Petition for the Determination of Nonregulated Status for Maize 32138 SPT Maintainer Used in the Pioneer Seed Production Technology (SPT) Process

We submit this petition under 7 CFR §340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article should no longer be regulated under 7 CFR §340.

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OECD Unique Identifier: DP-32138-1

June 18, 2009

NO CBI

Release of Information

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting the information in this assessment for review by the USDA as part of the regulatory process. By submitting this information, Pioneer does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., Section 552; USDA complies with the provisions of FOIA and USDA's implementation regulations (7 CFR Part 1.4); and this information is responsive to the specific request. Except in accordance with the FOIA, Pioneer does not authorize the release, publication, or other distribution of this information (including website posting) without Pioneer's prior notice and consent.

Pioneer authorizes the posting of the petition to the BRS Website at such time when the Pest Risk Assessment and the Environmental Assessment are published in the Federal Register for public comment.

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 petitioners, which are unfavorable to the petition.

Natalie Weber June 18 2009
Date

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Summary

Pioneer Hi-Bred International, Inc. (Pioneer), a DuPont Company, has developed a process called 'Seed Production Technology' (SPT) that facilitates large-scale, in-house production of male-sterile maize lines that are used as female inbred parents for subsequent hybrid seed production. Hybrid maize seed production requires crossing two inbred parent lines to produce hybrid seed sold to growers. The female inbred parent must be prevented from shedding pollen to avoid self- pollination that reduces hybrid seed quality and compromises hybrid yield potential. SPT offers a number of advantages over other approaches to controlling male-fertility in female inbred parents, such as detasseling (physical removal of the pollen-producing tassels) or cytoplasmic male-sterility (CMS). The SPT process requires no detasseling, works across all germplasm types, increases seed yields and seed quality, and produces F1 hybrid plants that are fully fertile. The female inbred parent seed produced using this technology does not contain the 32138 SPT insertion and consequently, hybrid seed produced using these male-sterile female inbred parents does not contain the 32138 SPT insertion and hence are non-transgenic for SPT.

Pioneer's SPT process is based on a transgenic maize event DP-32138-1, hereafter referred to as '32138 SPT maintainer'. The 32138 SPT maintainer is used as a pollinator to propagate the seed of male-sterile female inbred parent lines. The 32138 SPT maintainer is neither a commercial product on its own nor part of a commercial product; there is no intent for the 32138 SPT maintainer seed to enter commercial grain channels. The transgenic 32138 SPT maintainer is currently grown under USDA-APHIS permits on Company-controlled parent seed production fields that are reproductively isolated from other maize fields. In the future, 32128 SPT maintainer will be grown on a total of less than 5,000 acres/year in the U.S. Rather than obtaining a USDA-APHIS permit each year for these 32138 SPT maintainer acres, Pioneer is requesting that USDA-APHIS deregulate the 32138 SPT maintainer to facilitate its use in the Company's inbred seed production process.

In addition, Pioneer is supplying data to show that progeny derived from the SPT process are consistently and reliably non-transgenic for SPT genes. In order to address questions regarding the movement of progeny seed and grain in international commerce, we request that APHIS make a finding that SPT progeny lack the 32138 SPT insertion and are therefore non-transgenic for SPT.

The 32138 SPT maintainer was generated by *Agrobacterium*-mediated transformation of a genetically male-sterile (*ms45/ms45*) maize line with a plasmid designated PHP24597. Plasmid PHP24597 contains three gene expression cassettes essential for the functioning of the SPT system: *Ms45*, *zm-aa1*, and *DsRed2(Alt1)*[1].

1) *Ms45* is a maize gene that encodes the MS45 protein required for the production of fertile pollen and is controlled by a maize anther preferred promoter *5126*. Mutations in the *Ms45* gene result in male-sterile plants when in a homozygous recessive (*ms45/ms45*) state. A single copy of the *Ms45* gene can restore fertility in the male-sterile *ms45/ms45* genetic background.

2) *zm-aa1* is a maize gene that encodes the ZM-AA1 α-amylase protein and is controlled by the *Pg47* maize promoter. The α-amylases belong to a family of glycosyl-hydrolases catalyzing hydrolysis of (1-4)-α-D-glucosidic linkages in polysaccharide molecules, such as starch. Expression of ZM-AA1 α-amylase in developing pollen results in starch hydrolysis and depletion of starch reserves, and renders the pollen infertile[2].

[1] A variant of Clontech's (www.clontech.com) DsRed protein, isolated from a marine coral, *Discosoma sp.*
[2] Pollen infertility refers to the inability of pollen to germinate and fertilize the ovule.

