used for analyzing the data. Various comparisons were constructed to determine if the ASR368 genotypes were significantly different from B99061R or any of the conventional cultivars.

VIII.D.1.2. Results of greenhouse studies

Number of days required for inflorescence emergence (first head date)

The mean number of days required for inflorescence emergence (first head date) for the five batches (replications) of each genotype or population tested in 2001 and the specific comparisons of the control cultivars with ASR368 R0 and F1 are presented in Tables VIII-48 and VIII-49. The mean days to first head date for both ASR368 R0 and F1 were not significantly different from B99061R and Penn A-4. However, significantly more days were required to first head date by Crenshaw and Penncross than by ASR368 F1 progeny. Although Penncross took significantly longer to reach first head date than the ASR368 R0 plants, the days required for Crenshaw was not significantly different from that of ASR368 R0 plants.

Tables VIII-50 and VIII-51 depict the mean number of days required for inflorescence emergence for the five batches of each genotype tested in 2002 and the specific comparisons of the reference genotypes and cultivars with R0, F1 and F2 ASR368. As in 2001, the mean number of days to first head date for all plants derived from ASR368 was not significantly different from B99061R or Penn A-4. In addition, Penncross was not, while Crenshaw was, significantly different from ASR368 R0, F1 and F2 plants for days to first head date.

Table VIII-48. Number of observations, mean, standard deviation and the minimum and maximum values for the number of days required for first head date among ASR368 R0 and F1 progeny, B99061R and the three conventional cultivars grown in the greenhouse in 2001

Genotype	Number of observations	Mean (days)	Std deviation	Minimum value	Maximum value
Penn A-4	25	18.11	4.50	9	28
Crenshaw	25	18.92	3.93	13	28
Penncross	25	19.80	2.96	14	26
B99061R	15	16.40	2.67	11	19
ASR368 R0	15	17.33	3.15	13	25
ASR368 F1	15	16.92	4.56	10	29

Table VIII-49. Comparisons between ASR368 R0 or F1 progeny and B99061R or the three conventional cultivars for first head date in 2001

Contrast	Mean Difference	StdErr	Probt
B99061R vs. ASR368 R0	16.40 - 17.33 =-0.93	1.09	0.40
B99061R vs. ASR368 F1	16.40 - 16.92 =-0.52	0.99	0.60
Penn A-4-vs. ASR368 R0	18.11 - 17.33 = 0.78	0.92	0.40
Penn A-4 vs. ASR368 F1	18.11 - 16.92 = 1.19	0.80	0.14
Crenshaw vs. ASR368 R0	18.92 - 17.33 = 1.59	0.90	0.08
Crenshaw vs. ASR368 F1	18.92 - 16.92 = 2.00	0.78	0.01*
Penncross vs. ASR368 R0	19.80 - 17.33 = 2.47	0.91	0.01*
Penncross vs ASR368 F1	19.80 - 16.92 = 2.88	0.79	0.0004*

* Means are significantly different ($\alpha = 0.05$)

Table VIII-50. Number of observations, mean, standard deviation and the minimum and maximum values for the number of days required for first head date among ASR368 R0 and F1 and F2 progeny and B99061R and the three conventional cultivars grown in the greenhouse in 2002

Genotype	Number of observations	Mean (days)	Std deviation	Minimum value	Maximum value
Penn A-4	5	22.0	1.9	20	24
Crenshaw	5	27.8	4.4	20	30
Penncross	5	24.2	4.1	21	31
B99061R	3	18.7	1.5	17	20
ASR368 R0	3	19.3	4.0	17	24
ASR368 F1	5	22.4	1.8	20	25
ASR368 F2	5	20.8	4.1	14	24

Table VIII-51. Comparisons between ASR368 R0 and F1 or F2 progeny and B99061R or the three conventional cultivars for first head date in 2002

Contrast	Mean Difference	StdErr	Probt
B99061R vs. ASR368 R0	18.7– 19.3 =-0.7	2.76	0.81
B99061R vs. ASR368 F1	18.7– 22.4 =-3.7	2.47	0.14
B99061R vs. ASR368 F2	18.7– 24.2 =-2.1	2.47	0.40
Penn A-4-vs. ASR368R0	22.0– 19.3 = 2.7	2.47	0.29
Penn A-4 vs. ASR368 F1	22.0– 22.4 =-0.4	2.14	0.85
Penn A-4 vs. ASR368 F2	22.0– 20.8 = 1.2	2.14	0.58
Crenshaw vs. ASR368 R0	27.8– 19.3 = 8.5	2.47	0.002*
Crenshaw vs. ASR368 F1	27.8– 22.4 = 5.4	2.14	0.02*
Crenshaw vs. ASR368 F2	27.8– 20.8 = 7.0	2.14	0.003*
Penncross vs. ASR368 R0	24.2– 19.3 = 4.9	2.47	0.06
Penncross vs. ASR368 F1	24.2– 22.4 = 1.8	2.14	0.41
Penncross vs. ASR368 F2	24.2– 20.8 = 3.4	2.14	0.13

* Means are significantly different ($\alpha = 0.05$)

Number of days required for anthesis initiation:

Tables VIII-52 and VIII-53 contain the mean number of days required for anthesis initiation for the five batches (replications) of each genotype or population tested in 2001 and the specific comparisons between the ASR368 generation treatments and B99061R or the conventional cultivars. Tables VIII-54 and VIII-55 contain similar information for the 2002 experiment.

Plants derived from ASR368 fell within the range of the conventional creeping bentgrass cultivars for days required to reach anthesis in both 2001 and 2002. In 2001, the ASR368 R0 and F1 were not significantly different from B99061R, Penncross and Crenshaw but Penn A-4 required significantly fewer days for anthesis initiation than the ASR368 R0. In 2002, plants of ASR 368 R0, F1 and F2 were not significantly different from B99061R, Penncross and Penn A-4, but Crenshaw required significantly more days for anthesis initiation than the ASR368 R0 plants and the F1 and F2 progeny populations.

Table VIII-52. Number of observations, mean, standard deviation and the minimum and maximum values for the number of days required for anthesis initiation among ASR368 R0 and F1 progeny, B99061R and the three conventional cultivars grown in the greenhouse in 2001

Genotype	Number of observations	Mean estimate (days)	Std deviation	Minimum value	Maximum value
Penn A-4	25	30.09	4.13	20	39
Crenshaw	25	30.71	3.93	26	43
Penncross	25	31.62	2.98	23	37
B99061R	15	32.07	2.19	25	34
ASR368 R0	15	32.8	2.51	29	37
ASR368 F1	15	30.81	5.25	20	45

Table VIII-53. Comparisons between ASR368 R0 or F1 progeny and B99061R and the three conventional cultivars for number of days required for anthesis initiation in 2001

Contrast	Mean Difference	StdErr	Probt
B99061R vs. ASR368 R0	32.07- 32.80 =-0.73	1.39	0.59
B99061R vs. ASR368 F1	32.07- 30.81 = 1.26	1.24	0.31
Penn A-4-vs. ASR368 R0	30.09- 32.80 =-2.71	1.16	0.02*
Penn A-4 vs. ASR368 F1	30.09- 30.81 =-0.72	0.99	0.47
Crenshaw vs. ASR368 R0	30.71- 32.80 =-2.09	1.12	0.07
Crenshaw vs. ASR368 F1	30.71- 30.81 =-0.10	0.96	0.92
Penncross vs. ASR368 R0	31.62- 32.80 =-1.18	1.14	0.30
Penncross vs. ASR368 F1	31.62- 30.81 = 0.81	0.98	0.41

* Means are significantly different ($\alpha = 0.05$)

Table VIII-54. Number of observations, mean, standard deviation and the minimum and maximum values for anthesis initiation among ASR368 R0 and F1 and F2 progeny, B99061R and the three conventional cultivars grown in the greenhouse in 2002

Genotype	Number of observations	Mean (days)	Std deviation	Minimum value	Maximum value
Penn A-4	5	30.6	1.7	28	32
Crenshaw	5	38.8	5.0	30	42
Penncross	5	33.2	5.1	30	42
B99061R	3	31.0	2.0	29	33
ASR368 R0	3	29.0	3.0	26	32
ASR368 F1	5	30.6	1.5	29	33
ASR368 F2	5	29.0	2.9	25	33

Table VIII-55. Comparisons between ASR368 R0 and F1 or F2 progeny and B99061R and the three conventional cultivars for number of days required for anthesis initiation in 2002

Contrast	Mean Difference	StdErr	Probt
B99061R vs. ASR368 R0	31.0– 29.0 = 2.0	2.80	0.48
B99061R vs. ASR368 F1	31.0– 30.6 = 0.4	2.50	0.87
B99061R vs. ASR368 F2	31.0– 29.0 = 2.0	2.50	0.43
Penn A-4-vs. ASR368 R0	30.6– 29.0 = 1.6	2.50	0.53
Penn A-4 vs. ASR368 F1	30.6– 30.6 = 0.0	2.17	1.00
Penn A-4 vs. ASR368 F2	30.6– 29.0 = 1.6	2.17	0.47
Crenshaw vs. ASR368 R0	38.8– 29.0 = 9.8	2.50	0.0007*
Crenshaw vs. ASR368 F1	38.8– 30.6 = 8.2	2.17	0.0009*
Crenshaw vs. ASR368 F2	38.8– 29.0 = 9.8	2.17	0.0001*
Penncross vs. ASR368 R0	33.2– 29.0 = 4.2	2.50	0.11
Penncross vs. ASR368 F1	33.2– 30.6 = 2.6	2.17	0.24
Penncross vs. ASR368 F2	33.2– 29.0 = 4.2	2.17	0.07

* Means are significantly different ($\alpha = 0.05$)

Number of days from the beginning of anthesis to the end of anthesis of each genotype (anthesis duration):

ASR368 and its progeny were not significantly different from B99061R and at least one of the three conventional cultivars for duration of anthesis in both the 2001 and 2002 experiments. Tables VIII-56 and Table VIII-57 depict the mean number of days for anthesis duration for the five batches (replications) of each genotype tested and the specific comparisons between the ASR368 treatments with B99061R and the conventional cultivars in 2001. Tables VIII-58 and VIII-59 contain the same information for the experiment conducted in 2002. The p-values ($\alpha = 0.05$) indicate that the duration of anthesis for ASR368 R0 were not significantly different from B99061R or any of the three conventional cultivars in 2001. ASR368 F1 progeny were not significantly different from B99061R or Crenshaw, but were significantly different from Penncross and Penn A-4 during 2001. ASR368 R0, F1 and F2 plants were not significantly different from B99061R or any of the conventional cultivars during 2002.

Table VIII-56. Number of observations, mean, standard deviation and the minimum and maximum values for anthesis duration among ASR368 R0 and F1 progeny, B99061R and the three conventional cultivars grown in the greenhouse in 2001.

Genotype	Number of observations	Mean (days)	Std deviation	Minimum value	Maximum value
Penn A-4	25	11.77	7.05	3	27
Crenshaw	25	14.59	5.33	8	25
Penncross	25	13.19	6.76	4	28
B99061R	15	12.87	5.32	7	24
ASR368 R0	15	11.60	3.46	6	18
ASR368 F1	14	16.35	5.48	8	26

Table VIII-57. Comparisons between ASR368 R0 or F1 progeny and B99061R and the three conventional cultivars for number of days required for anthesis duration in 2001

Contrast	difference Mean	StdErr	Probt
B99061R vs. ASR368 R0	12.87 - 11.60 = 1.27	2.11	0.55
B99061R vs. ASR368 F1	12.87 - 16.35 =-3.48	1.92	0.08
Penn A-4-vs. ASR368 R0	11.77 - 11.60 = 0.17	1.78	0.92
Penn A-4 vs. ASR368 F1	11.77 - 16.35 =-4.58	1.55	0.004*
Crenshaw vs. ASR368 R0	14.59 - 11.60 = 2.99	1.73	0.09
Crenshaw vs. ASR368 F1	14.59 - 16.35 =-1.76	1.5	0.24
Penncross vs. ASR368 R0	13.19 - 11.60 = 1.59	1.76	0.37
Penncross vs. ASR368 F1	13.19 - 16.35 =-3.16	1.53	0.04*

* Means are significantly different ($\alpha = 0.05$)

Table VIII-58. Number of observations, mean, standard deviation and the minimum and maximum values for anthesis duration among ASR368 R0, F1 and F2 progeny and B99061R and the three conventional cultivars grown in the greenhouse in 2002

Genotype	Number of observations	Mean (days)	Std deviation	Minimum value	Maximum value
Penn A-4	5	27.4	3.4	23	32
Crenshaw	5	27.6	2.1	25	30
Penncross	5	25.4	7.9	12	32
B99061R	3	25.7	1.2	25	27
ASR368 R0	3	23.3	3.5	20	27
ASR368 F1	5	25.6	1.1	24	27
ASR368 F2	5	25.6	1.8	24	28

Table VIII-59. Comparisons between ASR368 R0 and F1 or F2 progeny and B99061R and the three conventional cultivars for number of days required for anthesis duration in 2002

Contrast	Mean Difference	StdErr	Probt
B99061R vs. ASR368 R0	25.7 – 23.3 = 2.3	3.14	0.46
B99061R vs. ASR368 F1	25.7 – 25.6 = 0.07	2.81	0.98
B99061R vs. ASR368 F2	25.7 – 25.6 = 0.07	2.81	0.98
Penn A-4-vs. ASR368 R0	27.4 – 23.3 = 4.1	2.81	0.16
Penn A-4 vs. ASR368 F1	27.4 – 25.6 = 2.0	2.43	0.47
Penn A-4 vs. ASR368 F2	27.4 – 25.6 = 1.84	2.43	0.47
Crenshaw vs. ASR368 R0	27.6 – 23.3 = 4.3	2.81	0.14
Crenshaw vs. ASR368 F1	27.6 – 25.6 = 2.0	2.43	0.42
Crenshaw vs. ASR368 F2	27.6 – 25.6 = 2.0	2.43	0.42
Penncross vs. ASR368 R0	25.4- - 23.3 = 2.1	2.81	0.47
Penncross vs. ASR368 F1	25.4 – 25.6 = -0.2	2.43	0.94
Penncross vs. ASR368 F2	25.4 – 25.6 = -0.2	2.43	0.94

VIII.D.2. Field studies

VIII.D.2.1. Experimental methods

In 2001, ASR368 R1 seedlings resulting from a cross of ASR368 R0 with Elite Parent Plants known to be segregating for GT and GS phenotypes were evaluated in trials at two locations in Franklin County, Washington (USDA # 00-220-02n and 01-177-02n). The ASR368 R1 population harvested from the R0 plant was expected to segregate in a 1:1 ratio for the GT and GS phenotypes (Figure V-17.).

In 2002, plants of ASR368 F1 and F2 progeny and three conventional creeping bentgrass cultivars Backspin, Crenshaw and Penn A-4 were evaluated at the two locations in Jefferson County, Oregon (USDA #01-177-02N). The ASR368 F1 progeny and the three conventional cultivars were compared in the first study location and the ASR368 F2 progeny and conventional cultivars were evaluated at the second study location.

2000 – 2001 trials in Franklin County, Washington

Plant Propagation

Seedlings were allowed to mature in a greenhouse in Marion County, Oregon until field release. Plantings were made at two locations in Franklin County, Washington. Site I was planted on October 17, 2000, and Site II was planted in on April 30, 2001.

Planting and establishment - Fall Planting

Sixty random ASR368 R1 plants were transferred to the field in Franklin County Washington on October 17, 2000 upon reaching the 2 to 3-tiller stage. The planting consisted of six rows with ten plants each on five foot spacing. Floral induction and vernalization occurred under natural environmental conditions for subsequent floral initiation and seed production during summer 2001.

Planting and establishment - Spring Planting

Plants intended for planting during the spring were transplanted to six-inch plastic pots containing an organic peat potting mix when plants reached the five to six tiller stage during fall 2000. All plants were maintained in Marion County, Oregon for floral induction and subsequently moved to the second field site in Franklin County, Washington on April 30, 2001 for floral initiation and seed production during the summer. Planting procedures were similar to the fall 2000 planting.

Determination of GT and GS subpopulations

ASR368 R1 plants in the fall planting (field planted in October 2000) were individually sampled and tested during March to May 2001 for the presence of the *cp4 epsps* gene using PCR (polymerase chain reaction) techniques. Those plants testing positive for the gene were assigned to the GT sub-population. Those plants testing negative for the presence of the gene were assigned to the GS sub-population. Plants of ASR368 R1 in the spring planting (field planted on April 30, 2001) were individually tested for presence or absence of the CP4 EPSPS protein with a non-destructive immuno-assay strip test (Strategic Diagnostics Inc, Newark, DE).

Data collection

Heading date was recorded at the two Washington locations as the date when the first three seedheads on a plant were exerted from the flag leaf collar. Anthesis date was recorded as the date when the first three seedheads on a plant had florets with anthers exerted from the glumes.

Statistical analysis

The flowering characteristics of the ASR368 R1 GT and GS were analyzed by pooling the data for each characteristic after having accounted for the potential variability contributed by location using Friedman's test ($\alpha = 0.05$).

2001 - 2002 trials in Jefferson County, Oregon ASR368 F1 trial

ASR368 F1 seed harvested the summer of 2000 was planted in the Marion County, Oregon poly-house in August 2000. The germinated seedlings were sprayed with Roundup Pro herbicide to remove the glyphosate susceptible segregates. Randomly selected ASR368 F1 GT seedlings were then transplanted and maintained in the poly-house until planting in the field the following year. Three conventional creeping bentgrass cultivars were concurrently cultivated from random seedlings and maintained in the same fashion as the ASR368 F1 plants. The ASR368 F1 field trial was field-planted with one-year old plants of all genotype populations.

ASR368 F2 trial

Seed of the ASR368 F2 GT were harvested, planted and selected during the summer of 2001 in the same manner as the F1 progeny in the previous year. The conventional conventional cultivars were also initiated from random seedlings at the same time and maintained the same as the ASR368 F2 plants. The ASR368 F2 trial was field-planted with mature plants with 3 to 4 tillers from each genotype population.

Data collection

Heading date was recorded at the two Oregon locations as the date when the first three seedheads on a plant were exerted from the flag leaf collar. Anthesis date was recorded as the date when the first three seedheads on a plant had florets with anthers exerted from the glumes. Anthesis end date was documented as the date when the last three seedheads on a plant had florets with anthers exerted from the glumes. Anthesis duration was calculated as the number of days from the beginning of anthesis to the end of anthesis. Panicle maturity date was documented as the date when the majority of the panicles were mature for the harvest process. Maturity duration period was calculated as the number of days from the beginning of anthesis to the panicle (seed head) maturity date.

Statistical analysis

The data from the ASR368 F1 and F2 evaluations were analyzed separately using the Kruskal-Wallis test ($\alpha = 0.05$) to compare the ASR368 flowering characteristics with each conventional cultivar (paired comparison). Fisher's LSD ($\alpha = 0.05$) was used post-hoc for pair-wise testing between groups.

VIII.D.2.2. Results of field studies 2000 - 2001

There were no significant differences in the median heading dates between the ASR368 R1 GT and GS segregants in either the fall 2000 or spring 2001 planting locations. There was also no significant effect of planting date/location (site) for heading date of plants. The latest heading dates for the ASR368 R1 GT (June 3) and GS (June 5) segregants differed by just two days. Median heading dates were May 24 for GS R1 progeny and May 25 for GT R1 progeny. The difference was not statistically significant (Table VIII-60) and unlikely to be of biological significance given the variability found among commercial cultivars during the 2001 - 2002 trials.

The range (earliest to latest) for anthesis initiation date among plants extended three days longer for the ASR368 R1 GT progeny (28 days) versus the GS progeny (25 days). However, median anthesis date for GS progeny (June 13) was 2 days later than for GT progeny (June 11), but the difference was not statistically significant (Table VIII-61).

Table VIII-60. Earliest, latest and median heading date for ASR368 R1 GR and GS segregants evaluated during 2001 in Franklin County, Washington

Genotype	n	Earliest date	Latest date	Median date	Day range	P-value	
All GS	46	20-May	03-Jun	24-May	12 days	**Site**	**Segregants**
All GT	67	20-May	05-Jun	25-May	14 days	0.392	0.440

Table VIII-61. Earliest, latest and median anthesis date for ASR368 R1 GT and GS segregants evaluated during 2001 in Franklin County, Washington

Genotype	n	Earliest Date	Latest Date	Median Date	Day Range	P-value	
All GS	47	01-Jun	25-Jun	13-Jun	25 days	**Site**	**Segregants**
All GT	67	01-Jun	28-Jun	11-Jun	28 days	0.5054	0.5723

2001 - 2002

Heading date for the ASR368 F1 GT population extended from June 2, 2002 to June 12, 2002. The F1 population (mean ~ June 8) was not significantly different from the mean heading dates of Backspin and Crenshaw but was significantly different from Penn A-4 (mean 7.3, Table VIII-62). The mean heading date for the ASR368 F2 progeny was ~ June 8 and ranged from June 3 to June 12, 2002 was not significantly different from Backspin and Penn A 4, but was significantly different from Crenshaw (Table VIII-62).

The date anthesis began for ASR368 F1 progeny ranged from June 10 to June 17 with a mean date of June 12, 2002, which was not significantly different from the mean anthesis begin dates of the three commercial cultivar populations (Table VIII-63). The date anthesis began for ASR368 F2 progeny ranged from June 9 to June 19 with a mean date of June 11, 2002. This duration was not significantly different from Backspin but significantly different from Crenshaw and Penn A 4 (Table VIII-63).

The date upon which anthesis ended for the ASR368 F1 ranged from July 3 to July 8 with a mean anthesis duration of 24 days, which was not significantly different from the mean days duration for the conventional cultivars (Table VIII-64). The date anthesis ended for the ASR368 F2 progeny ranged from July 4 to July 9, with a mean anthesis duration of 25.6 days, which was also not significantly different from the conventional cultivars (Table VIII-64).

The duration of anthesis to seedhead maturity for ASR368 F1 progeny extended from June 12 to July 18, 2002 with a mean duration of 34.4 days. This period was not significantly different from that of Crenshaw and Penn A-4 but significantly different from that of Backspin (Table VIII-65). The mean duration for anthesis to seedhead maturity for ASR368 F2 extended from July 11 to July 18, 2002 with a mean of 34.6 days. This period was not significantly different from any of the conventional cultivars (Table VIII-65).