3) *DsRed2 (Alt1)* is a marker gene that encodes a variant of the red fluorescent protein (DsRed2) with one alteration (*Alt1*) and is controlled by the *Ltp2* barley promoter. The expression of DsRed2 protein in seed imparts a pinkish-red coloration to the aleurone layer of maize seeds allowing for visual identification and efficient automated separation of 32138 SPT maintainer seed from yellow seed that are non-transgenic for SPT.

The expressed MS45 protein restores fertility in transgenic 32138 SPT maintainer by enabling pollen production. However the *Ms45* gene in 32138 SPT maintainer is hemizygous (*Ms45/-*) and as a result, only one half of the pollen produced contains the *Ms45* gene. That half of the pollen also contains the linked *zm-aa1* gene, so the expressed α-amylase enzyme degrades starch, rendering the transgenic *(Ms45/zm-aa1/DsRed2(Alt1))* pollen infertile. The remaining half of the pollen is non-transgenic for SPT, fertile, and carries the endogenous recessive *ms45* gene. Therefore, when 32138 SPT maintainer is used as a pollinator to propagate the seed of male-sterile female inbreds that are non-transgenic for SPT, the derived progeny retain their male-sterility genotype (*ms45/ms45*) and do not contain the 32138 SPT insertion and are therefore non-transgenic for SPT. Hence F1 commercial hybrids produced using these male-sterile progeny do not contain the 32138 SPT insertion and are also non-transgenic for SPT.

Molecular characterization of the DNA insertion in event DP-32138-1 confirmed that a single, intact copy of the PHP24597 T-DNA containing a single copy of each of the gene expression cassettes was inserted into the maize genome. Southern blot analysis confirmed the absence of any plasmid backbone sequences. The integrity and stability of the inserted PHP24597 T-DNA were verified across three breeding generations.

While it is expected and intended that 32138 SPT maintainer will not enter commercial grain channels, Pioneer evaluated the food and feed safety of 32138 SPT maintainer seed. Both MS45 and ZM-AA1 proteins are encoded by maize gene sequences and have no biologically significant homologies to known toxins or allergens. The DsRed2 protein is the only protein not derived from a maize gene sequence expressed in the 32138 SPT maintainer. A New Protein Consultation (NPC) safety package for the DsRed2 protein was submitted to FDA on October 11, 2006. Bioinformatic analyses revealed no biologically significant homologies to known or putative protein allergens or toxins. The DsRed2 protein is not glycosylated and is hydrolyzed rapidly within 30 seconds in simulated gastric fluid. There was no evidence of acute toxicity in mice for the DsRed2 protein at a dose of 1860 mg of protein per kg of body weight. Therefore, based on the expression levels of this protein in maize seed, a wide margin of safety exists for human and animal consumption of the DsRed2 protein.

The 32138 SPT maintainer has been field tested in the U.S. since 2005 under permit and notification granted by USDA-APHIS. Comprehensive agronomic performance and ecological assessments of the 32138 SPT maintainer were conducted in replicated field studies in the U.S. during the 2007 growing season. Agronomic characteristics such as emergence, seedling vigor, plant height, ear height, stalk lodging, root lodging, stay green, time to silking and pollen shed, disease incidence, and insect damage were measured. Field trials of 32138 SPT maintainer were observed for naturally occurring disease or insect biotic stressors. In laboratory experiments, information was also collected on seed germination, dormancy, and the duration of pollen viability. Analysis of agronomic and ecological data showed no biologically meaningful differences between 32138 SPT maintainer and the inbred control maize, indicating no plant pest characteristics for 32138 SPT maintainer. Likewise, assessment of the ecological data detected no biologically meaningful differences between 32138 SPT maintainer and the inbred control maize lines indicative of a selective advantage that would result in 32138 SPT maintainer having an increased invasiveness potential for natural habitats.

The data support a conclusion of agronomic comparability of 32138 SPT maintainer to conventional inbred maize with respect to the lack of increased weediness and plant pest potential. In addition, based on the compositional evaluation conducted in accordance with the

OECD consensus document on compositional considerations for new varieties of maize, 32138 SPT maintainer seed was found to be compositionally equivalent to seed from conventional maize inbreds.