Table VIII-62. Mean heading date for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars within the month of June 2002 in Jefferson County, Oregon

	ASR368 F1 Evaluation				ASR368 F2 Evaluation			
	June Heading date				Heading date			
	n	Earliest	Latest	Mean	n	Earliest	Latest	Mean
ASR368	37	Jun-02	Jun-12	Jun-8.5	40	Jun-03	Jun-12	Jun-7.8
Backspin	28	Jun-06	Jun-12	Jun-8.1	38	Jun-06	Jun-12	Jun-7.5
Crenshaw	35	Jun-04	Jun-12	Jun-8.1	38	Jun-02	Jun-10	Jun-6.6*
Penn A-4	25	Jun-06	Jun-08	Jun-7.3*	38	Jun-06	Jun-08	Jun-7.8
p value ($\alpha = 0.05$)				0.032				0.004

* Means are significantly different from ASR368 F1 or F2 according to Fisher's exact test ($\alpha = 0.05$)

Table VIII-63. Mean anthesis begin date for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars within the month of June 2002 in Jefferson County, Oregon

	ASR368 F1 Evaluation				ASR368 F2 Evaluation			
	Anthesis begin date				Anthesis begin date			
	n	Earliest	Latest	Mean	n	Earliest	Latest	Mean
ASR368	37	Jun-10	Jun-17	Jun-12.2	40	Jun-09	Jun-19	Jun-11.4
Backspin	29	Jun-09	Jun-18	Jun-13.1	38	Jun-09	Jun-18	Jun-11.3
Crenshaw	35	Jun-09	Jun-17	Jun-11.7	37	Jun-09	Jun-15	Jun-10.4*
Penn A-4	25	Jun-09	Jun-18	Jun-11.7	39	Jun-09	Jun-17	Jun-10.5*
p value (α = 0.05)				0.14				0.03
LSD (α = 0.05)				1.25				0.89

* Means are significantly different from ASR368 F1 or F2 according to Fisher's exact test (α = 0.05)

Table VIII-64. Anthesis ending date and mean anthesis duration in days for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars within the month of July 2002 in Jefferson County, Oregon

	ASR368 F1 Evaluation						ASR368 F2 Evaluation					
	Anthesis ending date			Anthesis duration (days)*			Anthesis ending date			Anthesis duration (days)*		
	n	Earliest	Latest	Mean	Min	Max	n	Earliest	Latest	Mean	Min	Max
ASR368	37	Jul-03	Jul-08	24.1	18	27	40	Jul-04	Jul-09	25.6	17	30
Backspin	29	Jul-04	Jul-08	24.1	18	29	38	Jul-04	Jul-09	25.6	19	29
Crenshaw	35	Jul-03	Jul-08	24.8	19	29	38	Jul-03	Jul-09	26.4	21	30
Penn A-4	24	Jul-02	Jul-09	25.5	20	31	39	Jul-03	Jul-09	26.3	21	31
p value (α = 0.05)				0.23						0.43		
LSD (α = 0.05)				1.40						1.07		

* Anthesis duration = the number of days from the beginning of anthesis to the end of anthesis.

Table VIII-65. Seedhead maturity (date) and anthesis to seedhead maturity duration[1] (days) for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars within the months of June and July 2002 in Jefferson County, Oregon

Genotype	ASR368 F1 Evaluation						ASR368 F2 Evaluation					
	Seedhead Maturity Date			Anthesis to Seedhead Maturity (Days)			Seedhead Maturity Date			Anthesis to Seedhead Maturity (Days)		
	n	Earliest	Latest	Mean	Min	Max	n	Earliest	Latest	Mean	Min	Max
ASR368	37	Jul-13	Jul-18	34.4	29	39	39	Jul-11	Jul-18	34.6	26	38
Backspin	29	Jul-11	Jul-17	32.9*	26	38	38	Jul-12	Jul-18	34.3	27	39
Crenshaw	35	Jul-12	Jul-18	35.0	29	39	39	Jul-12	Jul-18	35.7	31	40
Penn A-4	25	Jul-11	Jul-18	33.8	25	39	39	Jul-12	Jul-18	35.5	28	39
p value (α = 0.05)				0.05						0.08		
LSD (α = 0.05)				1.44						1.15		

[1] The maturity duration period was determined as the number of days from the beginning of anthesis to the seed head maturity date.

* Means are significantly different from ASR368 F1 or F2 according to Fisher's exact test (α = 0.05)

VIII.D.3. Conclusion for flowering studies

Flowering characteristics of plants derived from ASR368 were compared to several conventional cultivars, B99061R and/or null segregants (GS) in greenhouse and field experiments conducted in 2001 and 2002. No consistently significant differences were detected between ASR368 R0 plants or F1 and F2 GT progeny and conventional creeping bentgrass plants and populations for flowering characteristics including heading date, anthesis initiation dates, anthesis duration or maturity dates. In 2001, ASR368 R0 and F1 GT were not significantly different from B99061R and at least one of the conventional bentgrass cultivars for the days required for heading, anthesis initiation and anthesis duration. In addition, no significant differences between GT and GS progeny derived from ASR368 were observed for heading date or anthesis date in field trials conducted at two locations in Franklin County, Washington during 2001.

In 2002, ASR368 F1 and F2 progeny were not significantly different from B99061R and at least one of the conventional cultivars for beginning head date, anthesis initiation, and duration of anthesis at the two Jefferson County, Oregon locations. ASR368 F1 had a significantly earlier mean heading date than Penn A-4 and required significantly more days from anthesis to seedhead maturity than Backspin. ASR368 F2 had significantly earlier mean heading and anthesis begin dates than Crenshaw.

The scientific literature also provides evidence of considerable variability in the initiation and duration of inflorescence and flowering by species of *Agrostis*. Christoffer (2003) evaluated the flowering characteristics of ASR368 and a number of other *Agrostis* and *Polypogon* species. In this study, which spanned two years, the initiation of anthesis for ASR368 was not different from that of several conventional *A. stolonifera* genotypes in both years evaluated. Christoffer (2003) also cites a number of authors that reported bentgrass anthesis to begin during May and June and end from June to August depending upon environment and species. More specifically, Davies (1953) reported that it takes *A. stolonifera* approximately 22 days from the initiation of first inflorescence to initiation of

anthesis. These findings support the results of the greenhouse and field studies presented in this section.

The results of the flowering studies presented in this section, which are consistent with the scientific literature, demonstrate that the flowering characteristics of ASR368 and its progeny are within the normal range of the conventional creeping bentgrass cultivars or and should not be expected to flower differently from them. The results also support an overall conclusion that ASR368 is not different than conventional creeping bentgrass in terms of plant pest or weediness characteristics and is no more likely to pose a plant pest risk than conventional creeping bentgrass.

VIII.E. Pollen Size, Viability and Longevity

Creeping bentgrass is a highly self-incompatible, essentially obligate outcrossing and wind-pollinated species. Within the United States, the potential for outcrossing among *Agrostis spp.* is understood, and low levels of gene flow have been viewed as an acceptable risk in agricultural seed production (Knowles, 1966). Studies by Wipff and Fricker (2001), Belanger et al. (2003) and Christoffer (2003) demonstrate that a low level (ca. 0.02%) of creeping bentgrass outcrossing to conventional creeping bentgrass (*Agrostis stolonifera* to *A. stolonifera*) may occur at distances up to 354 meters from the pollen source. However, the potential for intraspecific or interspecific gene flow is influenced not only by genetic compatibility but also by characteristics of the pollen itself, such as its size and duration or longevity of viability. If these characteristics of ASR368 pollen are altered, these plants could potentially have an altered ability to outcross with *A. stolonifera* or other species with which it is known to interbreed. Therefore, in this section, the biology of ASR368 pollen is evaluated. To conduct these studies, pollen was collected from the same greenhouse-grown plants used to assess the flowering characteristics in 2001 discussed in the previous section (Table VIII-48). Pollen characteristics in 2002 were analyzed from plants vernalized and induced to flower in the field and moved to the greenhouse for final floral initiation and pollen production. These experiments were conducted under USDA # 00-220-02n, 01-017-14n, 01-177-01n and 00-177-02n.

VIII.E.1. Experimental methods

Plant propagation and establishment

A detailed description of the genotypes evaluated in 2001 is provided in Section VIII.D.1.1.

During 2002, the pollen size, viability and longevity of nine plants representing four ASR368 F2 lines and three plants each of the conventional cultivars Backspin, Crenshaw and Penn A-4 were evaluated. These plants were propagated from seed during late summer 2001 and random plants were transplanted to Jiffy pots in September. On September 25, 2001, plants were field-planted in Jefferson County, Oregon. Plants were treated in similar fashion and exposed to natural vernalization conditions and then removed and shipped to Iowa greenhouses on May 30, 2002 when plants were beginning floral initiation. Upon receipt in Iowa, plants were placed in an environment for continued floral initiation similar to that used for greenhouse plants from 2001 studies described in Section VIII.D.1.1 of this petition.

Pollen collection

In both 2001 and 2002, an artificial germination medium containing 0.5 M sucrose, 1 mM boric acid, 2 mM $CaCl_2$ and 0.3% phytogel was used for comparisons of ASR368 creeping bentgrass plants to B99061R and/or conventional cultivars.

Pollen was collected at 11:00 am of the collecting day from inflorescences of three plants of each genotype and placed in an empty Petri plate. One subsample was immediately dusted onto the germination media to determine the initial base-line germination. The remnant pollen was immediately stored and then sub-sampled over time as described below in order to establish the decline of pollen viability over time.

Pollen size

Thirty pollen grains from each of three inflorescences from each plant were measured in 2001 and 2002 using a compound microscope. The diameter of a pollen grain was recorded to the nearest 1 micron.

Pollen storage and germination

In both 2001 and 2002, pollen samples were stored in a desiccator sealed with silicon gel in the dark in a Percival® growth chamber at 21 C. The humidity within the desiccator was adjusted to 67% using saturated $NaNO_2$. Pollen storage conditions were designed to mimic environmental conditions common in the Pacific Northwest during typical dates of creeping bentgrass anthesis. Pollen sub-samples were removed for a germination test every 30 minutes for three hours or until pollen lost viability. Pollen germination was performed at 25^0 C. After 1 hour of germination, Petri plates containing germinating pollen were moved to a refrigerator at 4^0 C to slow further pollen tube elongation in order to simplify counts of germinated pollen later. In most cases during 2001, more than 300 pollen grains were counted for each observation using a Nikon® compound microscope. In 2002, fewer pollen grains were available for this experiment so the pollen from the four F2 progeny lines and the three conventional cultivars was pooled within genotypes.

Statistical analysis

As described in Section VIII.D.1.1 of this petition.

VIII.E.2. Results

Pollen size

The mean, standard deviation, and the minimum and maximum pollen diameter for the ASR368 genotypes, B99061R and the conventional genotypes evaluated in 2001 and 2002 are presented in Table VIII-66 and VIII-68, respectively. The specific comparisons in pollen size between plants derived from event ASR368 and the control genotypes in 2001 and 2002 are provided in Tables VIII-67 and VIII-69, respectively. The p-values ($\alpha = 0.05$) from these comparisons indicate that the size of pollen from ASR368 R0 and F1 plants was not significantly different from that of B99061R or the three conventional creeping bentgrass cultivars in 2001. The pollen diameter of ASR368 F2 plants was not significantly different from that of the three conventional cultivars during 2002.

Table VIII-66. Number of observations, mean, standard deviation, and minimum and maximum values for pollen diameter (µm) among ASR368 R0 and F1 progeny, B99061R and three conventional cultivars in 2001

Genotype	Number of observations	Mean diameter	Std deviation	Minimum value	Maximum value
Penn A-4	15	42.6	2.43	38.2	47.8
Crenshaw	15	41.96	1.87	38.3	45.3
Penncross	14	42.09	2.6	36.7	45.2
B99061R	9	42.11	4.09	37.2	47.7
ASR368 R0	9	41.53	3.91	33	46.5
ASR368 F1	9	41.68	3.17	36.3	45.3

Table VIII-67. Mean difference, standard error and p value (α = 0.05) associated with each comparison between ASR368 R0 or F1 progeny and B99061R and three conventional cultivars for pollen diameter (µm) in 2001

Contrast	Mean differenc	StdErr	Probt
B99061R vs. ASR368 R0	42.11- 41.53 = 0.58	1.27	0.66
B99061R vs. ASR368 F1	42.11- 41.68 = 0.43	1.3	0.74
Penn A-4 vs. ASR368 R0	42.60- 41.53 = 1.07	1.14	0.36
Penn A-4 vs. ASR368 F1	42.60- 41.68 = 0.92	1.18	0.44
Crenshaw vs. ASR368 R0	41.96- 41.53 = 0.43	1.14	0.71
Crenshaw vs. ASR368 F1	41.96- 41.68 = 0.28	1.18	0.81
Penncross vs. ASR368 R0	42.09- 41.53 = 0.56	1.16	0.63
Penncross vs. ASR368 F1	42.09- 41.68 = 0.41	1.19	0.73

Table VIII-68. Mean pollen diameter (µm) of four ASR368 F2 progeny lines' and three conventional cultivars evaluated in 2002

Genotype	Number of observations	Mean diameter (µm)	Std deviation	Minimum value	Maximum value
ASR368 F2 (13-2-2)	3	41.00	4.77	35.5	44.0
ASR368 F2 (14-2-6)	3	37.33	4.16	34.0	42.00
ASR368 F2 (15-2-5)	3	39.67	2.36	37.00	41.5
ASR368 F2 (16-2-2)	3	39.83	2.75	37.0	42.5
Penn A-4	3	40.00	1.80	38.0	41.5
Crenshaw	3	37.17	4.01	33.0	41.0
Backspin	3	38.83	1.76	37.0	40.5

13-2-2, 14-2-6, 15-2-5 and 16-2-2 refer to the maternal EPP of the respective ASR368 F2 progeny.

Table VIII-69. Mean difference, standard error and the p value (α = 0.05) associated with each comparison between four ASR368 F2 progeny lines' and B9906'R and three conventional cultivars for pollen diameter (µm) in 2002

Contrast	Mean difference	StdErr	Probt
Penn A-4-vs. ASR368 F2 (13-2-2)	40.00-41.00 = -1.0	2.68	0.72
Penn A-4 vs. ASR368 F2 (14-2-6)	40.00-37.33 = 2.7	2.68	0.34
Penn A-4 vs. ASR368 F2 (15-2-5)	40.00-39.67 = 0.3	2.68	0.90
Penn A-4 vs. ASR368 F2 (16-2-2)	40.00-39.83 = 0.2	2.68	0.95
Crenshaw vs. ASR368 F2 (13-2-2)	37.17-41.00 = -3.8	2.68	0.18
Crenshaw vs. ASR368 F2 (14-2-6)	37.17-37.33 = -0.2	2.68	0.95
Crenshaw vs. ASR368 F2 (15-2-5)	37.17-39.67 = -2.5	2.68	0.37
Crenshaw vs. ASR368 F2 (16-2-2)	37.17-39.83 = -2.7	2.68	0.34
Backspin vs. ASR368 F2 (13-2-2)	38.83-41.00 = -2.2	2.68	0.43
Backspin vs. ASR368 F2 (14-2-6)	38.83-37.33 = 1.5	2.68	0.59
Backspin vs. ASR368 F2 (15-2-5)	38.83-39.67 = -0.8	2.68	0.76
Backspin vs. ASR368 F2 (16-2-2)	38.83-39.83 = -1.0	2.68	0.72

13-2-2, 14-2-6, 15-2-5 and 16-2-2 refer to the maternal EPP of the respective ASR368 F2 progeny.

Pollen viability and longevity

In both 2001 and 2002, the longevity of pollen from plants derived from ASR368 was not significantly different from either B99061R or the conventional cultivars. Minimum and maximum pollen longevity among all populations tested was between 0.5 and 3.5 hours under the conditions provided in the laboratory. Mean longevity was approximately 1.5-2.5 hours. The means, standard deviations and the minimum and maximum values for pollen longevity for the ASR368 genotypes and the three conventional cultivars for the 2001 experiment are presented in Table VIII-70. The specific comparisons in pollen

viability between the ASR368 R0 and F1 and B99061R and the conventional cultivars are provided in Table VIII-71. The means, standard deviation and the minimum and maximum values for pollen longevity for the ASR368 F2 progeny and the three conventional cultivars for the 2002 experiment are presented in Table VIII-72.

Table VIII-70. Number of observations, mean, standard deviation, and the minimum and maximum values for pollen longevity (hours) among ASR368 R0 and F1 progeny, B99061R and three conventional cultivars in 2001

Genotype	Number of observations	Mean (hours)	Std	Minimum value	Maximum value
Penn A-4	7	1.55	0.85	0.5	3.0
Crenshaw	8	1.95	0.90	0.5	3.0
Penncross	6	1.48	0.98	0.5	3.0
B99061R	6	2.30	1.02	1.0	3.5
ASR368 R0	6	1.84	0.97	0.5	3.0
ASR368 F1	9	1.78	0.71	1.0	3.0

Table VIII-71. Mean difference, standard error and the p value (α = 0.05) associated with each comparison between ASR368 R0 or F1 progeny and B99061R and three conventional cultivars for pollen longevity in 2001

Contrast	Mean difference	StdErr	Probt
B99061R vs. ASR368 R0	2.30 - 1.84 = 0.46	0.57	0.43
B99061R vs. ASR368 F1	2.30 - 1.78 = 0.52	0.58	0.38
Penn A-4 vs. ASR368 R0	1.55 - 1.84 = -029	0.56	0.61
Penn A-4 vs. ASR368 F1	1.55 - 1.78 = -0.23	0.57	0.68
Crenshaw vs. ASR368 R0	1.95 - 1.84 = 0.11	0.57	0.85
Crenshaw vs. ASR368 F1	1.95 - 1.78 = 0.17	0.58	0.77
Penncross vs. ASR368 R0	1.48 - 1.84 = -0.36	0.58	0.54
Penncross vs. ASR368 F1	1.48 - 1.78 = -0.3	0.59	0.61

Table VIII-72. Number of observations, mean, standard deviation, and the minimum and maximum values for pollen longevity (hours) among pooled ASR368 F2 progeny lines and three conventional cultivars in 2002

Group	Number of observations	Mean (hours)	Std	Minimum value	Maximum value
Commercial cultivars	7	2.29	0.64	1.50	3.00
ASR368 F2	6	2.17	0.26	2.00	2.50
Pr > F (α = 0.05)		0.68 NS			

NS = no significant differences between groups detected by the F test (α = 0.05).

VIII.E.3. Conclusion for pollen studies

Considering the consistency of the 2001 and 2002 results of the pollen size, viability and longevity studies, which encompassed three generations of ASR368, it is not expected that pollen of ASR368 varieties would differ in their ability to disperse or outcross with other *A. stolonifera* or other species with which it can interbreed compared to conventional cultivars. The pollen longevity results are in general agreement with reports in the scientific literature regarding other grass species. The longevity of grass pollen is generally considered low. Maize pollen was reported to lose viability after 2 hours under field conditions (Luna et al., 2001) and the pollen viability of Sudan grass (*Sorghum vulgare* var *sudanense*) became negligible 5 hours after pollen shed (Hogg and Ahlgren, 1943).

Consequently, given the results from the ASR368 pollen experiments, which are consistent with reports in the scientific literature regarding the longevity of grass pollen, ASR368 pollen would not be expected to be viable for a longer period of time than pollen from conventional creeping bentgrasses. The results of these studies also indicate that the size of ASR368 pollen would not be expected to be different from pollen of conventional creeping bentgrass. Pollen characterization data contribute to the detailed phenotypic description of ASR368 compared to conventional creeping bentgrass. These findings support a conclusion that the potential for ASR368 to outcross with *A. stolonifera* or other species with which the species is known to interbreed would be no different than that of conventional creeping bentgrass. The results also support an overall conclusion that ASR368 is not different than conventional creeping bentgrass in terms of plant pest or weediness characteristics and is no more likely to pose a plant pest risk than conventional creeping bentgrass.

VIII.F. Fecundity

As discussed in Section II.E.4 of this petition, fecundity refers to both reproductive and/or vegetative success of an organism in nature (Barbour, 1987). Both aspects of fecundity are important when considering *A. stolonifera* because of its predominantly clonal expansion activity in stable environments and seed production under environmental stress. A decrease or increase in seed production may influence the plant's desirability by a seed producer or potentially impact the number of seed available

to germinate in managed or unmanaged environments. An increase in seed production could potentially enhance a plant's ability to persist and consequently its weediness.

The results from several greenhouse and field experiments conducted in 2001 and 2002 comparing the seed set, seed yield and vegetative biomass of ASR368 and several other conventional creeping bentgrass cultivars are presented in this section. These experiments were conducted under USDA # 00-220-02n, 01-017-14n, 01-177-01n and 00-177-02n.

VIII.F.1. Greenhouse evaluations

VIII.F.1.1. Experimental methods – greenhouse study

Plant propagation and establishment

The greenhouse plants consisting of ASR368 R0 and F1 progeny evaluated for flowering characteristics in 2001 as described in Section VIII.D.1 were evaluated for viable (based on germination test) seed set per seed head under open- and self- pollination conditions. In 2002, ASR368 F2 and the conventional cultivars Backspin, Crenshaw and Penn A-4 were evaluated for pollen size and viability in Section VIII.E.1 were also evaluated for open-pollinated seed set.

The methods of floral initiation in the Iowa State University greenhouse were the same in both years and are detailed in Section VIII.D.1.1 of this petition.

Seed production and collection

Self-pollination. Just prior to anthesis in 2001, three inflorescences at similar developmental stages from each plant were pushed inside a hybridization bag (Lawson #411). The opening of the bag was then folded diagonally around the culms and secured with a jumbo paper clip at the bottom to isolate the panicles from cross-pollination and to prevent loss of seeds due to seed shattering. Bags were tapped lightly every day to facilitate pollen movement and self-pollination within the bag. Panicles were harvested approximately one month after bagging when seedheads were mature. Self-pollination studies were not performed in 2002.

Open-pollination. About two weeks after bagging panicles for self-pollination and when anthesis on the plants was complete, three additional randomly selected inflorescence from each plant were bagged together in a fashion similar to that used for self-pollination in both 2001 and 2002.

Seeds were manually threshed out of panicles. The number of germinable seed per panicle were evaluated in 2001 whereas the number of mature seed per panicle were counted in 2002.

Germinable seeds per panicle, 2001. Seeds were pre-chilled at 5^0C and were placed in a plastic Petri plate containing wet filter paper. After one week of pre-chilling, seeds were transferred to a six-inch plastic pot with 1/3 volume of fine sand and 2/3 volume of soilless medium for germination. Germination took place at 20 to 21^0C. All pots were irrigated with 0.2% KNO_3 for one week and then irrigated to prevent moisture stress.

Seed set per panicle, 2002. The number of mature seeds from open-pollinated inflorescences were examined and recorded using a stereomicroscope when necessary. The criteria used for judging a seed being mature or not were seed color (dark brown for mature seeds) and grain fill (mature seeds appear plump).

Data collected

In 2001, seedling emergence was continuously monitored for one month and the number of seedlings that emerged were recorded. However, in 2002, fewer plants of ASR368 F2 and the conventional cultivars were evaluated for this experiment so they were pooled, as discussed in the previous section (Section VIII.E.1).

Statistical analysis

As described in Section VIII.D.1.1 of this petition.

VIII.F.1.2. Results of greenhouse study

The mean number of seeds per three seed heads obtained from open-pollinated inflorescences (panicles) of the ASR368 genotypes, B99061R and the commercial cultivars in 2001 and 2002 are provided in Tables VIII-73 and VIII-75, respectively. The specific comparisons between the ASR368 genotypes with B99061R and the commercial cultivars for seeds set per three inflorescences in 2001 are provided in Table VIII-74. The p-values ($\alpha = 0.05$) for 2001 indicate that the number of open-pollinated seed set by the ASR368 R0 and F1 plants was not significantly different from B99061R and the conventional cultivars, Penncross, Crenshaw and Penn A-4. In 2002, open-pollinated seed set of the ASR368 F2 plants was not significantly different from the combined conventional genotypes.

Self-pollinated seed set among all genotypes in 2001 was low, as expected, due to self-incompatibility systems known to exist in the *Agrostis* genus. Summary statistics are presented in Table VIII-76 for 2001; however, no comparisons of the genotypes were performed on this data due to the limited and variable seed set observed. The maximum viable seed set upon self-pollination occurred on the commercial cultivar, Crenshaw. The lowest viable seed set upon self-pollination occurred for both ASR368 R0 and B99061R. ASR368 F1 plants were intermediate to the three conventional cultivars.

Table VIII-73. Number of observations, mean, standard deviation and the minimum and maximum values for the number of germinable seeds set on every three inflorescences (panicles) that were open pollinated among ASR368 R0 and F1 progeny B99061R and three conventional cultivars in 2001

Genotype	Number of observations	Mean	Std.	Minimum value	Maximum value
Penn A-4	10	29.83	24.10	5	73
Crenshaw	10	43.26	53.16	14	174
Penncross	9	23.42	20.00	3	59
B99061R	6	19.33	7.69	6	27
ASR368 R0	6	14.83	10.83	1	30
ASR368 F1	6	39.53	17.83	9	62

Table VIII-74. Mean difference, standard error and the p value (α = 0.05) associated with each contrast between ASR368 R0 or F1 progeny and B99061R and three conventional cultivars for number of seeds formed through open-pollination in 2001

Contrast	Mean difference	StdErr	Probt
B99061R vs. ASR368 R0	19.33-14.83=4.50	21.45	0.84
B99061R vs. ASR368 F1	19.33-39.53=-20.2	19.97	0.33
PennA-4 vs. ASR368 R0	29.83-14.83=15.00	17.25	0.40
PennA-4 vs. ASR368 F1	29.83-39.53=-9.70	15.39	0.53
Crenshaw vs. ASR368 R0	43.26-14.83=28.43	17.48	0.12
Crenshaw vs. ASR368 F1	43.26-39.53=3.73	15.57	0.81
Penncross vs. ASR368 R0	23.42-14.83=8.59	17.46	0.63
Penncross vs. ASR368 F1	23.42-39.53=-16.11	15.55	0.31

Table VIII-75. Number of observations, mean, standard deviation and the minimum and maximum values for the number of seeds set on every three inflorescences that were open-pollinated among creeping bentgrasses in 2002

Group	Number of observations	Mean	Std deviation	Minimum value	Maximum value
Commercial cultivars	3	281.67	288.74	0	577.00
ASR368 F2	4	261.50	189.98	16	435.00
Pr > F (α = 0.05)		0.99			

NS = no significant differences between groups detected by the F test (α = 0.05).

Table VIII-76. Number of observations, mean, standard deviation and the minimum and maximum values for the number of seeds set on every three inflorescences that were self-pollinated among creeping bentgrasses in 2001

Genotype	Mean	Std.	Minimum value	Maximum value
Penn A-4	0.78	1.09	0	3
Crenshaw	3.67	5.36	0	17
Penncross	1.67	2.50	0	7
B99061R	0.17	0.41	0	1
ASR368 R0	0.17	0.41	0	1
ASR368 F1	1.33	1.63	0	4

VIII.F.1.3. Conclusion for greenhouse evaluation of seed set

Based on the viable seed set results from both open- and self-pollination studies in the greenhouse in 2001 and 2002, it is not expected that that ASR368 plants would be different in their ability to cross- or self-fertilize than B99061R or conventional creeping bentgrass cultivars. Therefore, based on the evaluated seed set characteristics, it is not expected that ASR368 would pose an additional weediness or plant pest risk as compared to conventional creeping bentgrass.

VIII.F.2. Field evaluations

Evaluations of the fecundity of ASR368 plants were performed under field conditions in both 2001 and 2002. In 2001, ASR368 R1 plants were evaluated in Franklin County, Washington at the two locations described in Section VIII.D.2.1. In 2002, ASR368 F1 and F2 plants were evaluated in separate experiments in Jefferson County, Oregon as described in Section VIII.D.2.1. For additional details of the methods of plant propagation and establishment of these experiments please refer to Section VIII.D.2.1.

VIII.F.2.1. Experimental methods

Seed harvest

Seeds per Five Panicles (Seed heads): In both 2001 and 2002, panicles were first harvested from each plant at maturity by removing the flowering culm above the plant canopy when the majority of the panicles including the upper third of the culm were brown. Five mature random panicles were harvested from each genotype.

Gross Seed Weight and Clean Seed Weight: Panicles remaining on the plants after the five panicles were removed were harvested separately and processed to determine gross seed weight and clean seed weight. Seed was liberated from the panicles by rubbing panicles across a plastic screen drawn taut over a wooden frame measuring 24" by 24", which was placed over a plastic collection bin. Plant material passing through the initial screen and falling into the plastic container was collected. Collected material was then sifted through a 1/8" metal screen followed by a 1/17" and 1/21" metal screens. Seed was separated from the majority of plant material through sifting and captured in a solid bottomed tray underneath the screens and weighed to assess gross seed weight.

Vegetative Biomass: Remaining vegetative material was harvested from each plant at both locations of the trial to a height of 2" above the soil surface to use in determination of plant biomass (dry weight) and to avoid including soil in tissue samples.

Data collected

Gross Seed Weight: The partially clean seed from each plant collected, following the rubbing and screening procedures, was weighed to the nearest 0.1 g by gravimetric analysis to determine Gross Seed Weight.

Clean Seed Weight: In addition to the seed cleaning and screening described above, seed from the gross seed weight determination was separated from screenings by cleaning on a tabletop Clipper® seed cleaner with a single 1/20" round hole screen with air flow to purify the seed. The cleaner and screens were cleaned between each plant sample of seed. Clean seed for each individual plant was weighed to the nearest 0.1 g by gravimetric analysis.

Thousand Seed Weight: Clean seed from each individual plant was further purified on a General Blower (New Jersey Sheet Metal Works, NJ) set at an airflow setting of 8.0 for two minutes. One thousand seed from each purified sample were counted using a Pfeuffer Contador (Pfeuffer GmbH, Kitzingen, Germany) seed counter. Thousand seed weight for each clean seed sample was measured to the nearest 0.001 g by gravimetric analysis as an indirect index of seed size.

Seed Set per Five Panicles: Five panicles from each GT or GS plant in each location were individually hand-rubbed between the thumb and forefinger over a small metal pan. Seed were screened through a 1/17 and 1/20" screen and purified with a General Blower as described for the 1000 seed weight. Pure seed were counted on a seed counter to determine seed set per five panicles through open pollination on each plant.

Statistical analysis

In 2001, vegetative biomass and the seed characteristics evaluated for the ASR368 R1 GT and GS were analyzed by pooling the data for each characteristic after having accounted for the potential variability contributed by location using Friedman's test (α = 0.05). In 2002, the data from the ASR368 F1 and F2 evaluations were analyzed separately using ANOVA (α = 0.05) to determine if any significant differences exist between the means of ASR368 and the collection of conventional cultivars. Fisher's LSD (α = 0.05) tests were run post-hoc if any ANOVA revealed the presence of significant differences.

VIII.F.2.2. Results of fecundity studies

2001

The results of the 2001 field study comparing vegetative biomass per plant, gross seed yield per plant, clean seed weight per plant and 1000 count seed weight evaluated for the ASR368 R1 GT and GS segregants are presented in Tables VIII-77 through VIII-81. The results for each characteristic at the first and second Franklin County, Washington sites were not significantly different according to Friedman's test (α = 0.05). Between the ASR368 R1 GT and GS progeny no statistically significant differences were identified for any of the seed productivity characteristics other than for vegetative biomass where GS plants had greater biomass than GT plants (Table VIII-78). No impact on a greater ability to establish or persist is associated with this result since the biomass for the ASR368 R1 GS segregant was greater than that of the GT segregant.

Table VIII-77. Number of seeds per five panicles for ASR368 R1 GT and GS segregants combined over the two Franklin County, Washington sites in 2001

Number of Seeds per 5 Panicles							
Segregant	**n**	**Mean**	**Min**	**Max**	**SD**	**P-value**	
GT	66	2,282	132	4,880	887.2	**Site[1]**	**Segregants[2]**
GS	46	2,496	422	6,345	1,183.5	0.784	0.405

[1] Difference in the number of seeds per five seed heads for ASR368 R1 GT and GS segregants at both Franklin County, Washington locations was non-significant according to Friedman's test (α = 0.05).

[2] Difference between the number of seeds per five seed heads for ASR368 R1 GT and GS segregants was not significant according to Friedman's test (α = 0.05).

Table VIII-78. Vegetative biomass (grams per plant) of ASR368 R1 GT and GS segregants combined over the two Franklin County, Washington sites in 2001

Plant Biomass (g)							
Segregant	n	Mean	Min	Max	SD	P-value	
GT	64	163.0	40	413	96.3	Site[1]	Segregants[2]
GS	48	215.6	39	884	157.1	0.516	0.007

[1] Difference in the plant biomass of ASR368 R1 GT and GS segregants at both Franklin County, Washington locations was not significant according to Friedman's test (α = 0.05).
[2] Difference in the plant biomass of ASR368 R1 GT and GS segregants was significant according to Friedman's test (α = 0.05).

Table VIII-79. Gross seed weight (grams per plant) of ASR368 R1 GT and GS segregants combined over the two Franklin County, Washington sites in 2001

Gross Seed Weight (g/plant)							
Segregant	n	Mean	Min	Max	SD	P-value	
GT	65	9.5	0.2	41.5	8.9	Site[1]	Segregants[2]
GS	42	14.8	0.2	71.4	14.8	0.294	0.102

[1] Difference in ASR368 R1 GT and GS segregant seed weight at both Franklin County, Washington locations was not significant according to Friedman's test (α = 0.05).
[2] Difference between ASR368 R1 GT and GS segregant seed weight was not significant according to Friedman's test (α = 0.05).

Table VIII-80. Clean seed weight (grams per plant) of ASR368 R1 GT and GS segregants combined over the two Franklin County, Washington sites in 2001

Clean Seed Weight (g/plant)							
Segregant	n	Mean	Min	Max	SD	P-value	
GT	68	3.7	0.0	19.4	3.9	Site[1]	Segregants[2]
GS	47	5.3	0.1	30.3	6.5	0.642	0.715

[1] Difference in ASR368 R1 GT and GS segregant clean seed weight at both Franklin County, Washington locations was not significant according to Friedman's test (α = 0.05).
[2] Difference between ASR368 R1 GT and GS segregant clean seed weight was not significant according to Friedman's test (α = 0.05).

Table VIII-81. One thousand seed weight (g) of ASR368 R1 GT and GS segregants combined over the two Franklin County, Washington sites in 2001

1000 Count Seed Weight (g)							
Segregant	**n**	**Mean**	**Min**	**Max**	**SD**	**P-value**	
GT	63	0.073	0.040	0.140	0.015	**Site[1]**	**Segregants[2]**
GS	42	0.072	0.030	0.150	0.019	0.354	0.274

[1] Difference in ASR368 R1 GT and GS segregant 1000 seed weight at both Franklin County, Washington locations was not significant according to Friedman's test ($\alpha = 0.05$).

[2] Difference between ASR368 R1 GT and GS 1000 segregant seed weight was not significant according to Friedman's test ($\alpha = 0.05$).

2002

ASR368 F1 and F2 progeny were not significantly different from at least one of the conventional creeping bentgrass cultivars for characters including: seed set per five panicles, vegetative biomass per plant, gross seed weight per plant, clean seed weight per plant, and total seed count per plant.

The ASR368 F1 plants produced more seed per five panicles than Crenshaw and Penn A4, but fewer seed than Backspin; however each of the numerical differences was not significant (Table VIII-82). The ASR368 F2 produced significantly more seed per five panicles than Crenshaw and Penn A-4 but no significant difference was found compared to Backspin (Table VIII-82). It is likely that any apparent increase in seed set per five panicles between ASR368 F1 and F2 progeny populations is due to the forward breeding process with ASR368 rather plant transformation since no significant differences were detected during the 2000-2001 field trials where seed set of GT and GS segregants from R1 generation seed were compared.

Vegetative biomass of ASR368 F1 and F2 progeny was not significantly different from that of Backspin (Table VIII-83). Vegetative biomass of ASR368 F1 and F2 progeny was significantly less than that of Penn A-4. Crenshaw was not significantly different from the F1 progeny but produced significantly more biomass than ASR368 F2 progeny. Therefore, ASR368 F1 and F2 progeny are similar to conventional creeping bentgrass cultivars in biomass productivity and are unlikely to be more competitive or invasive due to increased growth potential.

ASR368 F1 and F2 progeny were not significantly different from at least one of the conventional cultivars for the characters including gross seed weight and clean seed weight (Tables VIII-84 and VIII-85). Variation among conventional cultivars for clean seed weight is apparent, but clean seed weight from ASR368 F1 and F2 progeny were intermediate to that of the commercial cultivars. Interestingly, the relative rank of ASR368 and cultivar populations for clean seed weight was similar in both the F1 and F2 comparative tests.

In 2002, the weight of 1000 clean seed (1000 count seed weight) of both ASR368 F1 and F2 progeny plants was significantly lower than each of the three conventional cultivars

(Table VIII-86). However, minimum 1000 count seed weight values for individual plants within Backspin and Crenshaw cultivars were similar to minimums observed for ASR368 progeny. Additionally, the 1000 count seed weight from ASR368 R1 progeny evaluated in 2001 was not significantly different from that of the R1 plants for this characteristic. Furthermore, a decrease in seed weight is a reflection of smaller seed size, which would not be expected to contribute to greater seedling establishment in unmanaged environments. Although some significant differences were identified between ASR368 F1 and F2 progeny and one or more conventional cultivars for the other seed characteristics, there did not appear to be a trend other than for smaller seed size.

Overall, ASR368 F1 progeny produced significantly fewer seed per plant than Backspin and were not significantly different from Crenshaw and Penn A-4 (Table VIII-87). The ASR368 F2 progeny produced fewer total seed per plant than Backspin and Crenshaw but significantly more than Penn A-4. These results indicate that seed production is variable among the creeping bentgrass cultivars and that the ASR368 F1 and F2 progeny are within the range of the seed produced per plant of the conventional cultivars that are representative of *A. stolonifera.*

Table VIII-82. Mean number of seed per five panicles for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	Number of Seeds per Five panicles							Number of Seeds per Five panicles						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	34	1,245	152	2,847	661	1,014	1,475	40	1,841	92	3,804	997	1,522	2,160
Backspin	29	1,472	268	3,383	880	1,137	1,807	39	1,750	328	3,769	919	1,452	2,047
Crenshaw	35	1,161	331	2,947	661	934	1,388	38	1,316*	313	2,176	543	1,137	1,494
Penn A-4	27	1,078	50	2,347	636	826	1,330	37	911*	65	2,084	532	733	1,088
p value (α = 0.05)						0.26		<0.0001						
LSD (α = 0.05)						358.4		350.2						

* Means are significantly different from ASR368 F1 or F2, according to Fisher's LSD (α = 0.05).

Table VIII-83. Vegetative biomass (grams per plant) of ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation Plant biomass (g/plant)							ASR368 F2 Evaluation Plant biomass (g/plant)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	37	265	127	486	81	238	292	40	320	102	715	147	273	367
Backspin	30	280	55	592	120	236	325	39	492	94	954	227	418	565
Crenshaw	35	313	123	534	122	271	355	38	569*	113	4,471	701	338	799
Penn A-4	27	540*	75	1,207	244	444	637	39	505*	143	1,359	278	415	595
p value (α = 0.05)						<0.0001		<0.0001						
LSD (α = 0.05)						72.84		177.37						

* Means are significantly different from ASR368 F1 or F2, according to Fisher's LSD (α = 0.05)

Table VIII-84. Gross seed weight per plant (grams per plant) for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation Gross seed weight/plant (g/plant)							ASR368 F2 Evaluation Gross seed weight/plant (g/plant)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	37	105.5	30.8	184.6	37.0	93.1	117.8	40	149.4	45.2	317.4	62.7	129.4	169.5
Backspin	30	193.2*	34.2	362.4	77.7	164.2	222.2	37	142.4	27.0	410.0	86.5	113.6	171.3
Crenshaw	35	126.4	31.8	310.6	62.0	105.1	147.7	38	117.3	44.6	227.0	41.9	103.5	131.1
Penn A-4	25	105.1	41.4	233.6	57.7	81.2	128.9	36	88.1*	5.6	313.4	62.5	67.0	109.2
p value (α = 0.05)						<0.0001		<0.0001						
LSD (α = 0.05)						29.42		29.54						

* Means are significantly different from ASR368 F1 or F2, according to Fisher's LSD (α = 0.05)

Table VIII-85. Clean seed weight per plant (grams per plant) for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	Clean seed weight/plant (g)							Clean seed weight/plant (g)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	36	8.3	0.01	24.00	6.5	6.1	10.5	40	12.9	1.20	34.00	8.9	10.1	15.7
Backspin	30	22.1*	0.40	58.80	14.8	16.6	27.6	37	17.3*	0.60	55.40	12.2	13.2	21.4
Crenshaw	35	13.2	0.80	46.80	10.7	9.5	16.9	38	14.7	2.00	29.80	7.1	12.3	17.0
Penn A-4	25	7.6	0.40	17.00	5.2	5.4	9.7	36	4.5*	0.06	25.00	4.9	2.8	6.1
p value (α = 0.05)						<0.0001		0.0						
LSD (α = 0.05)						4.98		3.94						

* Means are significantly different from ASR368 F1 or F2, according to Fisher's LSD (α = 0.05)

Table VIII-86. One thousand seed weight (g) for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars in in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	1000 count seed weight (g)							1000 count seed weight (g)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	35	0.092	0.07	0.13	0.013	0.087	0.096	40	0.093	0.07	0.12	0.011	0.090	0.097
Backspin	30	0.101*	0.07	0.14	0.013	0.096	0.106	37	0.109*	0.08	0.14	0.013	0.104	0.113
Crenshaw	35	0.107*	0.07	0.14	0.018	0.101	0.113	38	0.108*	0.09	0.14	0.014	0.104	0.113
Penn A-4	25	0.131*	0.10	0.17	0.018	0.124	0.138	35	0.121*	0.09	0.16	0.019	0.114	0.127
p - value (α = 0.05)						<0.0001		<0.0001						
LSD (α = 0.05)						0.008		0.007						

* Means are significantly different from ASR368 F1 or F2, according to Fisher's LSD (α = 0.05)

Table VIII-87. Seed count per plant[1] for ASR368 F1 and F2 progeny and conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	Seed count/plant							Seed count/plant						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	35	90,765	6,667	242,500	65,307	68,331	113,199	40	138,019	13,333	377,778	92,823	108,333	167,706
Backspin	30	225,520*	3,636	588,000	151,873	168,809	282,230	37	161,901	6,000	554,000	115,016	123,552	200,249
Crenshaw	35	122,216	7,273	425,455	97,311	88,788	155,643	38	138,668	18,182	331,111	72,249	114,921	162,416
Penn A-4	25	58,866	3,333	141,667	41,367	41,790	75,941	35	38,554*	2,000	192,308	40,198	24,745	52,362
					p value (α = 0.05)	<0.0001				<0.0001				
					LSD (α = 0.05)	49,594				38640				

[1] Seed count per plant = [Clean seed weight per plant (g) / 1000 Count Seed Weight (g)] X 1000
* Means are significantly different from ASR368 F1 or F2, according to Fisher's LSD (α = 0.05)

VIII.F.3. Conclusion for fecundity studies

The purpose of these studies was to examine whether the insertion of the *cp4 epsps* gene into creeping bentgrass impacted the reproductive characteristics of ASR368 when compared to conventional creeping bentgrass genotypes such that they might have a competitive advantage in managed or unmanaged ecosystems.

Greenhouse evaluations of open-and self-pollinated seed set for ASR368 R0 and F1 plants displayed no statistically significant differences from B99061R or the conventional cultivars, Penncross, Penn A-4 or Crenshaw. Self-pollinated seed set for the ASR368 genotypes was low, as expected, due to self-incompatibility systems known to exist in the *Agrostis* genus.

The 2001 field trial study at two locations resulted in no statistically significant differences between ASR368 GT and ASR368 GS half-sib progeny populations for each of the following characteristics: gross seed yield per plant, clean seed weight per plant, 1000 count seed weight and seed set per five panicles. The vegetative biomass of ASR368 R1 GT and GS progeny were significantly different. However, significant differences between the ASR368 F1 or F2 GT progeny and conventional cultivars in the 2002 studies were not consistent as Backspin was not significantly different from ASR368 F1 or F2 progeny populations. Nonetheless, reduced biomass would be inconsistent with an increase in weed potential.