Since the 32138 SPT maintainer will not be commercially available, there will be no impact on the existing hybrid maize agronomic and production practices, market dynamics, and grower choice. The use of the SPT process in Pioneer hybrid seed production will have no impacts on organic or conventional farming or on raw or processed agricultural commodities, especially since it will be cultivated on a limited acreage (<5,000 acres) and reproductively isolated from other maize fields. There will be no impact on threatened or endangered species because the proteins encoded by the 32138 SPT insertion are not known to be toxic. Any exposure would be limited to only those organisms in or near 32138 SPT maintainer fields. In addition, 32138 SPT maintainer will be grown on existing agricultural land, controlled and managed by Pioneer. No new land will be put into agricultural production because of the deregulation of 32138 SPT maintainer.

Based on the safety assessment of 32138 SPT maintainer, we conclude that there will be no adverse effects on animal or human health. The 32138 SPT maintainer is not intended to enter the commercial food or feed supply, however, Pioneer has taken necessary steps to evaluate the food and feed safety of any small quantities of 32138 SPT maintainer seed and vegetative material that may be disposed of as animal feed. Pioneer has submitted New Protein Consultations to the FDA for both the DsRed2 and ZM-AA1 proteins encoded by the 32138 SPT insertion.

Within this petition, Pioneer has supplied data on the genetic efficiency and stability of the SPT system (on 2.5 million progeny seeds), detailed information on the accuracy of the mechanical color sorting of 32138 SPT maintainer, a molecular analysis to confirm absence of the 32138 SPT insertion in the SPT progeny, and quality system information on handling of SPT seed and inbred parent seed production practices. Pioneer requests that USDA-APHIS make a finding that the SPT process, as described and operated by Pioneer, consistently gives rise to progeny that lack the 32138 SPT insertion and which are, therefore, non-transgenic for SPT.

The genetic efficiency of the SPT system (*i.e.,* the reproducibility of the production of male-sterile progeny that lack the 32138 SPT insertion when using 32138 SPT maintainer as a pollinator) is greater than 99.999%. Expression of the DsRed2 protein in the 32138 SPT maintainer seed enables automated seed sorting using high speed commercial optical sorters to separate SPT seed from non-SPT seed (seed that lacks the 32138 SPT insertion). The accuracy of the optimized seed sorters to detect and separate fluorescent red 32138 SPT transgenic seed from yellow seed (non-transgenic for SPT) is >99.95%. Hence the combined genetic and mechanical efficiency of the SPT process is >99.9999995%.

Based on the data contained herein, Pioneer requests USDA-APHIS to make a determination that 32138 SPT maintainer and crosses of maize lines containing this event with non-regulated maize lines no longer be considered regulated articles under 7CFR 340.

Furthermore, in order to address questions regarding the movement of 32138 SPT maintainer-derived progeny seed and grain in international commerce, Pioneer is requesting that USDA-APHIS make an additional finding that the SPT process, as described and operated by Pioneer, consistently and reliably produces progeny that lack the 32138 SPT insertion and are therefore non-transgenic for SPT.

Abbreviations, Acronyms, and Definitions

~	Approximately
5126 promoter	Maize anther-preferred promoter (Cigan and Albertsen, 1997)
32138 SPT maintainer	Maize line containing the DP-32138-1 event
32138 SPT insertion	The DNA from plasmid PHP24597 that has been integrated into the genome of the 32138 SPT maintainer.
ADF	Acid detergent fiber
AOSA	Association of Official Seed Analysts
APHIS	Animal and Plant Health Inspection Service
Brittle-1	Amyloplast-targeting transit peptide from the maize *brittle-1* gene
bp	Base pair
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary DNA
cM	Centimorgan
CMS	Cytoplasmic male-sterility
Da	Dalton
dam	DNA adenine methylase
DIG	Digoxygenin
DP-32138-1	Event DP-32138-1, also known as 32138 SPT maintainer
DNA	Deoxyribonucleic acid
DsRed2(Alt1)	A specific fluorescent marker gene; a variant of Clontech's *DsRed* gene
DsRed2	A specific fluorescent protein expressed by *DsRed2(Alt1)* gene
E. coli	*Escherichia coli*
E score	Expectation score
ELISA	Enzyme linked immunosorbent assay
ERS	Economic Research Service, the primary source of economic and research information in the U.S. Department of Agriculture
FDA	Food and Drug Administration
FDR	False discovery rate
Genetic efficiency of SPT system	Using the SPT maintainer as a pollinator, the reproducibility of the production of male-sterile progeny that lack the 32138 SPT insertion.
GRAS	Generally recognized as safe
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
ILSI	International Life Sciences Institute
Inbred 705	A Pioneer elite inbred line
IPM	Integrated Pest Management
In2-1	Terminator sequence from maize *In2-1* gene
kb	Kilobase pair
kDa	Kilodalton
LB	Left Border
LC/MS	Liquid chromatography/mass spectrometry
LLOQ	Lower limit of quantitation
LOD	Limit of detection