The results of the 2002 ASR368 F1 and F2 trials demonstrated that ASR368 F1 and F2 GT progeny performance generally was within the range of values observed for conventional cultivars or was not significantly different from at least one of the conventional cultivars for characters including: seed set per five panicles, vegetative biomass per plant, gross seed weight per plant, clean seed weight per plant, and total seed count per plant. Seed of the ASR368 genotypes tended to be smaller than conventional cultivars as indicated by the 1000 count seed weight. However, there were individual conventional plants within Backspin and Crenshaw that had (minimum) 1000 count seed weights similar to the minimums observed from plants of ASR368 progeny. In addition, significant differences between conventional cultivars were evident as well and smaller seed is more likely to reduce establishment rate and competitive ability as seen for other grass species (Whalley et al., 1996). These results indicate that seed production is variable among the creeping bentgrass cultivars and that the ASR368 F1 and F2 are within the range of the seed produced per plant by these conventional cultivars that are representative of *A. stolonifera.*

Considering the 2001 and 2002 results of the fecundity studies, which encompassed three generations of ASR368, it is not expected that ASR368 varieties would differ in their ability to produce seed compared to conventional cultivars. This supports an overall conclusion that ASR368 is not different than conventional creeping bentgrass in terms of plant pest or weed characteristics and is no more likely to pose a plant pest risk than conventional creeping bentgrass.

VIII.G. Seed Physiology

USDA-APHIS considers the potential for weediness to constitute a plant pest factor (7 CFR § 340.6). Information on seed characteristics, particularly germination and dormancy characteristics, is useful when assessing a plant for increased weediness potential. Seed germination and dormancy mechanisms vary with species and their genetic basis tends to be complex. Seed dormancy (*e.g.*, hard seed) is an important characteristic that is often associated with plants that are considered weeds (Anderson 1996; Lingenfelter and Hartwig 2007). Seed viability, seedling vigor, dormancy and longevity of ASR368 seed were evaluated relative to that of conventional creeping bentgrass. Although seed persistence data from controlled laboratory or field research is not common, there are recognized tests and modifications of them useful to assess seed viability, seedling vigor, dormancy and longevity. These include: (1) standard germination test (SGT); (2) suboptimal temperature (SUB); (3) supra-optimal temperature (SuOP) and (4) an accelerated aging test (AAT).

These four tests were used to compare the relative seed and seedling performance of ASR368 R1 GT and GS segregants and two conventional bentgrass varieties, SR 1020 and Highland bentgrass (*A. castellana*).

AOSA Standard Germination Test

Seed viability was evaluated by the SGT as described in the Association of Official Seed Analysts Rules for Testing Seeds (1998). The SGT provides an ideal germination condition to obtain the maximum potential germination of the seed lot.

Total germination percentage (% viability) was used as an indicator of seed-lot quality and to provide a baseline for the ratio of GT to GS progeny to expect under ideal conditions. The percentage viability of each genotype in the SGT was also used as a baseline for comparing the percentage of the same seed germinating under the conditions of the SUB, SuOP and AAT tests.

Germination Rate (GR)

Germination rate or the speed of germination is determined by using the results of the SGT. The GR is considered one index of seed vigor and germination energy (Kulik and Yaklich, 1982; Tekrony and Elgi, 1977). Seeds with similar total germination percentage vary in their rate of germination and growth (i.e., speed of germination). Vigorous seeds germinate faster than medium or poor quality seeds. Germination rate calculations for each genotype were compared to determine if the seedlots were of similar vigor. Germination rate does not test for unintended effects of the *cp4 epsps* gene since the R1 seedlot is expected to segregate 1:1 for GT and GS phenotypes.

Sub-Optimal Temperature (SUB) and Supra-Optimal Temperature (SuOP) Germination Tests:

Vigorous seeds germinate under a wide range of extreme temperatures. Beard (1973) describes the optimum temperature range as 15 to 24°C for cool season turfgrass growth.

Therefore, the objective of these two stress tests was to evaluate the germination and development of seedlings of each genotype under continuous sub- and supra-optimal temperatures (14ºC and 32ºC, respectively). If the ratio of GT to GS changes significantly under varying environmental stresses (SUB and SuOP) compared to the ratio under SGT, then it could be concluded that the transgene imparts a selective advantage for germination under stress.

Accelerated Aging Test (AAT)

The AAT can be used as an indication of the potential relative longevity of seeds or as an indication of relative seed survivability or longevity in the soil (Delouche and Baskin, 1973). The test exposes the seeds to high temperature and high relative humidity (45ºC and near 100% relative humidity) for 30 hours. The decline in germination following this period of stress is proportional to the level of seed vigor and its potential physiological longevity.

VIII.G.1. Experimental methods

VIII.G.1.1. General test conditions

Each of the four germination tests was conducted with four replications each of 100 seeds of the three test genotypes; ASR368 R1, SR1020 and Highland bentgrass. Germination percentage was recorded each week for four weeks. Seedlings emerging during each weekly evaluation were marked with different colored pins and beads for later classification of germination rate (SGT only) or relative germination energy and seedling vigor. Seedlings were evaluated according to the AOSA Seedling Evaluation Handbook, 1992.

At the end of each test period (28 days), plants were moved to a greenhouse at 24°C ± 6°C and continuous light. Uniform nutrition and continuous irrigation were provided to all plants to prevent visible nutrient deficiencies and drought stress. Plants of each ASR368 R1 seed lot were sprayed with Roundup herbicide approximately two weeks after moving them to the greenhouse when the first 50% of all seedlings had reached the first tiller stage to determine the percentage of GT and GS seedlings.

VIII.G.1.2. Specific germination test conditions

Standard Germination Test (SGT)

Seed samples were evaluated for viability using the standard germination test as described in the AOSA Rules for Testing Seeds, 1998. Four replications of 100 seeds each were pre-chilled at 10°C for five days before moving to15/25°C for the 28-day test period. Seeds of each line were germinated in a growth chamber under alternating 15°C in the dark and 25°C in lighted conditions. Supplemental light with an intensity of 125 ft-c was provided by a cool white fluorescent source.

Germination Rate (GR)

Seedlings that germinated during each seven-day period during the four weeks of the SGT test were marked with different colored pins for easier identification. Germination counts from the standard germination test reported at each seven-day interval for 28 days were used to calculate the germination rate.

The following equation was used to calculate the rate (speed) of germination, GR;

$$GR = \frac{\text{number of normal seedlings}}{\text{days of first count}} + \ldots + \frac{\text{number of normal seedlings}}{\text{days of final count}}$$

Sub- and Supra-Optimal Temperature Tests (SUB and SuOP)

In the sub-optimal temperature germination test, seeds of each seed lot were germinated under constant 14°C for four weeks before moving them to the greenhouse for seedling vigor determinations. The test can identify if GT seeds are more likely to germinate than GS seed under sub-optimal temperatures. In the supra-optimal temperature germination test, seeds of each event or cultivar were germinated under constant 32°C for four weeks before moving seedlings to the greenhouse for seedling vigor determinations. The SuOP test can identify if GT seeds are more likely to germinate than GS seeds under supra optimal temperatures. High-pressure sodium bulbs provided supplemental light.

Accelerated Aging Test (AAT)

Seeds of each seed lot were exposed to 45ºC and near 100% relative humidity for 30 hours. The decline in germination following this stress is proportional to the level of seed vigor and its potential physiological longevity or dormancy. The test was performed using the wire-tray mesh method described by McDonald and Phaneendranath (1978) in the AOSA Seed Vigor Testing Handbook, 1983. Following AAT, seed were tested for viability under standard AOSA temperature conditions for 28 days. The AAT test can determine if GT seed are more likely to persist than GS seed.

VIII.G.1.3. Germination energy and seedling vigor

During the first two weeks in the greenhouse following each of the four test procedures, seedlings of each genotype within each test were marked and divided into sub-populations based on their relative germination energy (date of germination) and seedling vigor. The first 50% of the plants that reached the first tiller stage among all seedlings from the seven-day germination count were classified as having high germination energy and seedling vigor. Slower developing seedlings remaining from the seven-day count along with the seedlings germinating after the 14, 21 and 28-day counts, were classified as having low germination energy and seedling vigor.

Following the two-week greenhouse acclimation and the classification into high or low germination energy and seedling vigor, sub-populations of the ASR368 R1 plants were

sprayed with a 50:50 mixture of Roundup Pro and Roundup Accord herbicides (total rate of 1 gal/acre) in a greenhouse spray chamber. Percentage glyphosate tolerance was determined for each sub-population two weeks after spraying with glyphosate.

VIII.G.1.4. Data collected

Data for the different experiments included in the study were collected according to Figure VIII-2.

VIII.G.2. Data analysis

ASR368 R1 seed was expected to segregate 1:1 for glyphosate tolerant (GT) and glyphosate sensitive (GS) phenotypes. Consequently, it was possible to compare ASR368 GT and GS phenotypes in each experiment. Potential gene effects on seed viability or longevity under stress were evaluated by comparing the baseline percentage of GT progeny recovered from SGT (optimum germination conditions) to the percentage of GT progeny recovered from stress environments, SUB, SuOP or AAT. If there were an increase in the percentage of GT progeny among seedlings that germinate during a stress test compared to that found during the SGT, increased relative stress tolerance of ASR368 R1 GT seed could be indicated. Duncan's Multiple Range Test was used to compare the mean percentage GT recovered from SGT to that recovered from other stress tests.

As described above, seedlings that germinated during each test were further divided into sub-populations based on relative germination energy and seedling vigor. Seedlings with high germination energy and seedling vigor were identified as the first 50% of seedlings to develop a new tiller among all seedlings that germinated within the first seven days of the test. Seedlings with low germination energy and seedling vigor included all remaining seedlings from the seven-day count plus all of those that germinated by the end of the test (28 days). Potential gene effects on germination energy and seedling vigor were evaluated by comparing the percentage of GT in sub-populations categorized by high germination and seedling vigor vs. low germination energy and seedling vigor within each of the seed tests. A t-test was used to compare means between sub-populations within each test environment.

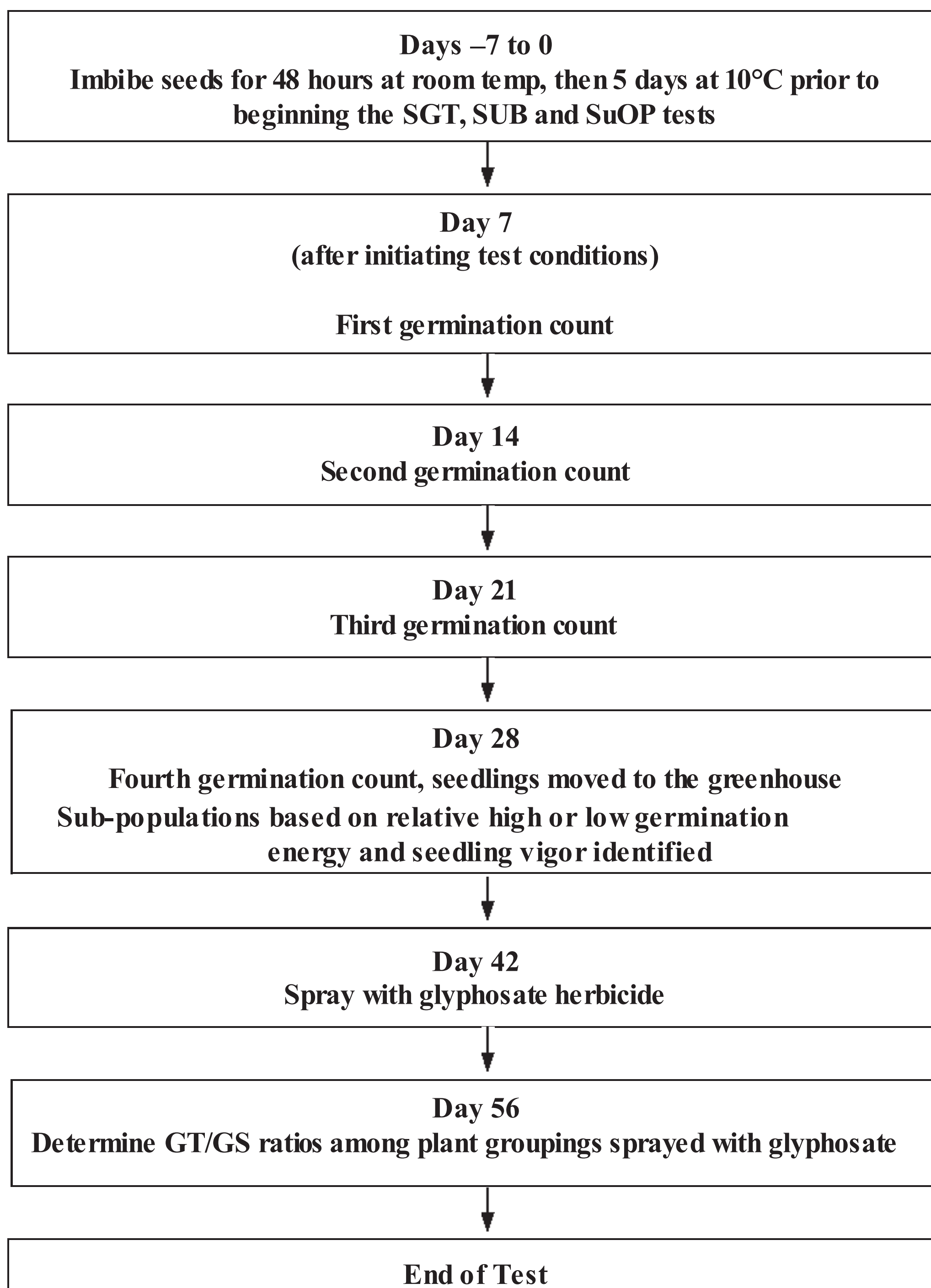

Figure VIII-2. Method of collecting data for the SGT, SUB, SuOP, and AAT seed physiology tests

VIII.G.3. Results

VIII.G.3.1. AOSA Standard Germination Test (SGT)

The percentage germination results under the standard AOSA germination test showed no statistically significant difference for seed viability between seed lots derived from ASR368, SR1020 and Highland (Table VIII-88). The germination percentages for these cultivars were 87.8, 89.8, and 94.5, respectively. Moreover, the viability of the ASR368 R1 seed lots met standards acceptable for certified creeping bentgrass seed (i.e., >85% germination).

Table VIII-88. Percentage germination of ASR368 R1 seed segregating for GT and GS progeny, and two conventional cultivars, SR 1020 and Highland, following four seed quality tests

Genotype	Germination (%)			
	SGT[2]	SUB[3]	SuOP[4]	AAT[5]
ASR368	87.8 bcd[1]	87.0 cde	81.5 de	88.5 abc
SR 1020	89.8 abc	93.5 abc	90.3 abc	73.3 f
Highland	94.5 ab	91.5 abc	80.8 e	90.3 abc

[1] Means followed by the same letter are not significantly different at P > 0.05according to Duncan's Multiple Range Test.
[2] Standard Germination Test
[3] Sub-optimal test at 14°C
[4] Supra-optimal test at 32°C
[5] Accelerated aging test at 45°C for 30h, final germination count was completed after five weeks.

The total percentage of GT plants among ASR368 R1 seedlings was 49.23% (173 GT of 351 total plants, Table VIII-89), which is consistent with the approximate expected 1:1 segregation of GT to GS within the R1 seed lots produced from the hemizygous R0 primary transformant of event ASR368 (see Figure V-17).

Table VIII-89. Percentage of ASR368 GT progeny recovered from segregating ASR368 R1 seed following four seed quality tests

Genotype	Mean % GT plants			
	SGT[2]	SUB[3]	AAT[4]	SuOP[5]
ASR368	49.23a[1]	51.10a	50.28a	39.53b

1 Means followed by the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's Multiple Range Test.
2 Standard Germination Test
3 Sub-optimal temperature germination test at constant 14°C
4 Accelerated aging test at 45°C for 30h
5 Supra-optimal temperature germination test at constant 32°C

The percentage of GT plants among seedlings with high germination energy and seedling vigor was not significantly different (α = 0.05) from the percentage of GT plants among seedlings with lower germination energy and seedling vigor within ASR368 R1 (Table VIII-90). Therefore, the *cp4 epsps* gene, its insertion and production of the CP4 EPSPS protein in ASR368 have no impact on germination energy or seedling vigor. Seed derived from ASR368 is no more likely to germinate or establish faster or be more competitive or invasive than seed that does not possess the glyphosate tolerance trait.

Table VIII-90. Comparison of the percentage of ASR368 GT recovered within two sub-populations of ASR368 R1 seedlings as characterized by high and low relative germination energy and seedling vigor following four seed quality tests

	Mean % GT Plants				
Seed Quality Test	High Vigor[1]	S.D.	Low Vigor[2]	S.D.	Probt[3]
SGT	49.7	0.77	49.05	3.79	0.78 ns
SUB	52.45	5.19	50.52	13.74	0.85 ns
SuOP	47.13	3.29	32.48	12.09	0.08 ns
AAT	50.00	5.80	50.45	4.09	0.92 ns

[1] % GT within the 50% of the plants that germinated by seven days and were first to reach the first tiller stage.
[2] % GT within plants that germinated and reached the first tiller stage later than those of the early developed plants.
[3] ns = not significant at α = 0.05

VIII.G.3.2. Germination rate

The germination rate of ASR368 (GR = 24.53) was not significantly different from that of SR 1020 (GR = 25.40) (Table VIII-91). However, both seed lots had a lower germination rate compared to Highland (GR = 27.95). These results suggest that the overall vigor or germination energy of the ASR368 R1 seed was not different from conventional creeping bentgrass seed.

Table VIII-91. Germination rate of ASR368, R1 seed segregating for GT and GS progeny and two conventional cultivars, SR 1020 and Highland

Genotype	Percent Germination								Germination rate[1]
	After 1 week				After 4 weeks				
	Mean	Min	Max	SD	Mean	Min	Max	SD	
ASR368	79	74	84	4.2	88	86	91	2.4	24.53
SR 1020	82	73	88	6.3	90	84	95	4.8	25.40
Highland	94	92	96	1.7	95	93	96	1.3	27.95
LSD (0.05)	8.5				4.63				1.86

[1] Germination rate was calculated according to the AOSA Seed Vigor Testing Handbook (1998) and is an indication of the speed of germination of each genotype. It may also be used as an index of seed vigor and germination energy, whereby seed germination, germination energy and seed vigor increase as the index value increases.

VIII.G.3.3. Sub-optimal temperature test

Germination percentage of ASR368 R1 seed did not differ significantly from that of the conventional bentgrass cultivars, SR 1020 and Highland during the sub-optimal temperature (14°C) test (Table VIII-88).

The percentage of ASR368 GT seedlings recovered under the SUB test was not significantly different from the respective percentage of GT seedlings recovered under standard AOSA conditions (SGT) (Table VIII-89). Therefore, it can be concluded that ASR368 seed will not germinate and establish differently under sub-optimal temperatures.

The percentage of ASR368 GT seedlings with high germination energy and seedling vigor (52.45%) was not significantly different ($p > 0.05$) from the percentage of GT seedlings with lower germination energy and seedling vigor (50.52%). Consequently, seeds and seedlings of ASR368 will not be expected to have different germination energy and vigor characteristics under low temperature stress than conventional bentgrass cultivars.

VIII.G.3.4. Supra-optimal temperature test

Under the high temperature (32ºC) stress conditions of the SuOP test the germination of ASR368 GT seed was not different from that of Highland bentgrass with 81.5 and 80.8% rates, respectively (Table VIII-88). However, in the same test, the germination percentage of SR 1020 was significantly greater than both ASR368 GT and Highland.

Mean germination (viability) of ASR368 GT seed under conditions of SuOP was not significantly different from the germination rates observed under standard AOSA conditions (Table VIII-88). However, the percentage of ASR368 GT progeny recovered following SuOP conditions was significantly lower than that recovered from standard AOSA conditions (Table VIII-89).

Results of the SuOP test indicate that the percentage of ASR368 GT among seedlings with high germination energy and seedling vigor was not significantly different ($p > 0.05$) from the percentage of ASR368 among seedlings with lower germination energy and seedling vigor (Table VIII-90). Therefore, supra-optimal temperatures do not appear to impact the germination energy and seedling vigor of ASR368 seedlings.

The poor germination of ASR368 GT seed during the SuOP test relative to the other seed tests may be attributed to the constant heat stress (32°C for 28 days) the young seedlings were exposed to in this study. Heat stress may have weakened seedlings and predisposed both the GT and GS plants to the post-SuOP test Roundup treatments at high rates. Beard (1973) describes the optimum temperature range for cool season turfgrass growth as 15 to 24°C. DiPaola and Beard (1992) also found that creeping bentgrass has a lower threshold for heat killing temperature and a lower time exposure threshold than several other cool season grasses. As a result, the ASR368 GT plants expected to survive following the application of glyphosate may have died due to heat stress, which ultimately decreased the potential percentage of GT seedlings and consequently, the apparent percentage of GS. GS seedlings that were expected to die following treatment with glyphosate may have died from both heat stress and the glyphosate treatment.

The higher percentage of ASR368 GT seedlings (47.13%) among high germination energy plants than among the low germination energy plants in this test suggests increased overall mortality from heat stress among the weak seedlings that germinated later (Table VIII-90). The percentage of GT seedlings within the high germination energy sub-population is very similar although lower as compared to the overall percentage of GT seedlings (49.23 – 51.10%) observed under each of the other test environments (Table VIII-89).

VIII.G.3.5. Accelerated Aging Test (AAT)

The stress conditions of the accelerated aging test (i.e., 45°C for 30 h at relative humidity of approximately 100%) reduced the percentage of seed germinating as well as the growth rate of seedlings throughout the test (Table VIII-91). Seedlings of all genotypes were smaller compared to the standard germination test. However, by the end of the test period, SR 1020 had significantly lower germination than either ASR368 GT and Highland (Tables VIII-88 and VIII-92). Seed longevity in the AAT was likely dependent upon the physiological condition of the seed at the time the test was performed. The work of Elias and Copeland (1997) support this conclusion. These authors reported that the tolerance of multiple lots of canola seed to the conditions of accelerated aging was a reflection of their physiological quality at the time they were tested.

Table VIII-92. Seed longevity of ASR368 R1 seed segregating for GT and GS progeny, and two conventional cultivars, SR 1020 and Highland as measured by percentage germination following the Accelerated Aging Test[1]

Genotype	Percent Germination[2]											
	After 1 week				After 2 weeks				After 5 weeks			
	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD
ASR368	19b[3]	14	28	6.4	75b	65	84	8.0	89a	86	91	1.1
SR 1020	28b	14	43	11.9	59c	40	76	14.7	70b	62	79	7.3
Highland	61a	55	63	3.7	88ab	85	91	2.5	90a	87	92	2.1

[1] Seeds were exposed to 45°C for 30 hours at relative humidity of near 100% before germinating the seeds. The accelerated aging test is used to predict the potential longevity of seeds.

[2] Slower growth rate of all genotypes was observed as a result of the stress conditions of the test compared to non-stressed seeds in the standard germination test.

[3] Means followed by the same letter in each column are not significantly different at $P< 0.05$ according to Duncan's Multiple Range Test.

The total percentage of GT seedlings from ASR368 R1 seed that germinated following exposure to the conditions of the AAT was not different from the respective percentages of ASR368 R1 GT seedlings from seed germinating under the SGT conditions (Table VIII-89).

The percentage of GT seedlings with high germination energy and seedling vigor was not different from the percentage of GT seedlings with lower germination energy and seedling vigor within ASR368 R1 progeny following the AAT test. Therefore, seed or seedlings of ASR368 would not be expected to have greater longevity, germination energy or seedling vigor than other bentgrasses.

VIII.G.3.6. Germination energy and seedling vigor

The percentages of GT plants identified within the sub-populations of ASR368 R1 seedlings characterized by either high or low germination energy and seedling vigor were not significantly different following any of the four germination tests (Table VIII-90). These results indicate that seed of the GT segregants from ASR368 R1 do not exhibit novel germination or developmental characteristics compared to GS segregants from the same R1 seed lot and seedling population. Therefore, seed or seedlings of ASR368 would not be expected to be more persistent, vigorous or invasive under diverse environmental conditions or stresses.

VIII.G.1. Conclusion for seed physiology (viability, vigor, dormancy and longevity)

The total germination percentage of ASR368 R1 seed during the SGT was not significantly different from those obtained during the SuOP, SUB and AAT tests (Table VIII-88). The percentage of GT progeny recovered from seedlings that germinated during the SGT was not significantly different from the percentage of GT observed

during both the SUB and AAT. Therefore, ASR368 seed and seedlings would not be expected to germinate and survive under suboptimal temperatures or to persist longer than those of conventional bentgrass cultivars.

The viability and longevity of ASR368 R1 GT and GS seed were not different across germination environments as indicated by the percentage of GT seedlings identified following the SGT, AAT and SUB tests. However, in the SuOP test, a reduction in the apparent viability of ASR368 GT seed was attributed to the constant heat stress the seedlings were exposed to in this test. Heat stress may have predisposed both the ASR368 GT and GS plants to increased mortality regardless of the post-test glyphosate treatments. Nonetheless, the apparent reduction in survival of ASR368 GT seedlings under conditions of the SuOP test would not contribute to increased invasiveness or persistence of glyphosate tolerant bentgrass.

Germination energy and seedling vigor (establishment) of ASR368 GT and GS seedlings were not different across germination environments as indicated by the percentages of GT seedlings with high or low germination energy and seedling vigor.

In summary, the data from these diverse germination studies provides sufficient evidence to support the conclusion that seed and seedlings of ASR368 would not be expected to demonstrate greater survival, longevity, dormancy or vigor in diverse environments than conventional bentgrass. These results support an overall conclusion that ASR368 is not different than conventional creeping bentgrass in terms of plant pest or weed characteristics and is no more likely to pose a plant pest risk than conventional creeping bentgrass.

VIII.H. Botanical Structures

A number of botanical structures are important characteristics when taxonomically identifying a plant as *A. stolonifera*. These characteristics help establish familiarity with the species, which can be used as a means of comparing plants of ASR368 with other conventional creeping bentgrass cultivars. Familiarity is in essence a baseline or the expected variability common to plants of the same species for a particular plant characteristic.

In this section, a number of vegetative and floral structures of ASR368 F1 and F2 plant populations, Elite Parent Plants (EPPs) and conventional cultivars were measured in the field and greenhouse in 2001 and 2002 (USDA # 00-220-02n, 01-017-14n, 01-177-01n and 00-177-02n). The characteristics evaluated included: the presence or absence of stolons, nodes, flag leaf, floral panicles, ligules, prominent venation on the abaxial leaf surface and vernation of the bud leaf. These features are characteristic in the taxonomic diagnosis of creeping bentgrass and may also contribute to the dispersal and invasiveness of a creeping bentgrass plant.

In addition, in 2001 and 2002, data on the mean number of florets per inflorescence and inflorescence (panicle) length (2002 only) were taken from plants grown in the Iowa State University greenhouse study discussed in Section VIII.D – VIII.F.

VIII.H.1. Experimental methods - field studies

Field studies were established in October 2000 in Franklin County, Washington and Jefferson County, Oregon and in September 2001 in Jefferson County, Oregon. The botanical characteristics of these plants were evaluated in 2001 and 2002, respectively.

Creeping bentgrass genotypes

In 2001, the botanical characteristics of ASR368 R1 progeny (GT) and Elite Parent Plants (GS) were evaluated. The botanical characteristics of ASR368 F1 and F2 plants were evaluated in separate experiments in 2002 at two locations in Jefferson County, Oregon as described above in Section VIII.F.2. Three conventional creeping bentgrass cultivars were used as comparators in the 2002 experiments.

Plant propagation and establishment

2001 – Study: Seed of the above genotypes was germinated in a poly-house in Marion County, Oregon. Two weeks after germination, the F1 seedlings were sprayed with Roundup Pro herbicide to remove the GS segregates and surviving GT plants were transplanted to Jiffy plugs. Upon growth to the two to three tiller stage, the plantlets were transferred to the field sites in Jefferson County, Oregon on October 6, 2000 and Franklin County, Washington on October 17, 2000. Upon arrival at the field sites the plants were space-planted in rows with seven plants per row. Elite Parent Plants (GS) were planted in alternating rows with the ASR368 F1 (GT) progeny. All plants were irrigated as needed to prevent plant stress and fertilized to maintain good plant vigor for seed production and to avoid visual nutrient deficiencies. Weeds were controlled through both post emergent herbicide applications and hand weed control cultivation.

2002 – Study: Botanical characteristics were evaluated on the same plants from which flowering and fecundity data were taken for Section VIII.D.2. The methods used to propagate and establish the plants in these experiments are described in Section VIII.D.2.1.

Sample Collection

In both 2001 and 2002, tillers with panicles in late anthesis were randomly harvested at the second node subtending the inflorescence. All samples were air dried in a laboratory prior to botanical evaluations.

Data collected

All botanical and morphological observations were conducted and recorded on the dried plant material in an enclosed laboratory in 2001 and 2002. Observed and recorded botanical and morphological characters included: presence of panicle and panicle length, presence or absence of anthers, stolons with nodes, flag leaf length and width, flag leaf sheaf length, ligule length, venation of abaxial leaf blade surface, and vernation of bud shoots. In addition, the number of florets per panicle was recorded in 2001.

Statistical analysis

In 2001, the botanical characteristics of the ASR368 F1 (GT) and EPP (GS) were analyzed by pooling the data for each characteristic after having accounted for the potential variability contributed by location using Friedman's test (α = 0.05). In 2002, the data from the ASR368 F1 and F2 evaluations were analyzed separately using ANOVA (α = 0.05) to determine if any significant differences exist between the means of ASR368 and the collection of conventional cultivars. Fisher's LSD (α = 0.05) tests were run post-hoc if any ANOVA revealed the presence of significant differences.

VIII.H.2. Results – field studies

2001 Results

The mean and p-value (α = 0.05) for the ASR368 GT and EPP (GS) populations combined across sites for the botanical characteristics evaluated in 2001 are presented in Tables VIII-93 through VIII-98. The analysis revealed no significant differences between the ASR368 GT and GS populations for each of the characteristics with the exception of ligule length. However, ligule length observed for both GT and GS phenotypes is within the range of values attributed to *A. stolonifera* in published botanical manuals (Hitchcock, 1951). All botanical features were observed to exist as a normal plant character in both the GT and GS populations at both locations.

Table VIII-93. Panicle length (cm) among ASR368 F1 GT and GS EPP plants evaluated in Jefferson County, Oregon and Franklin County, Washington in 2001

Panicle Length (cm)	n	Mean	Min	Max	SD	P-value	
GT	51	9.20	5.80	11.80	1.63	Site[1]	RR/RS[2]
GS	37	9.50	6.00	14.10	2.21	0.103	0.237

[1] Difference in ASR368 R1 GT and GS EPP panicle length at Franklin County, Washington and Jefferson County, OR locations was not significant according to Friedman's test (α = 0.05).
[2] Difference between ASR368 R1 GT and GS EPP panicle length was not significant according to Friedman's test (α = 0.05).

Table VIII-94. Flag leaf length (cm) among ASR368 F1 GT and GS EPP plants evaluated in Jefferson County, Oregon and Franklin County, Washington in 2001

Flag Leaf Length (cm)	n	Mean	Min	Max	SD	P-value	
GT	50	6.17	2.50	9.60	1.63	Site[1]	RR/RS[2]
GS	36	5.66	2.00	9.30	1.96	0.201	0.419

[1] Difference in ASR368 R1 GT and GS EPP flag leaf length at Franklin County, Washington and Jefferson County, OR locations was not significant according to Friedman's test (α = 0.05).
[2] Difference between ASR368 R1 GT and GS EPP flag leaf length was not significant according to Friedman's test (α = 0.05).

Table VIII-95. Flag leaf width (cm) among ASR368 F1 GT and GS EPP plants evaluated in Jefferson County, Oregon and Franklin County, Washington in 2001

Flag Leaf Width (cm)	n	Mean	Min	Max	SD	P-value	
GT	50	0.31	0.10	0.50	0.08	Site[1]	RR/RS[2]
GS	36	0.29	0.10	0.50	0.10	0.115	0.562

[1] Difference in ASR368 R1 GT and GS EPP flag leaf width at Franklin County, Washington and Jefferson County, OR locations was not significant according to Friedman's test ($\alpha = 0.05$).
[2] Difference between ASR368 R1 GT and GS EPP flag leaf width was not significant according to Friedman's test ($\alpha = 0.05$).

Table VIII-96. Flag leaf sheath length (cm) among ASR368 F1 GT and GS EPP plants evaluated in Jefferson County, Oregon and Franklin County, Washington in 2001

Flag Leaf Sheath Length (cm)	n	Mean	Min	Max	SD	P-value	
GT	51	8.18	5.00	10.50	1.27	Site[1]	RR/RS[2]
GS	36	8.59	6.00	12.20	1.74	0.059	0.098

[1] Difference in ASR368 R1 GT and GS EPP flag leaf sheath length at Franklin County, Washington and Jefferson County, OR locations was not significant according to Friedman's test ($\alpha = 0.05$).
[2] Difference between ASR368 R1 GT and GS EPP flag leaf sheath length was not significant according to Friedman's test ($\alpha = 0.05$).

Table VIII-97. Ligule length (cm) among ASR368 F1 GT and GS EPP plants evaluated in Jefferson County, Oregon and Franklin County, Washington in 2001

Ligule Length (cm)	n	Mean	Min	Max	SD	P-value	
GT	50	0.24	0.10	0.35	0.05	Site[1]	RR/RS[2]
GS	36	0.27	0.10	0.40	0.08	0.034	0.003

[1] Difference in ASR368 R1 GT and GS EPP ligule length at Franklin County, Washington and Jefferson County, OR locations was significant according to Friedman's test ($\alpha = 0.05$).
[2] Difference between ASR368 R1 GT and GS EPP ligule length was significant according to Friedman's test ($\alpha = 0.05$).

Table VIII-98. Number of florets per panicle among ASR368 F1 GT and GS EPP plants evaluated in Jefferson County, Oregon and Franklin County, Washington in 2001

Number Florets per Panicle	n	Mean	Min	Max	SD	P-value	
GT	51	443	185	852	154	Site[1]	RR/RS[2]
GS	37	497	146	764	148	0.119	0.391

[1] Difference in ASR368 R1 GT and GS EPP number of florets per panicle at Franklin County, Washington and Jefferson County, OR locations was not significant according to Friedman's test ($\alpha = 0.05$).
[2] Difference between ASR368 R1 GT and GS EPP number of florets per panicle was not significant according to Friedman's test ($\alpha = 0.05$).

2002 Results

The mean, p–value (α = 0.05) and LSD (α = 0.05) for the ASR368 F1 and F2 comparisons to the conventional cultivars in 2002 are presented in Tables VIII-99 through VIII-103. The ASR368 F1 was not significantly different from the conventional cultivars for any of the botanical characteristics with the exception of panicle length. The panicle length of the ASR368 F1 plants was significantly shorter than the conventional cultivars. However, the panicle lengths of both GT and GS phenotypes are within the range of those published in botanical manuals (Hitchcock, 1951). The flag leaf width of ASR368 F2 was significantly narrower than Backspin and Penn A-4, flag leaf sheath length was significantly longer than Crenshaw and Penn A-4 and ligule length was significantly shorter than Backspin. Despite these differences, all of the botanical features evaluated were observed to exist as a normal plant character in both the ASR368 F1 and F2 populations during 2002.

Table VIII-99. Panicle length (cm) of ASR368 F1 and F2 progeny and three conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	Panicle length (cm)							Panicle length (cm)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	38	8.14	6.33	10.75	1.04	7.80	8.48	40	8.64	4.50	11.25	1.47	8.17	9.10
Backspin	28	9.37*	6.67	13.00	1.58	8.76	9.98	37	8.34	5.83	11.50	1.52	7.83	8.85
Crenshaw	34	9.76*	6.67	13.50	1.82	9.13	10.40	38	8.95	6.17	12.17	1.41	8.48	9.41
Penn A-4	26	9.33*	5.83	13.00	1.73	8.63	10.03	38	8.25	6.00	13.50	1.46	7.78	8.73
p value (α = 0.05)	<0.0001							0.15						
LSD (α = 0.05)	0.76							0.66						

* Means are significantly different from ASR368 F1 or F2 according to Fisher's LSD (α = 0.05)

Table VIII-100. Flag leaf length (cm) of ASR368 F1 and F2 progeny and three conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	Flag leaf length (cm)							Flag leaf length (cm)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	38	5.25	3.00	7.75	1.02	4.92	5.59	40	5.44	2.75	9.00	1.50	4.97	5.92
Backspin	28	6.79*	2.45	9.00	1.40	6.24	7.33	36	5.50	1.50	8.50	1.44	5.01	5.99
Crenshaw	34	6.17*	4.00	11.50	1.62	5.60	6.73	38	5.14	3.00	6.83	1.07	4.79	5.49
Penn A-4	26	5.30	3.00	8.00	1.16	4.83	5.77	36	5.03	2.75	8.50	1.19	4.62	5.43
p value (α = 0.05)	<0.0001							0.33						
LSD (α = 0.05)	0.65							0.60						

* Means are significantly different from ASR368 F1 or F2 according to Fisher's LSD (α = 0.05).

Table VIII-101. Flag leaf width (mm) of ASR368 F1 and F2 progeny and three conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	Flag leaf width (mm)							Flag leaf width (mm)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	38	2.38	1.60	3.70	0.50	2.21	2.55	40	2.48	1.27	4.10	0.60	2.29	2.68
Backspin	28	2.85*	1.23	3.87	0.55	2.64	3.07	36	2.89*	1.60	3.93	0.59	2.69	3.09
Crenshaw	34	2.51	1.45	4.25	0.70	2.26	2.75	38	2.49	1.57	3.97	0.58	2.30	2.68
Penn A-4	26	2.55	1.60	4.00	0.55	2.33	2.77	38	2.84*	1.53	4.55	0.74	2.60	3.08
p value (α = 0.05)						0.01		0.003						
LSD (α = 0.05)						0.29		0.29						

* Means are significantly different from ASR368 F1 or according to Fisher's LSD (α = 0.05).

Table VIII-102. Flag leaf sheath length (cm) of ASR368 F1 and F2 progeny and three conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	Flag Leaf Sheath length (cm)							Flag Leaf Sheath length (cm)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	38	7.94	5.50	9.33	0.86	7.65	8.22	40	8.19	5.83	9.83	1.03	7.86	8.52
Backspin	28	8.35	6.33	10.33	1.10	7.92	8.78	37	7.85	5.00	11.50	1.39	7.39	8.32
Crenshaw	34	8.11	6.33	11.33	1.30	7.66	8.56	38	7.46*	6.17	9.50	0.81	7.19	7.73
Penn A-4	26	8.10	5.83	11.83	1.64	7.44	8.76	38	7.35*	4.67	10.50	1.20	6.96	7.75
p value (α = 0.05)						0.44		0.002						
LSD (α = 0.05)						0.61		0.51						

* Means are significantly different from ASR368 F1 or F2 according to Fisher's LSD (α = 0.05).

Table VIII-103. Ligule length (mm) of ASR368 F1 and F2 progeny and three conventional creeping bentgrass cultivars in Jefferson County, Oregon in 2002

	ASR368 F1 Evaluation							ASR368 F2 Evaluation						
	Ligule Length (mm)							Ligule Length (mm)						
	n	Mean	Min	Max	SD	95% CI of Mean		n	Mean	Min	Max	SD	95% CI of Mean	
ASR368	38	2.13	1.50	2.95	0.38	2.00	2.25	40	2.05	1.30	2.80	0.36	1.94	2.17
Backspin	28	3.16*	2.47	4.33	0.46	2.99	3.34	37	2.61*	1.45	3.57	0.49	2.44	2.77
Crenshaw	34	2.27	1.47	3.23	0.44	2.12	2.42	38	2.02	1.43	2.73	0.35	1.90	2.13
Penn A-4	26	2.17	1.50	3.70	0.45	1.99	2.35	38	1.98	1.17	2.70	0.35	1.86	2.09
p value (α = 0.05)							<0.0001	<0.0001						
LSD (α = 0.05)							0.21	0.18						

* Means are significantly different from ASR368 F1 or F2 according to Fisher's LSD (α = 0.05).

VIII.H.3. Experimental methods – greenhouse studies

Plant propagation and establishment

See Section VIII.D.1.1 for details of the genotypes and plant propagation and establishment methods used in both 2001 and 2002.

Data collected

In 2001 and 2002, data for the number of florets per inflorescence were obtained on three panicles harvested from plants and fixed (Carnoy's solution: 3 volumes 95% ethanol plus 1 volume of glacial acetic acid) for later observation. These observations were made from three batches (repetitions) of plants shipped from Marion County, Oregon to an Iowa greenhouse as described in Section VIII.D.1.1.

In 2002, inflorescence length was measured from the base of the basal whorl of panicle branches to the tip of the inflorescence in centimeters. Measurements were made on three fixed panicles from each creeping bentgrass plant.

Statistical analysis

As described in Section VIII.D.1.1 of this petition.

VIII.H.4. Results - greenhouse studies

In 2001, the R0 plants of ASR368 were not significantly different from B99061R and the three conventional cultivars, Penncross, Penn A-4 and Crenshaw, for florets per panicle (Tables VIII-104 and VIII-105). The ASR368 F1 GT progeny had significantly more florets per panicle than B99061R and the three conventional cultivars tested. However, mean florets per panicle among ASR368 F1 GT progeny were intermediate to the minimum and maximum number of florets per panicle detected for the conventional cultivars. The difference detected between the ASR368 F1 GT progeny and B99061R was attributed to the genes contributed by the maternal elite parent plants (EPP). In support of this supposition, when maternal elite parent plants were compared directly to the F1 GT progeny in the WA and OR locations described in section VIII.F, no significant difference was observed for the number of florets per panicle. In addition, these figures are not out of the ordinary for florets per panicle, since seed set per five panicles indicates means exceeding 450 per panicle for GT and GS phenotypes (Table VIII-77). The number of florets per panicle presented in Table VIII-106 provides additional evidence that 160 florets per panicle is not atypical of creeping bentgrass.

Consistent with the 2001 results, the number of florets per panicle for the four ASR368 F2 GT progeny lines was not significantly different from the three conventional cultivars in 2002 (Table VIII-106 and VIII-107). Several other morphological traits including the

number of anthers and stigmas, the number of glumes, paleas and lemmas were also examined and no unusual characteristics were observed.

Tables VIII-108 and VIII-109 provide the mean, standard deviation, minimum and maximum values and specific comparisons between the genotypes tested for panicle length in 2001. Length of the ASR368 R0 was not significantly different from B99061R but was significantly smaller than the conventional cultivars. The ASR368 F1 GT progeny were not significantly different from the control genotypes with regard to panicle length.

Table VIII-110 contains the mean, standard deviation, minimum and maximum values for inflorescence length taken in 2002. Table VIII-111 provides the specific comparisons between the genotypes tested for inflorescence length in 2002. The inflorescence length of ASR368 F2 were significantly shorter than those of the conventional cultivars Backspin and Crenshaw but not significantly different from those of Penn A-4.

Table VIII-104. Mean, standard deviation and the minimum and maximum values for the number of florets per panicle for ASR368 R0 and F1 progeny, B99061R and three conventional creeping bentgrass cultivars in 2001

Genotype	Number of observations	Mean	Std.	Minimum value	Maximum value
Penn A-4	15	79.78	39.45	29	199
Crenshaw	15	103.75	54.27	44	259
Penncross	14	109.6	45.38	37	197
B99061R	9	99.33	46.18	52	192
ASR368 R0	9	81.84	31.18	46	146
ASR368 F1	9	160.17	60.03	80	250

Table VIII-105. Comparisons between ASR368 R0 or F1 progeny and B99061R and three conventional creeping bentgrass cultivars for number of florets per panicle in 2001

Contrast	Mean difference	StdErr	Probt
B99061R vs. ASR368 R0	99.33-81.84 = 17.49	17.61	0.340
B99061R vs. ASR368 F1	99.33-160.17 = -60.84	17.82	0.002*
Penn A-4 vs. ASR368 R0	79.78-81.84 = -2.06	15.68	0.900
Penn A-4 vs. ASR368 F1	79.78-160.17 = -80.39	15.86	<0.0001*
Crenshaw vs. ASR368 R0	103.75-81.84 = 21.91	15.60	0.180
Crenshaw vs. ASR368 F1	103.75-160.17 = -56.42	15.87	0.001*
Penncross vs. ASR368 R0	109.6-81.84 = 27.76	15.89	0.100
Penncross vs. ASR368 F1	109.6-160.17 = -50.57	16.05	0.003*

* Means are significantly different ($\alpha = 0.05$)

Table VIII-106. Mean, standard deviation, minimum and maximum values for the number of florets per inflorescence between ASR368 F2 GT progeny lines[1] and three conventional creeping bentgrass cultivars in 2002

Genotype	Number of observations	Mean	Std.	Minimum value	Maximum value
Penn A-4	3	335.67	187.40	160	533
Crenshaw	3	316.00	14.73	300	329
Backspin	3	192.00	41.22	162	239
ASR368 F2 13-2-2	3	309.00	37.40	284	352
ASR368 F2 14-2-6	3	325.33	115.01	211	441
ASR368 F2 15-2-5	3	241.33	25.97	213	264
ASR368 F2 16-2-2	3	189.00	15.39	172	202

13-2-2, 14-2-6, 15-2-5 and 16-2-2 refer to the maternal EPP of the respective ASR368 F2 progeny.