Abbreviations, Acronyms, and Definitions (continued)

Ltp2	Promoter from barley lipid transfer protein gene
Mb	Megabase
Ms45	A specific (dominant) male-fertility gene in maize
MS45	A specific protein expressed by the *Ms45* gene in maize
ms45	A mutant allele (recessive) of the *Ms45* gene in maize
MALDI-MS	Matrix assisted laser desorption ionization mass spectrometry
NCBI	National Center for Biotechnology Information
NDF	Neutral detergent fiber
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
PAT	Phosphinothricin acetyltransferase
PVDF	Polyvinylidene fluoride
PCR	Polymerase chain reaction
Pg47	A pollen-specific polygalacturonase gene from maize
pinII	Proteinase inhibitor II from *Solanum tuberosum*
RB	Right Border
RFP	Red fluorescent protein
RFU	Relative fluorescent unit
RNA	Ribonucleic acid
Roguing	Removal of undesirable off-types or atypical plants after a systematic evaluation of seed production fields.
SOP	Standard operating procedure
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sorter accuracy	The accuracy of mechanical seed color sorter to remove any transgenic 32138 SPT-maintainer maize seed from non-transgenic male-sterile progeny maize
SPT	Seed Production Technology
SPT system	A genetic system comprising a transgenic 32138 SPT maintainer used to increase non-transgenic male-sterile female inbred parent seed
SPT process	A seed production process comprising the SPT system combined with color-based seed sorting to insure genetic purity
SPT progeny	Male-sterile female inbred seed derived from using the 32138 SPT maintainer as a pollen source and also the commercial hybrid grain derived thereafter from using the SPT process
TSS	Tissue-specific sterility
USDA	United States Department of Agriculture
UTR	Untranslated region
zm-aa1	A specific α-amylase gene in maize
ZM-AA1	A specific α-amylase protein expressed in 32138 SPT maintainer and comprised of maize endogenous protein sequences from the Brittle-1 transit peptide and the ZM-AA1 protein.
zm-bt-1	A transit peptide gene sequence from the maize *brittle-1* gene

*Abbreviations of units of measurement and of physical and chemical quantities are done according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/)..

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I. Introduction to Seed Production Technology (SPT)

I-A. Advantages of the Pioneer SPT Process

Maize plants bear both male flowers (located on the tassel) and female flowers (located on the ear), and plants can both self-pollinate and cross-pollinate. Hybrid maize seed production requires crossing of two inbred parent lines to produce the F1 hybrid seed sold to growers. In the hybrid seed production field, the female inbred seed parent must be prevented from shedding pollen to avoid self- or sib-pollination that reduces seed quality and compromises yield potential. In order to produce hybrid seed, it is first necessary to increase the seed of the inbred parents (Figure 1). Pioneer has developed a novel process called Seed Production Technology (SPT) that facilitates the increase of large quantities of male-sterile female inbred parent seed. Fertility is completely restored in F1 hybrids plants generated using the male-sterile female inbred parent seed generated using SPT. This technology offers the following advantages over other approaches to maize fertility control:

- The need to remove pollen bearing tassels from female inbred parent lines by hand or mechanical detasseling is eliminated resulting in:
    - higher quality seed products for growers
    - increased female inbred parent seed yields
    - increased production efficiency and reduced costs
    - increased worker safety
- The process works across all germplasm types so it can be used to produce any maize hybrid.
- Increasing male-sterile female inbred lines is a simple process achieved *via* cross-pollination with a transgenic 32138 SPT maintainer.

I-B. Maize Hybrid Seed Production

Hybrid maize (*Zea mays* L.) is grown on more than 80 million acres in the United States (USDA-National Agricutural Statistics Service (NASS), 2008), making it the largest producer of hybrid maize. The seed used to produce hybrid maize is generated by crossing two unique inbred parental lines referred to as the ‘male inbred parent’ (the plant that is the pollinator) and ‘female inbred parent’ (the plant on which the seed is produced).

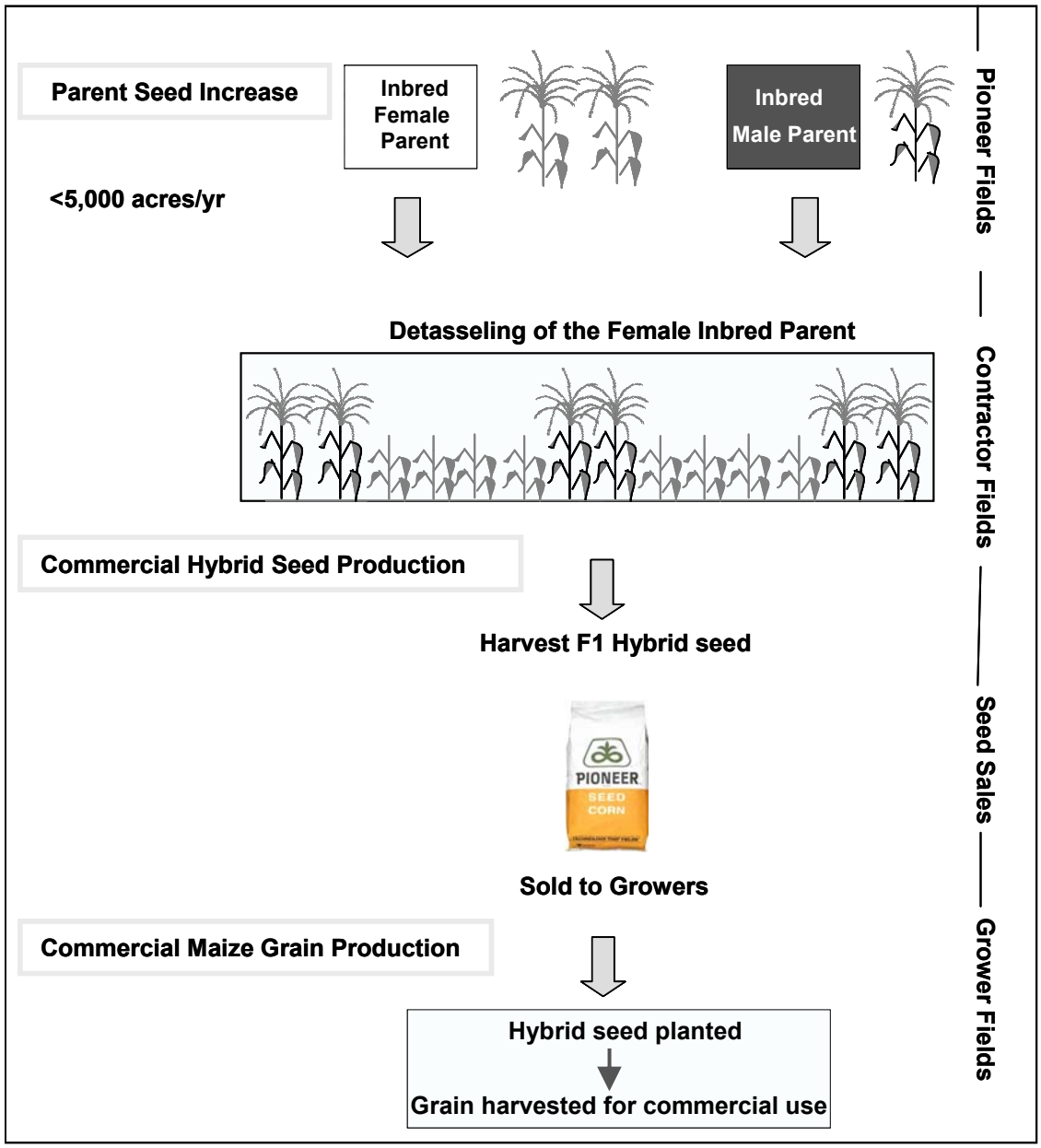
For inbred parent seed increase, male and female inbred parental lines are grown separately and allowed to self-pollinate in Pioneer fields that are reproductively isolated from other maize plants in order to maintain genetic purity and increase the quantity of the inbred parents (Figure 1). In order to produce hybrid seed, male and female inbred parent lines are inter-planted in adjacent rows (*e.g.,* 2 rows male, 4 rows female, 2 rows male) in reproductively isolated contractor fields and allowed to open pollinate.

To produce pure hybrid seed, the male inbred parent must cross pollinate the female inbred parent and the female inbred parent must be prevented from self-pollinating. The most common practice to accomplish this is to cut off the pollen-bearing tassels (*i.e.,* detasseling) on the female inbred parent (Figure 1).

In the hybrid seed production fields, the ears of the detasseled female inbred parent can only be fertilized with pollen from the male inbred parent planted in adjacent rows. The male inbred parent is removed from the fields before the ears reach maturity. Thus, the seed produced on the

female seed parent will be uniformly hybrid. The F1 hybrid seed is harvested, cleaned, conditioned, and bagged for sale to growers. When planted in growers' fields, the hybrid seed will produce a uniform F1 crop. The grain harvested from growers' fields is used for feed and fuel production, processed into food products and industrial materials, or exported.