Table VIII-107. Estimate difference, standard error and the associated p values of comparisons between ASR368 F2[1] progeny lines and three conventional creeping bentgrass cultivars for the number of florets per panicle in 2002

Contrast	Mean difference	StdErr	Probt
Penn A-4-vs. ASR368 F2 13-2-2	335.67-309.00 = 26.7	70.77	0.71
Penn A-4 vs. ASR368 F2 14-2-6	335.67-325.33 = 10.3	70.77	0.89
Penn A-4 vs. ASR368 F2 15-2-5	335.67-241.33 = 94.3	70.77	0.20
Penn A-4 vs. ASR368 F2 16-2-2	335.67-189.00 = 146.7	70.77	0.06
Crenshaw vs. ASR368 F2 13-2-2	316.00-309.00 = 7.0	70.77	0.92
Crenshaw vs. ASR368 F2 14-2-6	316.00-325.33 = -9.3	70.77	0.90
Crenshaw vs. ASR368 F2 15-2-5	316.00-241.33 = 74.7	70.77	0.31
Crenshaw vs. ASR368 F2 16-2-2	316.00-189.00 = 127.0	70.77	0.09
Backspin vs. ASR368 F2 13-2-2	192.00-309.00 = -117.0	70.77	0.12
Backspin vs. ASR368 F2 14-2-6	192.00-325.33 = -133.3	70.77	0.08
Backspin vs. ASR368 F2 15-2-5	192.00-241.33 = -49.3	70.77	0.50
Backspin vs. ASR368 F2 16-2-2	192.00-189.00 = 3.00	70.77	0.97

13-2-2, 14-2-6, 15-2-5 and 16-2-2 refer to the maternal EPP of the respective ASR368 F2 progeny.

Table VIII-108. Number of observations, mean, standard deviation and the minimum and maximum values for inflorescence length for ASR368 R0 and F1 progeny, B99061R and three conventional creeping bentgrass cultivars in 2001

Genotype	Number of observations	Mean (cm)	Std	Minimum value	Maximum value
Penn A-4	15	5.16	1.22	2.9	8.0
Crenshaw	15	5.24	1.26	3.4	7.5
Penncross	14	5.32	1.15	3.0	7.7
B99061R	9	4.98	1.08	3.6	6.5
ASR368 R0	9	4.23	0.62	3.0	5.0
ASR368 F1	9	5.36	0.92	3.9	6.6

Table VIII-109. Mean difference, standard error and the p value associated with each comparison between ASR368 R0 or F1 progeny and B99061R and three conventional creeping bentgrass cultivars for inflorescence length in 2001

Contrast	Mean difference	StdErr	Probt
B99061R vs. ASR368 R0	4.98-4.23 = 0.75	0.51	0.16
B99061R vs. ASR368 F1	4.98-5.36 = -0.38	0.47	0.42
Penn A-4 vs. ASR368 R0	5.16-4.23 = 0.93	0.42	0.04*
Penn A-4 vs. ASR368 F1	5.16-5.36 = -0.20	0.37	0.59
Crenshaw vs. ASR368 R0	5.24-4.23 = 1.01	0.42	0.02*
Crenshaw vs. ASR368 F1	5.24-5.36 = -0.12	0.37	0.76
Penncross vs. ASR368 R0	5.32-4.23 = 1.09	0.42	0.02*
Penncross vs. ASR368 F1	5.32-5.36 = -0.04	0.37	0.93

* Means are significantly different ($\alpha = 0.05$)

Table VIII-110. Mean, standard deviation, minimum and maximum values for inflorescence length between ASR368 F2 progeny lines[1] and three conventional creeping bentgrass cultivars in 2002

Genotype	Number of observations	Mean (cm)	Std	Minimum value	Maximum value
Penn A-4	3	7.83	2.00	5.9	9.9
Crenshaw	3	7.90	0.85	7.1	8.8
Backspin	3	8.07	0.78	7.2	8.7
ASR368 F2 13-2-2	3	6.70	0.36	6.4	7.1
ASR368 F2 14-2-6	3	6.10	0.75	5.4	6.9
ASR368 F2 15-2-5	3	6.40	1.35	4.9	7.5
ASR368 F2 16-2-2	3	6.80	0.78	5.9	7.3

13-2-2, 14-2-6, 15-2-5 and 16-2-2 refer to the maternal EPP of the respective ASR368 F2 progeny.

Table VIII-111. Mean difference, standard error and the associated p values of comparisons between four ASR368 F2 progeny lines[1] and three conventional creeping bentgrass cultivars for inflorescence length in 2002

Contrast	Mean difference	StdErr	Probt
Penn A-4- vs. ASR368 F2 13-2-2	7.83-6.70 = 1.13	0.90	0.23
Penn A-4 vs. ASR368 F2 14-2-6	7.83-6.10 = 1.73	0.90	0.07
Penn A-4 vs. ASR368 F2 15-2-5	7.83-6.40 = 1.43	0.90	0.13
Penn A-4 vs. ASR368 F2 16-2-2	7.83-6.80 = 1.03	0.90	0.27
Crenshaw vs. ASR368 F2 13-2-2	7.90-6.70 = 1.20	0.90	0.20
Crenshaw vs. ASR368 F2 14-2-6	7.90-6.10 = 1.80	0.90	0.06
Crenshaw vs. ASR368 F2 15-2-5	7.90-6.40 = 1.50	0.90	0.12
Crenshaw vs. ASR368 F2 16-2-2	7.90-6.80 = 1.10	0.90	0.24
Backspin vs. ASR368 F2 13-2-2	8.07-6.70 = 1.37	0.90	0.15
Backspin vs. ASR368 F2 14-2-6	8.07-6.10 = 1.97	0.90	0.05*
Backspin vs. ASR368 F2 15-2-5	8.07-6.40 = 1.67	0.90	0.08
Backspin vs. ASR368 F2 16-2-2	8.07-6.80 = 1.27	0.90	0.18

13-2-2, 14-2-6, 15-2-5 and 16-2-2 refer to the maternal EPP of the respective ASR368 F2 progeny. * Means are significantly different ($\alpha = 0.05$)

VIII.H.5. Conclusion for botanical characteristic studies

In field trials at two locations, ASR368 F1 and F2 GT progeny were significantly different from populations of conventional creeping bentgrass cultivars on few occasions for each of the parameters measured or observed within the Washington and Oregon locations other than for ligule length. However, the ligule length observed for all of the plants in the study was typical of *A. stolonifera,* according to Hitchcock (1950) and not likely to affect the weed or plant pest potential of ASR368. Visual inspection and evaluation of typical botanical features expected for creeping bentgrass indicate no gross aberration or deviation in any plant morphological feature between the ASR368 genotypes and conventional cultivars. In the Iowa State University greenhouse study, differences were not detected in the number of florets per panicle between ASR368 R0, B99061R and the conventional cultivars.

The results of these studies demonstrate that the measured botanical characteristics of ASR368 and its progeny are within the normal range of conventional creeping bentgrass cultivars and should not be expected to be different from them. These results support an overall conclusion that ASR368 is not different than conventional creeping bentgrass in terms of plant pest or weed characteristics and is no more likely to pose a plant pest risk than conventional creeping bentgrass.

VIII.I. Disease and Pest Susceptibility of ASR368

Observations of plant growth and insect and disease susceptibility were made during field releases conducted between 1999 through 2002. Data collected from these trials demonstrated that plants of ASR368 grew normally and exhibited the expected morphology, phenotype, and physical characteristics of conventional creeping bentgrass. Susceptibility to diseases and insects remained unchanged compared to conventional creeping bentgrasses. Observations on plant growth characteristics and weediness potential provided evidence that ASR368 does not pose a plant pest risk, or exhibit enhanced weediness characteristics.

The first field release of ASR368 occurred in the United States during 1999. The notification number and release site information for ASR368 can be found in Table VIII-112. Field data reports were submitted to USDA-APHIS for field trials conducted in 1999 through 2003 as required.

In all field releases of ASR368 conducted under notification, researchers were requested to monitor field sites for pest susceptibility, aberrant plant characteristics, or changes in the weediness characteristic of ASR368. Field observations were also made on the incidence of beneficial organisms. Visual observations were made while walking the fields and, in almost all circumstances; the observations were qualitative rather than quantitative.

Plant health management in professionally managed turf is distinct from that of row crops, trees, nuts, and vines. Golf course superintendents are trained and expert in the identification of potential problems in golf course turfgrass. They consider injury or damage caused by biotic agents as unacceptable, while in traditional agriculture the need for intervention is a decision driven more by economic injury levels. When considering that every golf superintendent desires a uniform and consistent playing surface of creeping bentgrass, the list of potential plant pests is relatively long.

The major turf diseases of consistent concern affecting creeping bentgrass performance are dollar spot (*Sclerotinia homocarpa*), brown patch (*Rhizoctonia solani*), snow mold (*Myriosclerotinia borealis, Typhula incarnata)*, leafspots (*Helminthosporium sp.*, *Dreschelera* sp.*, Septoria* sp.), take-all patch (*Gaeumannomyces graminis*), copper spot (*Gloeocercospora sorghil*), leaf rust (*Puccinia* sp.), bentgrass dead spot (*Leptosphaeria narmari*), and pythium (*Pythium* sp).

Insect pests of major importance affecting the performance of creeping bentgrass are chinch bugs (*Blissus leucopterus*), various beetle grubs (*Popillia* spp.), sod webworms (*Crambus* spp.), cutworms (*Agriotis ipsilon and Peridroma saucia*), armyworms (*Spodoptera* spp.), billbugs (*Spheophorus* spp.), mole crickets (*Scapteristicus* spp.), and aphids (*Aphidius* spp.).

The diseases and insects observed during these field trials are summarized in Table VIII-112. No discernible differences in plant growth, disease severity or insect infestation were detected between ASR368 and conventional control plants in these trials. Although this information is qualitative, many of the research cooperators conducting these

experiments were turf scientists or turfgrass managers experienced in the evaluation of new turfgrass varieties and performance of bentgrasses. Many of them have participated in the National Turfgrass Evaluation Program trials.

Finally, information gathered over three years of monitoring ASR368 field trials demonstrated ASR368 varieties exhibited morphology, phenotype, and physical characteristics and disease and pest susceptibility no different than that of conventional creeping bentgrass cultivars. Thus, based on these results of field observations, ASR368 is not different than conventional creeping bentgrass, with the exception of the glyphosate tolerance trait, and is no more likely to pose a plant pest/weediness risk or have environmental impacts than conventional creeping bentgrass.

Table VIII-112. Differences in disease and insect susceptibility and plant growth characteristics observed between ASR368 and conventional bentgrasses

USDA	Year	State	County	Disease[1]	Insect[2]	Growth[3]
99-203-04n	1999-2000	NJ	Middlesex	no	no	no
99-203-04n	1999-2000	OH	Union	no	no	no
99-203-04n	1999-2000	OR	Marion	no	no	no
00-159-02n	2000-2001	IL	Clinton	no	no	no
00-159-02n	2000-2001	MI	Ottawa	no	no	no
00-201-03n	2000-2001	NJ	Middlesex	no	no	no
00-201-03n	2000-2001	OH	Union	no	no	no
00-201-03n	2000-2001	OR	Marion	no	no	no
00-220-02n	2000-2001	OR	Jefferson	no	no	no
00-220-02n	2000-2001	WA	Franklin	no	no	no
00-224-01N	2000-2001	AL	Baldwin	no	no	no
00-224-01n	2000-2001	CO	Larimer	no	no	no
00-224-01n	2000-2001	IN	Tippecanoe	no	no	no
00-224-01n	2000-2001	KY	Fayette	no	no	no
00-224-01n	2000-2001	MA	Franklin	no	no	no
00-224-01n	2000-2001	MD	Prince George	no	no	no
00-224-01n	2000-2001	MI	Ingham	no	no	no
00-224-01n	2000-2001	NJ	Middlesex	no	no	no
00-224-01n	2000-2001	NY	Tompkins	no	no	no
00-224-01n	2000-2001	OH	Franklin	no	no	No
00-224-01n	2000-2001	OR	Linn	no	no	No
00-224-01n	2000-2001	OR	Marion	no	no	no

[1] Diseases observed for included: dollar spot, brown patch, snow mold, leaf spot, take-all patch, copper spot, rust, spring dead spot, Pythium.
2 Δ = difference observed between transgenic and control plants
[3] Insects observed for included: chinchbugs, grubs, sod webworms, cutworms, armyworms, billbugs, mole crickets, aphids, lady beetles, spiders, honeybees.
[4] Plant growth characteristics observed for turf included: rate of germination and establishment and flowering when mowed and plant growth. Characteristics observed for seed production included: rate of establishment, spread, flowering and yield.

Table VIII-112. Differences in disease and insect susceptibility and plant growth characteristics observed between ASR368 and conventional bentgrasses (continued)

USDA	Year	State	County	Disease[1]	Insect[2]	Growth[3]
00-224-01n	2000-2001	OR	Umatilla	no	no	no
00-224-01n	2000-2001	VA	Montgomery	no	no	no
00-224-01n	2000-2001	WI	Dane	no	no	no
00-272-05n	2000-2001	NE	Saunders	no	no	No
01-064-02n	2001-2002	IL	Champaign	no	no	no
01-064-02n	2001-2002	NC	Wake	no	no	no
01-151-02n	2001-2002	IL	Clinton	no	no	no
01-151-02n	2001-2002	MI	Ottawa	no	no	no
01-177-01n	2001-2002	OR	Jefferson	no	no	no
01-177-01n	2001-2002	WA	Franklin	no	no	no
01-177-02n	2001-2002	AL	Baldwin	no	no	no
01-177-02n	2001-2002	FL	Orange	no	no	no
01-177-02n	2001-2002	IA	Story	no	no	no
01-177-02n	2001-2002	IL	Champaign	no	no	no
01-177-02n	2001-2002	IN	Tippecanoe	no	no	no
01-177-02n	2001-2002	KY	Fayette	no	no	no
01-177-02n	2001-2002	MD	Prince George	no	no	No
01-177-02n	2001-2002	MI	Ingham	no	no	no
01-177-02n	2001-2002	NC	Wake	no	no	no
01-177-02n	2001-2002	NE	Saunders	no	no	no
01-177-02n	2001-2002	NJ	Middlesex	no	no	no

[1] Diseases observed for included: dollar spot, brown patch, snow mold, leaf spot, take-all patch, copper spot, rust, spring dead spot, Pythium.
2 Δ = difference observed between transgenic and control plants
[3] Insects observed for included: chinchbugs, grubs, sod webworms, cutworms, armyworms, billbugs, mole crickets, aphids, lady beetles, spiders, honeybees.
[4] Plant growth characteristics observed for turf included: rate of germination and establishment and flowering when mowed and plant growth. Characteristics observed for seed production included: rate of establishment, spread, flowering and yield.

Table VIII-112. Differences in disease and insect susceptibility and plant growth characteristics observed between ASR368 and conventional bentgrasses (continued)

USDA	Year	State	County	Disease	Insect	Growth
01-177-02n	2001-2002	NY	Tompkins	no	no	no
01-177-02n	2001-2002	OH	Franklin	no	no	no
01-177-02n	2001-2002	OH	Union	no	no	no
01-177-02n	2001-2002	OR	Marion	no	no	no
01-177-02n	2001-2002	OR	Linn	no	no	no
01-177-02n	2001-2002	OR	Umatilla	no	no	no
01-177-02n	2001-2002	SC	Pickens	no	no	No
01-177-02n	2001-2002	VA	Montgomery	no	no	no
01-177-02n	2001-2002	WA	Franklin	no	no	no
01-177-02n	2001-2002	WI	Dane	no	no	no
01-199-06n	2001-2002	IA	Polk	no	no	no
01-199-06n	2001-2002	IL	Cook	no	no	no
01-199-06n	2001-2002	KY	Boone	no	no	no
01-199-06n	2001-2002	MD	Baltimore	no	no	no
01-199-06n	2001-2002	NJ	Union	no	no	no
01-199-06n	2001-2002	NY	Broome	no	no	no
01-199-06n	2001-2002	OH	Delaware	no	no	no
01-199-06n	2001-2002	OH	Fairfield	no	no	no
01-199-06n	2001-2002	OH	Union	no	no	no
01-199-06n	2001-2002	WI	Sheboygen	no	no	no
01-214-01n	2001-2002	DE	New Castle	no	no	no
01-214-01n	2001-2002	MD	Montgomery	no	no	no

[1] Diseases observed for included: dollar spot, brown patch, snow mold, leaf spot, take-all patch, copper spot, rust, spring dead spot, Pythium.
[2] Δ = difference observed between transgenic and control plants
[3] Insects observed for included: chinchbugs, grubs, sod webworms, cutworms, armyworms, billbugs, mole crickets, aphids, lady beetles, spiders, honeybees.
[4] Plant growth characteristics observed for turf included: rate of germination and establishment and flowering when mowed and plant growth. Characteristics observed for seed production included: rate of establishment, spread, flowering and yield.

VIII.J. Behavior of glyphosate tolerant creeping bentgrass in the environment

In 2003, 421 acres of ASR368 were planted at 12 locations within the Oregon Control Area near Madras in Jefferson County. The Control Area was established by the state of Oregon in 2002 to physically separate biotechnology-enhanced creeping bentgrass from conventional creeping bentgrass. A strong wind in mid-August 2003 spread ASR368 seed in a generally southwest direction from the seed fields. The pollen from the 421 acres of ASR368 fields and the seed scatter event provided an opportunity for researchers to evaluate the establishment and persistence of ASR368 in an unmanaged ecosystem.

Dispersal and establishment of ASR368 has been rigorously studied in Jefferson County, Oregon for over 11 years. Three groups (two research groups led by Oregon State University and EPA , and The Scotts Company) have conducted surveys for ASR368 in and around Madras, OR. Data generated over that time have shown ASR368 being primarily limited to irrigation channels within the general vicinity of the production fields. Seed, not pollen, was the primary means of dispersal.

Watrud et al. (2004) published the first data on creeping bentgrass pollen flow from a large pollen source (421 acres) that flowered in 2003. Seeds were isolated from both naturally occurring resident plants found in mesic areas around the Control Area and plants maintained as sentinels in a regularly spaced 1.6 km grid. The resident plants were primarily located in nonagronomic habitats such as irrigation ditches and ponds. Most of the glyphosate tolerant seedlings occurred within 2 km consistent with the direction of prevailing winds. The furthest glyphosate tolerant seedlings were identified 21 and 14 km from sentinel and resident plants, respectively. Of the 565,000 seedlings isolated from seeds of resident creeping bentgrass plants, 157 were tolerant to glyphosate (0.03% of seedlings screened), 625 of 32,000 sentinel derived seedling were glyphosate tolerant (2%) and159 out of 397,000 redtop bentgrass seeds were glyphosate tolerant (0.04%).

Reichman et al. (2006) built upon their pollen flow study with field work to perform a robust analysis of glyphosate tolerant seedlings that established via pollen or seed. The researchers surveyed edges of feral populations that had minimal vegetative competition. The "disturbed microsites" were searched for "juvenile plants" that were "not in flower" and evaluated for the presence of the CP4 EPSPS protein with TraitChek^{TM} from Strategic Diagnostics. From this selective population, only 9 plants of 20,400 (0.04%) evaluated were positive for the CP4 EPSPS protein. All 9 plants were found in mesic habitats. Utilizing molecular markers, all 9 positive plants were confirmed to be creeping bentgrass, *A. st*olonifera. Three of the glyphosate tolerant positive plants were shown to be from seed scatter with the remaining 6 resulting from pollination with ASR368 plants. No hybrids with either redtop (*A. gigantea*) or rabbitsfoot grass (*Polypogon monspeliensis*) were identified.

Bollman et al. (2012) also conducted an extensive survey in central Oregon that included canals, ditches, creeks and the Deschutes River running from Culver north to South

Junction that includes Madras between the these two towns. Of the 13 creek plots evaluated, only one contained a creeping bentgrass plant, while five of the 15 Deschutes River plots contained creeping bentgrass plants. No ASR368 plants were found along the Deschutes River or the creeks southeast of the Control Area which is in the direction of the prevailing winds. The identified ASR368 plants were found in predominantly disturbed locations in either ditches or canals.

Zapiola (2008) conducted a 4-year study to assess gene flow from the 421 acres of ASR368 in and around the Control Area. Beginning in 2003 (the year the 421 acre fields flowered and produced seed) through 2006, the banks of irrigation canals, ditches, ponds, roadsides, and pipeline sides were surveyed and resident creeping bentgrass and related species plants were tested for the presence of CP4 EPSPS protein. A total of 610 km (379 miles) were surveyed over the 4-year study period. In all four years, no surveyed rabbitsfoot grass or redtop bentgrass plant were found to express the CP4 EPSPS protein. In the first study year (2003) no resident creeping bentgrass plants contained the CP4 EPSPS protein. In 2004, 2005, and 2006, 138 out of 148 (93%), 521 out of 968 (54%), and 360 out of 585 (62%) tested resident creeping bentgrass plants were CP4 EPSPS positive, respectively. The farthest CP4 EPSPS-positive plant identified was 4.6 km from the original ASR368 field found in 2006. These results are expected considering the biology of creeping bentgrass.

While conducting the in situ survey of resistant and susceptible plants, panicles were collected from Agrostis spp. and rabbitsfoot grass (Polypogon monspeliensis) plants from each of the four years (Zapiola, 2008). Seeds from the panicles were planted in a greenhouse and seedlings were screened using glyphosate to assess the occurrence of pollen-mediated gene flow. Low levels of pollen-mediated gene flow from ASR368 was observed in each of the four years. Of the 15,963 seedlings tested from seed originating from susceptible plants in 2003, 0.363% were glyphosate tolerant. The percentage of glyphosate resistant seedlings from seed originating from susceptible plants was 0.032%, 0.036% and 0.006% for 2004, 2005 and 2006, respectively. While, no glyphosate-tolerant seedlings derived from rabbitsfoot grass (Polypogon monspeliensis) plants were identified for any of the four years, a few glyphosate-tolerant seedlings derived from Agrostis spp were identified.