Figure 1. Stages in the Production of Hybrid Maize Seed



I-C. Maize Fertility Control in Hybrid Maize Production

I-C.1. Detasseling

The physical removal of the pollen-producing tassels (detasseling) has been the most widely used method of preventing female inbred parent self-pollination since maize hybrids were first produced in the 1920s (Wych, 1988). Less common or experimental methods include the application of chemical sterilants, the use of biological systems based on cytoplasmic male-sterility, and systems to control male-fertility using biotechnology (Refer to Appendix 1 for additional information on these systems).

Detasseling can be accomplished manually or mechanically with tassel-cutting or tassel-pulling machines. Thousands of temporary workers are employed to detassel maize hybrid seed production fields. The detasseling process is not entirely reliable, and on occasion a female plant will escape complete detasseling. For example, natural variation in plant development can result in the emergence of tassels after the detasseling process is complete or a mechanical detasseler can fail to remove a tassel completely. Incomplete detasseling results in pollen shed by the female inbred parent plant and self-pollination. When this occurs, seed of the female inbred parent is harvested along with the hybrid seed which results in reduced seed quality, lowered trait purity, and lost yield potential. Mechanical detasseling is faster than hand detasseling, but causes more damage to the plants and results in up to 40% reduction in seed yield (Wych, 1988). Today, a combination of mechanical and hand detasseling is often used; no form of detasseling is entirely satisfactory. Therefore, a need exists for an alternative method of pollen control that is safe, efficient, and highly effective.

I-C.2. Genetic Male-Sterility

Male-sterility in maize refers to the failure to produce functional pollen. Male-sterile maize plants can neither self-pollinate, nor sib-pollinate neighboring plants and can serve as female inbred parents in the production of hybrid maize seed. Male-sterility traits display either nuclear (also known as genetic) or cytoplasmic inheritance (Refer to Appendix 1 for details).

Genetic male or pollen sterility in maize was first described by Eyster (1921) that it is due to a single recessive gene, designated as *ms*. Since then, many different genetic male-sterility loci have been identified in maize. The chromosome number and map position of nearly all presently identified male-sterility genes are well characterized (Neuffer *et al*., 1997). Sterility phenotype is determined by a single gene and the allele for male-sterility is recessive (*ms*), although a small number of dominant male-sterile alleles have been described (Neuffer *et al*., 1997). In maize, a male-sterility plant trait is expressed only by maintenance of a homozygous recessive (*ms/ms*) condition for the fertility gene.

Limitations to the Use of Homozygous Recessive (*ms/ms*) Lines as Male-Sterile Parents

Increase of male-sterile (*ms/ms*) inbred parent seed is not possible *via* self-pollination as the homozygosity of the recessive gene (*ms*) results in male-sterility.

One way to increase the seed of the male-sterile inbred parent is to cross-pollinate male-sterile plants (*ms/ms*) with male-fertile plants which are heterozygous for the male-sterility gene (*Ms/ms*). The progeny from such a cross-breeding are approximately fifty percent male-sterile (*ms/ms*) and fifty percent male-fertile (*Ms/ms)*. Since the male-sterile seed can not be separated from the male-fertile seed, this is not a practical way to increase the male-sterile parent seed.

Recovery and maintenance of pure male-sterile (*ms/ms*) inbred populations using traditional breeding methods requires: a) additional maize lines for the maintenance of the male-sterility trait,

and b) several intermediate crosses and selection methods that are time consuming. Therefore, it is not practical for use in commercial hybrid maize production.

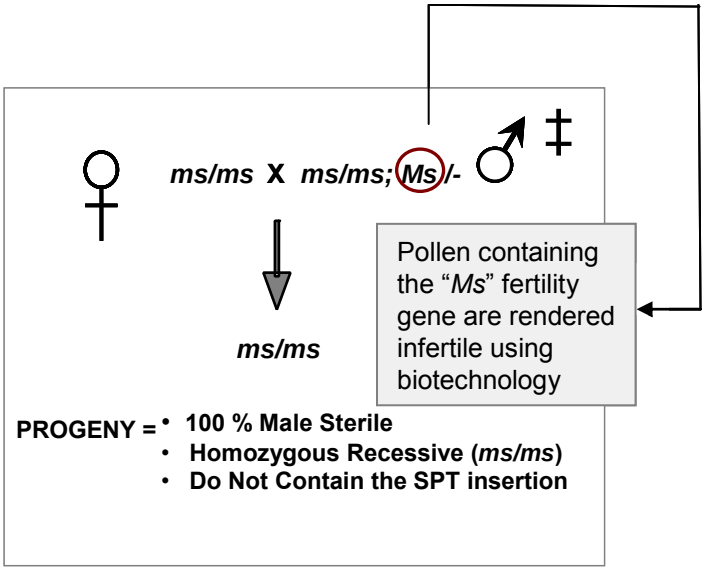
Thus, a need exists for a convenient and effective process for generating male-sterile inbred female parent lines.

I-C.3. Basis for the Development of Seed Production Technology

Section I-C.2 described how cross-pollinating the male-sterile (*ms/ms*) plants with heterozygous (*Ms/ms*) male-fertile plants yields a mix of male-sterile and male-fertile genotypes. However, if all pollen containing the fertility restoration gene (*Ms*) could be eliminated from the heterozygous male parent (*Ms/ms*) thereby ensuring pollination of male-sterile (*ms/ms*) parent only with pollen carrying the recessive *ms* allele, the male-sterile progeny would retain their homozygous (*ms/ms*) condition and could be used as male-sterile female inbred parent in hybrid seed production. This is the basis for the Pioneer Seed Production Technology (SPT) process utilizing a transgenic 32138 SPT maintainer (Figure 2).

Pioneer's novel SPT process generates male-sterile female inbred parent lines that maintain the homozygous recessive state (*ms/ms*) without any intermediate crosses. Male-sterile female inbred parent seed can be multiplied, while maintaining the homozygous recessive state of the male-sterility gene and providing for full restoration of fertility in the F1 hybrid plants grown in commercial fields.

Figure 2. Schematic Diagram of the Basis for SPT



‡ = Represents 32138 SPT maintainer.

The SPT construct containing three gene cassettes [*Ms45, zm-aa1, and DsRed2(Alt1)*] was inserted into a male-sterile maize line that is homozygous recessive for *ms45* (*ms45/ms45*) shown as *ms/ms* in Figure 2. The SPT insertion was integrated in the genome at a locus that segregates independently from the endogenous *ms45* locus (data not shown). Therefore, 32138 SPT maintainer is homozygous for the *ms45* recessive allele and hemizygous for the recombinant *Ms45* gene from the SPT insertion, indicated as *Ms/-* in Figure 2. A single copy of *Ms45* gene from the 32138 SPT insertion restores fertility in the male-sterile (*ms45/ms45*) line (Figure 7B). 32138 SPT maintainer sheds two different types of pollen in a 1:1 ratio: a) non-transgenic fertile pollen (with the *ms45* allele) that do not contain the SPT insertion, and b) transgenic infertile* pollen (with both the *ms45* allele and the transgenic *Ms45* allele) that contain the 32138 SPT insertion (Figure 5). The transgenic pollen carrying *Ms45* are rendered infertile due to the α-amylase gene (Refer to Sections 1-D and 1-H.2 for more details) in the SPT insertion (Figure 9).

Therefore, homozygous recessive (*ms45/ms45*) male-sterile female inbred parent seed can be increased by utilizing the pollen from an isogenic SPT maintainer (Figure 3, Step II and Figure 5).

***=** Pollen infertility refers to the inability of pollen to germinate and fertilize the ovule.

I-D. Description of the SPT Process

Seed Production Technology is a process rather than a trait or a product. The SPT process works across all germplasm, requires no detasseling, and produces hybrid seed that are fully fertile. The male-sterile female inbred parent increase process is entirely controlled by Pioneer and the total acreage in the US planted to the transgenic 32138 SPT maintainer and SPT derived male-sterile female inbred parent seed increase each year will be less than 5,000 acres.

32138 SPT Maintainer

The SPT process uses a transgenic 32138 SPT maintainer in an *ms45/ms45* genetic background (Figure 3, Step I). The 32138 insertion in SPT maintainer contains three gene expression cassettes essential for the functioning of the SPT system: *Ms45*, *zm-aa1*, and *DsRed2(Alt1)* (Figure 6).

The 32138 SPT maintainer is homozygous for the endogenous *ms45* recessive mutant allele and hemizygous for the SPT insertion (*i.e*., *ms45/ms45; SPT/-*) and therefore contains a single copy of the *Ms45* gene. Expression of a single copy of *Ms45* gene in the *ms45/ms45* genetic background restores male-fertility and enables pollen production in the 32138 SPT maintainer.