Zapiola and Mallory-Smith (2012) studied the potential hybridization of ASR368 to a related species rabbitsfoot grass (Polypogon monspeliensis). Out of 123,226 rabbitsfoot grass seedlings evaluated in and around the Control Area, no hybridization between rabbitsfoot grass (maternal parent) and ASR368 (pollen donor) and was observed. The study found a single hybrid between ASR368 (maternal parent) and rabbitsfoot grass (pollen donor). The researchers proposed that rabbitsfoot grass's highly self-pollinating nature as the reason for not finding rabbitsfoot grass × ASR368 creeping bentgrass hybrids.

Since 2004, personnel from Scotts, three independent herbicide applicator companies, as well as an independent mapping company have surveyed an extensive area in Jefferson County. Beginning in 2014, the boundaries of the survey areas were extended to include all of the irrigated areas where ASR368 may establish within three counties. This

encompassed 86,263 acres in Jefferson County, 82,182 acres in Malheur County, and 10,743 acres in Canyon County. Surveys have also been conducted along the Deschutes and Snake Rivers, where no ASR368 has been found. The data generated over this time has shown ASR368 being primarily limited to the general vicinity of the production fields, mainly in irrigation channels. No reports have been received that the environment or agriculture has been negatively impacted by the presence of ASR368. Mitigation of ASR368 has effectively reduced ASR368 plants to a very low level. In spring 2015, only 116 plants were found within the 86,263 acres surveyed in Jefferson County (0.001 plants/acre); 478 plants were found within the 82,182 acres surveyed in Malheur County (0.006 plants/acre); and 30 plants were found within the 10,743 acres surveyed in Canyon County (0.003 plants/acre).

The strong winds that blew seeds from ASR368 fields in 2003 resulted in volunteer ASR368 plants becoming established in Kentucky bluegrass seed production fields. While rogueing and herbicide applications were employed to mitigate the ASR368 volunteers, many ASR368 plants remained. To ensure ASR368 was not present in the final seed product, all seed lots from fields containing ASR368 plants were quarantined and evaluated for the presence of ASR368 seeds. After four years of testing, no ASR368 seeds were identified in a total of 102 seed lots analyzed by a registered, independent seed lab (Table VIII-113). ASR368 plants were also found in a Kentucky bluegrass seed production field in 2013. Finished seed from this field was also tested and confirmed negative for ASR368 seeds.

The lack of ASR368 seeds in Kentucky bluegrass seed lots can be attributed to the differences in flowering timing and seed size. Kentucky bluegrass flowers and matures 3-4 weeks sooner than creeping bentgrass which significantly interferes with creeping bentgrass ability to develop and mature. Kentucky bluegrass seeds are also 3.5-6 times larger than creeping bentgrass seeds (Pacific Northwest Extension Publication, PNW299). Creeping bentgrass seed at 0.07 mg per seed is among the smallest of the grass seeds commercially produced. Consequently, it is easily separated from Kentucky bluegrass seed as part of routine seed cleaning processes.

Table VIII-113. USDA Quarantine Testing Program Data

	2004	2005	2006	2007	**Total**
Number of Kentucky bluegrass seed lots examined	11	20	24	47	**102**
Number of glyphosate tolerant bentgrass identified	0	0	0	0	**0**

VIII.K. Conclusion

The experiments and observations described in Section VIII.A through VIII.J were performed to help establish greater familiarity with glyphosate tolerant creeping bentgrass ASR368 and to better understand its plant pest or weed potential in comparison to conventional creeping bentgrasses representing the range in variability common to *A. stolonifera.* All stages of the creeping bentgrass life cycle that could enhance the weed or plant pest potential of ASR368 compared to conventional creeping bentgrasses were examined. Comparisons were made with regard to: seed and vegetative establishment, plant growth, flowering, pollen viability and longevity, fecundity, seed germination and seedling vigor, botanical characteristics, insect resistance, and disease susceptibility. University scientists in addition to those of Scotts performed more than ninety individual experiments between 1999 and 2003 at 65 field locations representing the northern or cool, southern or warm and transition zone climates of turfgrass adaptation (Beard, 1982). The environments where these experiments were performed consisted of both managed and unmanaged ecosystems with variations of light, moisture, soils, nutrition, competition and temperature extremes. Studies of feral ASR368 in the environment confirm that ASR368 plants are primarily limited to disturbed, mesic sites near production fields and behaves similar to conventional creeping bentgrass.

The results of these studies provide a solid basis for determining that the weed or plant pest potential of ASR368, and its progeny, is not different from that of conventional creeping bentgrasses. The following paragraphs briefly summarize the findings reported in the Section VIII.

The results of bare soil and competitive turf seed establishment studies conducted in 2001 and 2002 at four total locations encompassing irrigated and non-irrigated conditions, variation in competition and seasonal establishment were presented in Section VIII.A. These studies demonstrated that: (1) the establishment and persistence of ASR368 tended to fall within the range of the conventional cultivars, (2) the establishment and persistence of ASR368 was generally low for all genotypes when seeded in bare soil (< 30%) and (3) the establishment and persistence of seedlings of all genotypes was unsuccessful when seeded into an existing competitive situation. Given the results from these experiments, which further confirm reports in the scientific literature cited in Section VIII.A, seed of ASR368 would not be expected to germinate, establish or persist in unmanaged competitive and non-competitive ecosystems differently from conventional creeping bentgrasses. These findings support a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on these seed establishment characteristics.

The results of several vegetative establishment studies conducted in 2001 and 2002 at six different locations including irrigated and non-irrigated conditions were presented in Section VIII.B. These studies demonstrated that: (1) the vegetative establishment and persistence of ASR368 tended to fall within the range of the conventional genotypes, (2) ASR368 plants are not different from conventional creeping bentgrass cultivars in their ability to produce new tillers from viable stolon nodes and (3) the establishment of all creeping bentgrass genotypes was much reduced under non-irrigated versus irrigated

conditions. Given the results from these experiments, which further confirm reports in the scientific literature cited in Section VIII.B, ASR368 would not be expected to vegetatively establish or persist differently from conventional creeping bentgrasses. These findings further support a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on its ability to vegetatively establish.

The growth of ASR368 was compared to conventional bentgrasses in bare soil and competitive turf at eleven locations representing the northern (cool), southern (warm) and transition climate zones in 2000, 2001, 2002 and 2003 in Section VIII.C. These studies demonstrated that: (1) ASR368 displayed no increase in vegetative growth, aggressiveness, invasiveness or relative fitness compared to conventional creeping bentgrass cultivars when established in bare soil with no competition or with competition from other turfgrasses in cool, warm or transition climates, (2) ASR368 displayed no competitive advantage in comparison to conventional creeping bentgrass cultivars in direct sun vs. shade or reduced light and (3) the relative growth of ASR368 is within the normal range for other conventional creeping bentgrass cultivars. Given the results of these experiments, which further confirm reports in the scientific literature cited in Section VIII.C, ASR368 and its progeny would not be expected to grow in a different manner in either managed or unmanaged ecosystems from conventional creeping bentgrass cultivars. Consequently, these findings support a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on its vegetative growth.

The flowering characteristics of ASR368 and conventional creeping bentgrass genotypes evaluated in the greenhouse and at four different field locations in 2001 and 2002 were reported in Section VIII.D. These studies demonstrated that: (1) ASR368 genotypes were within the range of B99061R and the conventional cultivars for date of first inflorescence, anthesis initiation, anthesis completion and the duration of anthesis; (2) were consistent with the findings of Christoffer (2003) who observed that the flowering characteristics of ASR368 and a number of other *Agrostis* species were not different in Washington in 2001 and 2002; and (3) ASR368 and its progeny should not be expected to flower differently from conventional creeping bentgrasses. Given the results from these experiments, which further confirm reports in the scientific literature cited in Section VIII.D, ASR368 would not be expected to flower differently from conventional creeping bentgrasses. Consequently, these data and information support a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on its flowering characteristics.

The size and longevity of pollen collected from plants of ASR368 and conventional creeping bentgrass genotypes, grown in the greenhouse and field, evaluated in 2001 and 2002 were reported in Section VIII.E. These studies demonstrated that: (1) the diameter of pollen from ASR368, B99061R and conventional creeping bentgrass cultivars was not significantly different; (2) the longevity of pollen from ASR368, B99061R and conventional creeping bentgrass cultivars was not significantly different and (3) the size and longevity of pollen from ASR368 or its progeny are within the normal ranges of these characteristics compared to conventional creeping bentgrass cultivars. Given the

results from the ASR368 pollen experiments, which are consistent with reports in the scientific literature cited in Section VIII.E regarding the longevity of grass pollen, ASR368 pollen would not be expected to be larger, smaller, or viable for a longer period of time than pollen from conventional creeping bentgrasses. These findings support a conclusion that the potential for ASR368 to outcross with *A. stolonifera* or other species with which the species is known to interbreed would be no different than that of conventional creeping bentgrass. The findings also support a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on the size and longevity of its pollen.

Results of the fecundity characteristics of ASR368 evaluated in the greenhouse and at four different field locations in 2001 and 2002 were reported in Section VIII.F. These studies demonstrated that: (1) ASR368 open-and self- pollinated seed set is not significantly different from B99061R or the conventional cultivars; (2) ASR368 self-pollinated seed set is low, as expected, due to self-incompatibility systems known to exist in the *Agrostis* genus and (3) seed production is variable among creeping bentgrass cultivars and that the ASR368 genotypes are within the range of the conventional cultivars evaluated that are representative of *A. stolonifera*. Given the results of the 2001 and 2002 fecundity studies, which encompassed three generations of ASR368, it is not expected that ASR368 and its progeny would differ in their ability to produce seed compared to conventional cultivars. This supports a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on its seed production characteristics.

Physiological characteristics of the seed of ASR368 were compared to conventional genotypes in a number of germination tests performed in the laboratory. The results of these evaluations were reported in Section VIII.G. These studies demonstrated that: (1) ASR368 GT seed does not germinate differently than non-transgenic ASR368 GS seed under, standard, suboptimal and supra-optimal conditions; (2) the survival and germination of ASR368 GT seed were not different than those of ASR368 GS seed under the stressful conditions of the Accelerated Aging Test, which is also a measure of seed longevity and (3) the germination energy and seedling vigor of ASR368 GT seed were not significantly different across germination conditions from those of the ASR368 GS. Given the results of the seed physiology studies, it is not expected that seed of ASR368 would differ in their ability to germinate under stressful conditions or have greater longevity than seed of conventional creeping bentgrasses. This supports a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on its seed characteristics.

The results of the ASR368 botanical characteristic evaluations conducted in the greenhouse and at four different field locations in 2001 and 2002 are presented in Section VIII.H. These studies demonstrated that: (1) the visual inspection and evaluation of a number of botanical characteristics indicate no gross aberration or deviation between the ASR368 genotypes and conventional cultivars and (2) insertion and expression of the *cp4 epsps* gene did not alter the morphology, floral or vegetative features of ASR368 in a significant way. Given the results from these experiments, which further confirm reports in the scientific literature cited in Section VIII.H, the botanical characteristics of ASR368

would not be expected to be different from conventional creeping bentgrasses. This supports a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on its botanical characteristics.

In Section VIII.I, the observations of plant growth, and insect and disease susceptibility conducted for 65 field releases performed between 1999 and 2002 are summarized. These observations demonstrate that: (1) there are no discernible differences in plant growth between ASR368 and conventional creeping bentgrass plants; (2) there are no discernible differences in disease severity between ASR368 and conventional creeping bentgrass plants and (3) there are no discernible differences in insect infestation between ASR368 and conventional creeping bentgrass. This supports a conclusion of no increased weed or plant pest potential of ASR368 and its progeny as compared to conventional creeping bentgrass based on field observations of plant growth and disease and pest susceptibility.

Finally, Section VIII.J describes a strong empirical record of evaluating feral glyphosate tolerant creeping bentgrass plants in Jefferson County and Malhuer County, Oregon and Canyon County, Idaho. Over eleven years of evaluations demonstrate that, as expected, ASR368 creeping bentgrass plants are primarily limited to disturbed, mesic sites near production fields and behave similar to conventional creeping bentgrass. In addition, even when ASR368 established in Kentucky bluegrass seed production fields, ASR368 seed was not present in the final Kentucky bluegrass seed product.

In summary, the phenotypic and agronomic assessments were evaluated to characterize ASR368, and to assess whether the trait introduced in ASR368 alter the plant pest or weediness potential compared to conventional creeping bentgrasses. The evaluation, using a weight of evidence approach, considered the reproducibility, magnitude, and direction of detected differences between ASR368 and conventional controls, and comparison to the range of the conventional reference cultivars. Results from the phenotypic and agronomic assessments and evaluations of ASR368 plants in managed and unmanaged ecosystems demonstrate that ASR368 does not possess increased weediness characteristics, increased susceptibility or tolerance to specific abiotic stressors, diseases or insect pests, or characteristics that would confer a plant pest risk as compared to conventional creeping bentgrass and are unlikely to increase the weediness of any other cultivated plant or native wild species with which *A. stolonifera* can interbreed.

IX. POTENTIAL OF GENE TRANSFER FROM ASR368 TO RELATED SPECIES

IX.A. Introduction

As discussed in Section II, creeping bentgrass is a highly self-incompatible, essentially obligate outcrossing and wind-pollinated species. Consequently, within the United States, the potential for outcrossing among *Agrostis spp.* is recognized and low levels of gene flow have been viewed as an acceptable risk in seed production (Knowles, 1966). Studies by Wipff and Fricker (2000) and Belanger *et al.* 2003) demonstrate that a low level of creeping bentgrass outcrossing to creeping bentgrass (*Agrostis stolonifera* to *A. stolonifera*) occurs between cultivars at distance. Wipff and Fricker (2000) identified a single intraspecific hybrid plant among more than a thousand at a distance of 958 feet from the pollen source.

There have been reports of inter-specific crossing within the genus *Agrostis* by a number of authors (Davies, 1953; Jones 1956 a, b, c; Bradshaw, 1958; Tutin, 1980; Wipff and Fricker, 2000; Belanger et al. 2003). In the studies conducted by Davies (1953), Jones (1956a,b, c), Bradshaw (1958) and Tutin (1980), these hybrids were artificially produced and were typically of intermediate morphology between the two parents. In addition, they either had a complete loss of or much reduced fertility. Inter-generic hybrids have also been reported between *A. stolonifera* and *Polypogon spp.* but the hybrids were sterile (Björkman, 1960).

The potential for ASR368 to hybridize with other species and the impact of that hybridization on weediness has been studied, as discussed below.

IX.B. ASR368 transgene flow to *A. stolonifera* and related species

To evaluate the potential for pollen-mediated transgene flow from event ASR368 to other related species, a two-year study was initiated with researchers at Washington State University in Franklin County, WA during 2000 – 2002 (USDA# 00-224-01n and 01-177-02n). The goals of this study were to examine: (1) the potential for pollen-mediated event ASR368 intraspecific, interspecific and intergeneric transgene flow and (2) the effect of distance from the "pollen (transgene) donor" on hybridization potential as compared with conventional *Agrostis spp.* and *Polypogon spp.* A summary of the results of this study is provided below and was excerpted from the Masters of Science thesis by Chistoffer (2003).

IX.B.1. Experimental methods

To evaluate the potential for ASR368 pollen-mediated transgene flow, a 50-ha irrigated field was planted in late August 2000 with 'Chateau' Kentucky bluegrass (*Poa pratensis* L.) in Franklin County, WA. In early October 2000, glyphosate at 841 g ae ha^{-1} was sprayed directly onto the Kentucky bluegrass where the ASR368 pollen source and the 15 species used as pollen receptors were to be planted. All plots were later tilled to loosen the soil for planting.

On October 24, 2000, a central planting (13.7-m radius) of approximately 400, two-month old seedlings of F1 ASR368 progeny hemizygous for the *cp4 epsps* gene were planted as a pollen (transgene) donor. Seedlings of 12 different *Agrostis spp.* and three *Polypogon spp.* served as "pollen (transgene) receptor" plants. These species were planted as a five-plant

row within 24 × 14 m plots (78.7 × 46 feet) at 50, 274 and 354 m (164, 899 and 1161 feet) from the edge of the ASR368 pollen donor plants along six axes radiating from the field center at 60-degree intervals. A single plot was also established in the direction of the prevailing wind 0.5 m from the ASR368 on the 0 line (0-line 2 m plot). All plots were approximately 23 × 14 m (Figure IX-1).

On March 21, 2001, separate five-plant rows of bentgrass cultivars Crenshaw and SR1020 were planted into new 8- × 8-m transgene receptor plots 185 m from the transgene donor ASR368 at all axes to supplement the study. Between the Crenshaw and SR1020 rows, 'Southshore', 'Pennlinks', 'Putter', and 'Providence' *A. stolonifera* were added as single five-plant rows. On April 13, 2001 additional receptor and ASR368 plants were added to supplement the study, or as replacements for winter-killed plants. The additional ASR368 plants were placed 0.5 m inside the first ASR368 circle. For the same reasons, additional plants were transplanted in many of the plots during March to May 2002 (Figure IX-1).

Plants were harvested from late June until early August of 2001 and 2002 when visual inspection demonstrated that greater than 65% of the panicles were mature. Panicles were cut near the base, placed into a paper bag, and each five-plant row within a plot was placed into a separate cloth bag. Seed from each plant were planted in the greenhouse, seedlings counted and then sprayed with glyphosate, nine days after emergence and again at 28 days after planting. The percentage of surviving seedlings was used to determine intraspecific, interspecific, and intergeneric transgene flow from event ASR368. Surviving seedlings were also transplanted to evaluate the vegetative and plant growth characteristics of these hybrids in comparison to the parental species.

IX.B.2. Results

In both 2001 and 2002, the percentage of intraspecific ASR368 progeny recovered within the *A. stolonifera* pollen receptor plants was greatest on the 0 and 60-degree lines (Tables IX-1 and IX-2). The percentage of ASR368 progeny recovered decreased with greater distance from the ASR368 pollen source. In 2001, intraspecific ASR368 progeny recovery was 48.9% at one to three meters and decreased to 0.07% at 354 meters on the single line aligned with the prevailing wind (Table IX-1). In 2002, intraspecific ASR368 progeny recovery was 26.6% at 1 to 3 meters, 0.94% and 2.74% at 50 meters and 0.01% and 0.15% at 354 meters on the 0 and 60-degree lines, respectively (Table IX-2). The percentage of intraspecific ASR368 progeny formation is consistent between years and with the findings of Belanger *et al.* (2003) described in Section II.E.6 of this petition.

In both 2001 and 2002, interspecific and intergeneric transgene flow were considerably less than intra-specific transgene flow and both also decreased significantly with distance from the pollen source plants. In 2001, event ASR368 formed interspecific and intergeneric hybrids with *A. trinii*, *A. pallida*, *A. idahoensis*, *A. pallens*, *A. capillaris, A. gigantea*, *A. canina*, *A. hyemalis var. scabra, A. vinealis*, *Polypogon monspeliensis*, *P. fugax,* and *P. viridis* at one to three meters from the event ASR368 pollen source (Table IX-3). No ASR368 hybrids were formed with *A. castellana* or *A. nebulosa* in 2001. At 50 meters, ASR368 hybrids were recovered from *A. capillaris*, *A. hyemalis var. scabra*, *A. idahoensis*, *P. fugax,* and *P. viridis* at less than 0.05% and only at the 0-line 50-m plot (Table IX-4). No interspecific or intergeneric progeny were recovered beyond 50 m.

In 2002, at 1 to 3 meters, event ASR368 formed interspecific and intergeneric hybrids with *A. idahoensis*, *A. pallens*, *A. capillaris*, *A. gigantea*, *A. canina*, and, *P. monspeliensis*, *P. fugax* and *P. viridis* (Table IX-3). No ASR368 hybrids were formed with *A. trinii, A. castellana* and *Apera interrupta* at this distance. At 50 meters on the 0-line, ASR368 hybrids were recovered from *P. fugax, P. monspeliensis, P. viridis, A. capillaris,* and *A. gigantea* (Table IX-4). ASR368 progeny were also recovered from *A. idahoensis* at the 60- and 300-lines 50-m plot at 0.004 and 0.003%, respectively. No interspecific or intergeneric progeny were recovered beyond 50 m.

IX.B.3. Conclusions from hybridization studies

These data suggest that the potential for interspecific and intergeneric hybridization beyond 50 m is low and much less likely to occur than intraspecific hybridization. These rates of interspecific or intergeneric hybridization are also consistent with the findings of Belanger et al. (2003; described in Section II.E.6 of this petition) and supports a conclusion that ASR368 has not greater potential for pollen-mediated gene flow to sexually compatible species than conventional creeping bentgrass.

IX.C. Characteristics of hybrids between ASR368 and related species

Comparative evaluation of ASR368 to conventional creeping bentgrass demonstrated no selective advantage conferred by the introduced glyphosate tolerance trait (Section VIII). Thus, it would be expected that no advantage would be conferred to a hybrid formed between ASR368 and a related species with which it can interbreed. However, confirmatory assessments of hybrid growth and development were conducted and are presented herein.

The glyphosate tolerant hybrid seedlings from several representative plants of each species and the parent species were transplanted to individual plastic pots. Three replications, each containing 20 plants of each seedling lot were arranged in a randomized complete block design. The plants were allowed to develop in a poly-house in Marion County, Oregon prior to estimation of ground coverage, growth habit and tiller density. The conventional parent species used as reference comparisons for the recovered transgenic hybrids are provided in Table IX-5.

Data were collected for the following parameters: (1) percent ground cover, (2) tillers per plant and (3) plant growth habit. Vegetative spring growth was estimated as percent cover of each container by the plant represented by the parental species collections or the recovered hybrids. Tiller capacity per plant was estimated using a scale of 1-5 where, 1 = 1-5 tiller, 2 = 6-20 tillers, 3 = 21-50 tillers, 4 = 51-90 tillers and 5 = > 90 tillers. An average for each population was then calculated prior to statistical analysis. Plant type was also estimated using a categorical 1-3 scale where, 1 = bunch, tufted, erect or non-spreading, 2 = pseudo erect, not strongly bunched, tufted nor decumbent and 3 = spreading, decumbent or prostrate. All observations were made 91 days after transplanting (180 days after seeding).

The recovered interspecific and intergeneric ASR368 hybrids were either equivalent to or considerably less robust than their respective maternal and paternal parent species collections with regard to ground cover, growth habit or tillering capacity (Table IX-6).

In 2002, these same hybrids were evaluated in the field to confirm that the recovered ASR368 hybrids remained comparable to or less robust than their respective maternal and parental species with respect to growth rate. Vegetative tillers of each hybrid and parent were transplanted in the field into cultivated soil on August 15 and 16, 2002 in Marion County, Oregon. Each ASR368 hybrid was represented by four replications of three or four plants arranged in a randomized complete block design. Soil fertility and moisture were maximized to ensure an optimum growing environment throughout the study. The diameter of each plant canopy was recorded on October 13, 2002 as a measurement of plant diameter (cm). Plant diameter was then transformed to percent ground cover based on the field space provided per individual plant.

The plant diameter and percent ground covered by the ASR368 hybrids were either not significantly different from or smaller than their related conventional parent species (Table IX-7.

After two years of studying these hybrids, the results further demonstrate that resulting hybrids from outrcossing with ASR368 would likely be no more weedy or invasive than plants that exist among the *Agrostis* and *Polypogon* genera today.

IX.D. Overall conclusions of ASR368 gene flow and hybrid evaluation studies

The results of pollen-mediated gene flow and hybrid evaluation studies with ASR368 are consistent with the findings of researchers evaluating conventional creeping bentgrass: (1) similar to conventional creeping bentgrass, ASR368 can form intraspecific, interspecific and intergeneric hybrids with several other *Agrostis* spp. and *Polypogon* spp.; (2) outcrossing frequency falls precipitously as distance increases from the pollen source; (3) prevailing wind influences the frequency of outcrossing and (4) hybrids between ASR368 and several related species were no more competitive than the populations representing the parent species.

Therefore, interspecific and intergeneric hybridization with ASR368 confers no novel growth characteristics that would make hybrid plants any more invasive than the respective parent species or than hybrids with conventional creeping bentgrass. This is consistent with the results of extensive phenotypic and agronomic assessments of ASR368 that demonstrate ASR368 does not possess increased weediness characteristics, increased susceptibility or tolerance to specific abiotic stressors, diseases or insect pests, or characteristics that would confer a plant pest risk compared to conventional creeping bentgrass.

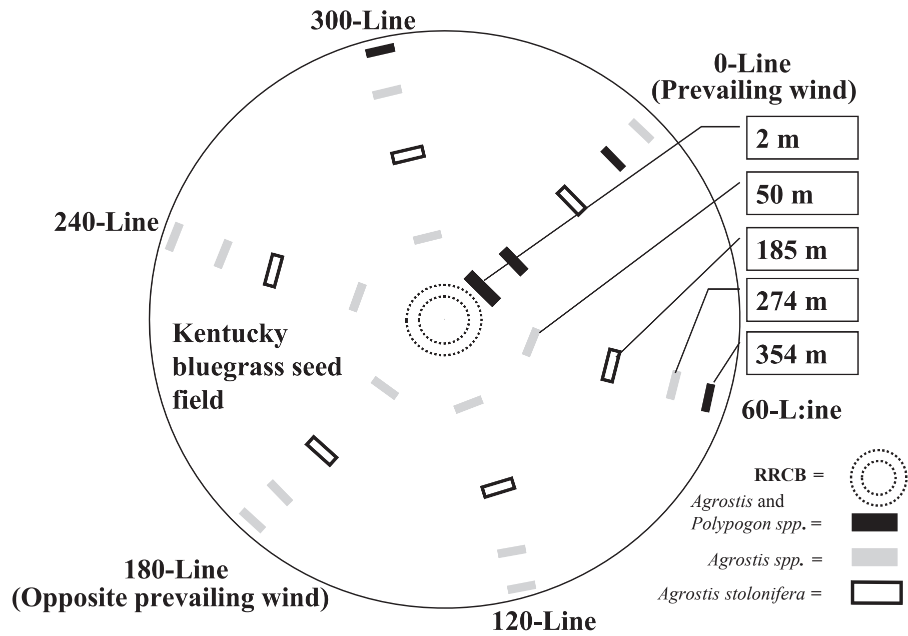

Figure IX.C.1.a. 2001

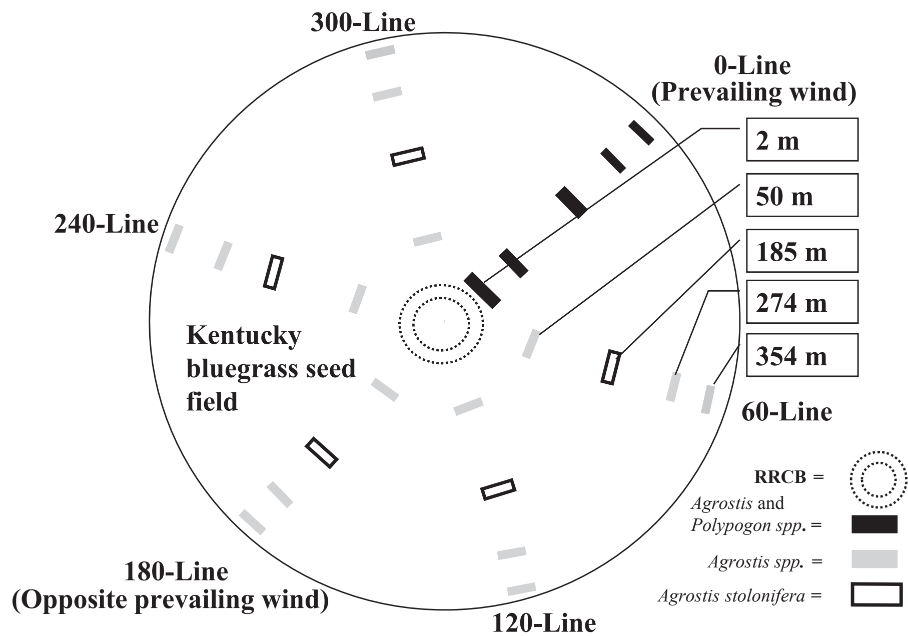

Figure IX.C.1.b. 2002

Figure IX-1. Plot design for pollen-mediated intraspecific, interspecific and intergeneric transgene flow study conducted in Franklin County, WA from 2001 and 2002

Table IX-1. Percentage of event ASR368 intraspecific *A. stolonifera* progeny recovered from plants at different line and distance plots in 2001 at Franklin County, WA

		2001				
		Distance (m) from ASR368 pollen (transgene) donor				
		2[1]	50	185	274	354
Line	0[2]	48.87	0.56	0.38	0.15	0.07
	60		0.27	0.03	0	<0.01
	120		0.02	0	0	0
	180		0[3]	0	0	0
	240		0.07	0	0	0
	300		0.59	0.03	0.03	0.03

[1] Only a single plot was planted 2 m from the ASR368 transgene donors (0-line 2-m plot).
[2] The 0-line was aligned with the prevailing wind direction. All other lines indicate compass degrees clockwise from the 0-line.
[3] All receptor *A. stolonifera* died following glyphosate application by a custom applicator.

Table IX-2. Percentage of event ASR368 intraspecific *A. stolonifera* progeny recovered from plants at different line and distance plots in 2002 at Franklin County, WA

		2002				
		Distance (m) from ASR368 pollen (transgene) donor				
		2[1]	50	185	274	354
Line	0[2]	26.63	0.94	0.19	0.02	0.01
	60		2.74	0.16	0.11	0.15
	120		0.85	0.02	0.01	0
	180		0.75	<0.01	<0.01	0
	240		0.08	<0.01	0	0
	300		0.39	0.01	<0.01	0

[1] The plot 2 m from the ASR368 transgene donors was only present on the 0-line (0-line 2-m plot).
[2] The 0-line was aligned with the prevailing wind direction. All other lines indicate compass degrees clockwise from the 0-line.

Table IX-3. Hybrid formation between event ASR368 and related species placed 1 to 3 m along the direction of the prevailing wind from event ASR368 (pollen donor) during 2001 and 2002 at Franklin County, WA

	2001			2002		
Species	n[1]	ASR368 progeny recovery (%)	Std. dev.	n	ASR368 progeny recovery (%)	Std. dev.
A. stolonifera	7	48.87	22.29	11	26.63	13.85
A. trinii	2	10.56[2]	13.34	1	0	0
P. monspeliensis	1	1.58	0	4	0.74	0.45
A. pallida	3	1.45	2.32	*	*	*
P. fugax	3	1.42	1.01	2	2.68	1.01
A. idahoensis	5	0.8	1.01	5	0.39	0.53
P. viridis	3	0.69	0.22	2	1.3	0.93
A. pallens	4	0.52	0.01	6	0.01	0.03
A. capillaris	5	0.37	0.55	10	0.4	0.51
A. gigantea	8	0.05	0.04	8	0.17	0.18
A. canina	5	0.03	0.16	4	0.11	0.23
A. vinealis	6	0.01	0.03	*	*	*
A. hyemalis	5	0.002	0.006	*	*	*
A. castellana	5	0	0	10	0	0
A. nebulosa	5	0	0	*	*	*
Apera interrupta	*	*	*	5	0	0

[1] Number of subsamples for each species. The percentage ASR368 progeny recovery for each subsample (plant) was determined and then a mean percentage ASR368 progeny recovery calculated across subsamples.

[2] Species identity of single plant harvested as *A. trinii* is considered to be a creeping bentgrass impurity that perished following pre-emergent herbicide application following the first year harvest.

* Species was not included during this year of the study.

Table IX-4. Hybrid formation between event ASR368 and related species placed 50 m along the direction of the prevailing wind (0^0 axis) from event ASR368 (pollen donor) during 2001 and 2002 at Franklin County, WA

Species	% ASR368 Progeny Recovered	
	2001	**2002**
P. fugax	0.006	0.021
P. monspeliensis	0.00	0.017
P. viridis	0.023	0.012
A. capillaris	0.026	0.011
A. castellana	0.000	0.000
A. gigantea	0.000	0.008
A. hyemalis	0.005	np
A. idahoensis (0^0 axis)	0.017	0.000
A. idahoensis (60^0 axis)	0.000	0.004
A. idahoensis (300^0 axis)	0.000	0.003
A. idahoensis (300^0 axis)	0.000	0.003
A. pallens	0.000	0.000

np not planted in 2002

Table IX-5. Conventional (non-transgenic) parent species used as reference comparisons for event ASR368 hybrids of the same parent species in 2001 and 2002

Scientific Name	Common Name	Plant Type Hybrid or Conventional*
Agrostis stolonifera L.	Creeping Bentgrass	C
Agrostis capillaris L.	Colonial Bentgrass	C, H
Agrostis gigantea Roth.	Redtop Bentgrass	C, H
Agrostis idahoensis Nash.	Idaho Bentgrass	C, H
Agrostis pallens	Dunes Bentgrass	C, H
Agrostis trinii Turcz.	Brown Bentgrass	C, H
Polypogon monspeliensis L. Desf.	Rabbitsfootgrass	C, H
Polypogon viridis (Gouan) Breistr.	Watergrass	C, H
Polypogon fugax Nees es Steud.	Ditch *Polypogon*	C, H
Agrostis castellana Boiss.& Reut.	Dryland Bentgrass	C,
Agrostis canina L.	Velvet Bentgrass	C,
Agrostis vinealis Schreb.	Brown Bentgrass	C,
Agrostis sp.	Rhode Island Bent	C,

* Where C = conventional and H = hybrid formed between ASR368 and conventional plants

Table IX-6. Plant characteristics of hybrids recovered from event ASR368 and related species in 2001

Plant Population[1]	Percent Ground Cover[2]	Tillers/Plant (1-5[3])					Growth Habit (1-3[4])		
		% Occurrence per Rating Unit							
		1	2	3	4	5	1	2	3
A. stolonifera (Creeping Bent) Conventional	20.47 d	0	17	67	17	0	44	27	27
A. capillaris (Colonial Bent) Hybrid	14.33 h-k	0	33	67	0	0	67	33	0
A. capillaris (Colonial Bent) Conventional	15.50 e-h	0	33	44	22	0	56	11	33
A. gigantea (Redtop) Hybrid	15.00 f-i	0	50	50	0	0	75	17	8
A. gigantea (Redtop) Conventional	10.65 jkl	22	44	33	0	0	83	17	0
A. idahoensis (Idaho Bent) Hybrid	9.43 l	11	56	22	11	0	67	33	0
A. idahoensis (Idaho Bent) Conventional	14.53 g-j	0	44	44	11	0	22	22	56
A. pallens (Dunes Bent) Hybrid	10.75 jkl	0	58	33	8	0	67	17	17
A. pallens (Dunes Bent) Conventional	29.33 a	0	0	67	33	0	33	67	0
P. monspeliensis (Rabbitsfootgrass) Hybrid	19.43 de	0	0	67	33	0	78	22	0
P. monspeliensis (Rabbitsfootgrass) Conventional	18.30 d-g	22	44	22	11	0	89	11	0
P. fugax Hybrid	20.13 d	0	44	56	0	0	100	0	0
P. fugax Conventional	21.80 cd	0	56	33	11	0	89	11	0
P. viridis (Watergrass) Hybrid	15.23 f-i	0	67	33	0	0	100	0	0
P. viridis (Watergrass) Conventional	25.13 bc	0	67	22	11	0	22	78	0
A. castellana (Dryland Bent) Conventional	28.37 ab	6	17	39	39	0	50	50	0
A. canina (Velvet Bent) Conventional	18.57 def	0	22	44	22	11	44	22	33
A. trinii Hybrid (Brown Bent)	5.33 m	36	64	0	0	0	100	0	0
A. trinii Conventional	10.40 kl	0	89	11	0	0	100	0	0
A. vinealis (Brown Bent) Conventional	10.50 kl	0	44	56	0	0	67	22	11
A. sp. (Rhode Island Bent) Conventional	19.53 d	0	44	44	11	0	67	33	0
LSD (p=0.05%)	3.964								
Stn. Deviation	3.433								

[1] Conventional or hybrid formed between ASR368 and conventional plants

[2] Means followed by the same letter are not significantly different (LSD, p = 0.05)

[3] Tillers/plant: 1 = 1 to 5; 2 = 6 to 20; 3 = 21 to 50; 4 = 51 to 90; 5 = greater than 90

[4] Growth habit: 1 = bunch, tufted, erect, not spreading; 2 = pseudo-erect, not strongly bunched, tufted or decumbent; 3 = spreading, decumbent, prostrate

Table IX-7. Plant characteristics in 2002 of conventional *Agrostis* and *Polypogon* species used as comparators and interspecific and intergeneric hybrids with creeping bentgrass ASR368 recovered from related *Agrostis* and *Polypogon* species in 2001

Plant Population	Relationship	n	Plant Diameter (cm)[2]	Ground Cover (%)[3,4]
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
A. capillaris (Colonial Bent) Hybrid	Hybrid	12	33.3fgh	57.1def
A. capillaris (Colonial) Conventional	Parent	12	38.7def	75.5def
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
A. gigantea (Redtop) Hybrid	Hybrid	16	39.6def	78.6de
A. gigantea (Redtop) Conventional	Parent	20	44.5cde	103.1cd
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
A. idahoensis (Idaho Bent) Hybrid	Hybrid	12	24.3hi	31.5ef
A. idahoensis (Idaho Bent) Conventional	Parent	12	23.4hi	27.9ef
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
A. pallens (Dunes Bent) Hybrid	Hybrid	16	36.1efg	63.9def
A. pallens (Dunes Bent) Conventional	Parent	12	39.5def	76.4def
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
P. monspeliensis (Rabbitsfootgrass) Hybrid	Hybrid	12	39.9def	77.5de
P. monspeliensis (Rabbitsfootgrass) Conventional	Parent	12	58.5ab	187.3b
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
P. fugax Hybrid	Hybrid	12	40.1def	84.9de
P. fugax Conventional	Parent	12	67.9a	267.2a
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
P. viridis (Watergrass) Hybrid	Hybrid	12	47.3cd	109.5cd
P. viridis (Watergrass) Conventional	Parent	12	53.2bc	147.bc
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
A. trinii Hybrid (Brown Bent)	Hybrid	16	35.9efg	78.7de
A. trinii Conventional	Parent	12	25.3ghi	31.5ef
A.stolonifera (Creeping Bentgrass) Conventional	Parent	24	69.3a	249.2a
A. pallida	Hybrid	16	36.6def	77.8de
LSD (p=0.05%)			11.17	60.44
Std Deviation			7.9	42.74

[1] conventional or hybrid formed between ASR368 and conventional plants
[2] Means followed by same letter are not significantly different (LSD, p = 0.05)
[3] Means followed by same letter are not significantly different (LSD, p = 0.05)
[4] Percent ground cover based on plant spacing of 45.7cm (1.5 ft)

X. Plant Pest Assessment

USDA-APHIS has responsibility, under the Plant Protection Act (PPA) (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. Regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

A plant pest, as defined in the PPA, is the living stage of any of the following, or a similar article, that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; (G) an infectious agent or other pathogen, or (H) any article similar to or allied with any of the articles specified in the preceding subparagraphs (7 U.S.C. § 7702[14]). Data presented in Sections V through IX of this petition confirm that ASR368, with the exception of glyphosate tolerance, is not fundamentally different from conventional creeping bentgrass, in terms of plant pest potential. Scotts and Monsanto are not aware of any study results or observations associated with ASR368 that would suggest an increased plant pest risk would result from its presence in the environment.

The plant pest assessment was based on multiple lines of evidence developed from a detailed characterization of ASR368 compared to conventional creeping bentgrass, followed by a risk assessment on detected differences. The plant pest risk assessment in this petition was based on the following lines of evidence: 1) a single, intact copy of the T-DNA inserted in a single locus within the creeping bentgrass genome; 2) characterization and safety of the expressed CP4 EPSPS protein; 3) compositional equivalence of ASR368 forage compared to a conventional creeping bentgrass; 4) phenotypic, agronomic, and environmental interaction characteristics demonstrating no increased plant pest potential or weediness as compared to conventional creeping bentgrass; 5) interspecific and intergeneric hybridization with ASR368 confers no novel growth characteristics that would make hybrid plants any more weedy than the respective parent species; and 6) negligible risk to non-target organisms. Using the assessment above, the data and analysis presented in this petition lead to a conclusion that ASR368 is not expected to be a plant pest, and therefore should no longer be subject to regulation under 7 CFR § 340.

XI. References

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Appendix A: Reports from the literature citing putative and confirmed hybrids between *A. stolonifera* and other related species and genera

Latin Name	Synonyms	Common Name	2	Adaptation	Cross Frequency or ratio	Confirmed Hybrid Reference	Other Reference. Putative + Fail	Barriers to crossing and persistence
Agrostis stolonifera L.	*A. palustris*	Creeping bentgrass	28	Marshy, fertile lowlands				Distance, pollen competition.
A. capillaris	*A. tenuis* Sibth. *A. vulgaris*	Colonial bentgrass Browntop	28	Variable Davies, 1953.	NA	Davies, 1953	Jones, 1956 examines meiosis of Davies hybrids. Bradshaw, 1958 examines morph. of Davies hybrids and field collection.	Anthesis time of parents Davies, 1953. Philipson, 1937 First anthers of hybrid fail to dehisce. Davies, 1953 Meiotic irregularity. Jones, 1953., 1956. Low fertility of hybrids. Davies,
					NA	Wipff, Pure Seed Testing, 1999 Wipff		
					0.1 / panicle	Belanger, 2000		
					0.00044	Belanger, 2001		
							Fouillade, 1932 Putative	
							Hegi, 1935. Putative	
A. castellana Bois. et Reut.		Dryland bentgrass 'Highland'	42		0.07 / panicle	Belanger, 2000		Anthesis date (Faith Belanger, 2000) Chromosome number
					0.000015	Belanger, 2001, 2002		
					NA	Wipff, Pure Seed Testing, 1999, Wipff		
A. alba L.	*A. gigantea* Roth.	Redtop	42	Damp shaded and arable land.	0		Davies, 1953 Failed forced cross	Anthesis date (Belanger, 2000) Anthesis time Davies, 1953. Philipson, 1937 Meiotic irregularity. Poor fitness of aneuploid hybrids makes field
					0.4 seed / panicle	Belanger, 2000		Anthesis date, cross incompatibility
					NA	Wipff, Pure Seed Testing,		

Latin Name	Synonyms	Common Name	2	Adaptation	Cross Frequency or ratio	Confirmed Hybrid Reference	Other Reference. Putative + Fail	Barriers to crossing and persistence
						Wipff and Fricker, 2001		
					0.000000		Belanger, 2001, 2002	Anthesis nick date and time of day
A. canina var. *fascicularis*	*A. canina canina*	Velvet bentgrass	14	Wet damp soils Davies, 1953.			Davies, 1953	Anthesis time Davies, 1953 Philipson, 1937. Low fertility of hybrids with
							Clapham, et al 1952 Putative ref.	
							Philipson, 1937 Putative ref.	
					0.046 seed / panicle	Belanger, 2000		Anthesis date, incompatibility
					NA	Wipff, Pure Seed Testing, 1999 Wipff and Fricker, 2001		
					0.000		Belanger, 2001, 2002	Anthesis date and time of day
A. canina var. *arida*	*A. vinealis* Schreber. *A. canina montana* Hartm. *A. trinnii*	Brown bent	28				Davies, 1953	Anthesis time of day. Davies, 1953 Meiotic irregularity of hybrids. Jones, 1953. Sterility of hybrids. Jones, 1956.
A. canina							Philipson, 1937	
A. exarata	*A. ampla, A. longiligula*	Spike bentgrass	56	Native Wetland Water limits colonization in West. Lamson Scribner, 1897	NA		Carlbom, 1967 Putative Welsh et al, 1987 Putative	Cleistogamous. Carlbom, 1967. Chromosome # 42 and 56.
A. pallens		Dune bent Seashore bent	42	Coastal	5/57	Wipff and Fricker, 2001		Coastal endemic
A. scabra:		Rough bent	42	Med. to high elevation	NA		Welsh et al., 1987 Putative	Allogamous

Latin Name	Synonyms	Common Name	2	Adaptation	Cross	Confirmed Hybrid Reference	Other Reference. Putative + Fail	Barriers to crossing and persistence
Polypogon monspeliensis		Annual rabbitsfoot grass		Wet ground	NA		Camus, 1958	
P. fugax	*A. stolonifera X P. monspeliensis* *A littoralis P littoralis*			Wet ground	NA		Hubbard, applies name for putative hybrids of *A. stolonifera* with *P. monspeliensis*	
P. littoralis	*P. fugax* *A littoralis,* *A. stolonifera X*			Wet ground	NA		Philipson, 1937 reports herbarium specimen appears to be a hybrid of *A. stolonifera* and *P. monspeliensis* .	
P. semiverticill a ta	*P. viridis* *A verticillata* *A semiverticillata*	Water bent	42	Wet ground	NA		Welsh et al., 1987 reports putative hybrid sighting.	
P. viridis	*A verticillata* *A semiverticillata*	Water bent	42	Wet ground	NA			
P. elongatus				Wet ground	NA		Parodi, 1951 Putative	

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