The 32138 SPT maintainer sheds two different types of pollen in a 1:1 ratio: a) fertile, does not contain SPT insertion, and b) infertile, does contain 32138 SPT insertion (Figure 5). Pollen containing the 32138 SPT insertion is rendered infertile due to the action of ZM-AA1 α-amylase protein. Expression of ZM-AA1 α-amylase in 32138 SPT maintainer pollen results in the depletion of starch (Figure 9) and depriving the SPT pollen of the energy reserves required for successful pollen germination and fertilization. Hence, any pollen containing the 32138 SPT insertion is made infertile.

A fluorescent color marker (*DsRed2(Alt1)*) gene is linked to the other two SPT genes (*Ms45* and *zm-aa1*) and it confers a pinkish red phenotype to any seed expressing the 32138 SPT insertion (Figures 4 and 10). The DsRed2 protein exhibits a high fluorescent intensity and emits a strong red fluorescence under appropriate illumination so seeds containing the SPT insertion can be readily detected and separated from the progeny seeds that do not contain the 32138 SPT insertion in a highly efficient manner using mechanical color sorting (Figure 3, Color Sort A and B; Refer to Appendix 3).

Step I: 32138 SPT Maintainer Seed Increase

32138 SPT maintainer seed is replicated *via* self-pollination (Figure 3 Step I and Figure 4). The SPT insertion is transmitted maternally. Upon self-pollination, 32138 SPT maintainer produces two different types of seed (yellow: pinkish red) in a 1:1 ratio (Figures 4 and 10). Yellow seed is non-transgenic for SPT (does not contain the 32138 SPT insertion). Pinkish red seed that fluoresces a bright red color under appropriate illumination is transgenic for SPT (contains the 32138 SPT insertion).

Color Sort A

Self-pollinated 32138 SPT maintainer seed containing a mixture of yellow and pinkish red (SPT maintainer) seed in a 1:1 ratio is passed thru mechanical color sorters twice (Refer to Appendix 3 for details) to separate the 32138 SPT maintainer seed from the yellow seed (Figure 3, Color Sort A and Figure 4). Color sorters detect the fluorescent red 32138 SPT maintainer seed and separate it from the yellow/non-transgenic for SPT seed. Pure seed of 32138 SPT maintainer is then available for the propagation of non-transgenic male-sterile female inbred parent seed (Figure 3, Step I). Therefore, color sort A is for the purification of 32138 SPT maintainer seed. Yellow seed collected from color sort A will be discarded (Refer to Appendix 3 for more details).

Step II: Male-Sterile Female Inbred Parent Seed Increase

For seed increase of male-sterile (*ms45/ms45*) female inbred parent, 32138 SPT maintainer will be planted in either a 2:2 or 4:4 or 4:2 planting pattern (male-sterile female: 32138 SPT maintainer rows). Fertile pollen (that does not contain the 32138 SPT insertion and carry the *ms45* recessive allele) from the 32138 SPT maintainer plants will cross-pollinate and fertilize nearly isogenic male-sterile (*ms45/ms45*) female inbred parent plants (Figure 3, Step II and Figure 5). The inbred seed produced on the male-sterile female inbred plants will maintain their homozygous recessive (*ms45/ms45*) state and genetic purity. All male-sterile progeny seed harvested will be yellow in color and will not contain the 32138 SPT insertion (Figure 5 and Refer to Appendix 4 for more details).

Color Sort B

The SPT system is highly genetically efficient (Refer to Appendix 4 and 5) and virtually all of the fertile pollen from 32138 SPT maintainer should be non-transgenic for SPT and therefore all of the male-sterile inbred progeny seed made using the pollen from 32138 SPT maintainer should also be non-transgenic for SPT. The purpose of the color sort B is to ensure the purity of the male-sterile female inbred parent by removing any seeds of 32138 SPT maintainer. Each batch of male-sterile female inbred parent seed is passed twice through the color sorter (Figure 3, Color Sort B and Figure 5). This step helps to assure that no seed of the 32138 SPT maintainer will be planted in the F1 hybrid seed fields that produce seed sold to growers (Figure 3, Step III).

Steps III. Commercial Maize Hybrid Seed Production

Commercial maize hybrid seed production (Figure 3, Steps III) using the male-sterile progeny seed derived using SPT technology will take place normally. The purity of the male-sterile female inbred progeny derived from the SPT process is insured by passing the seed twice through the mechanical color sorters (Refer to Appendix 3 for details on the color sorter). Therefore, the F1 hybrid seed harvested and sold to growers for commercial grain production is non-transgenic for SPT and the resulting F1 hybrid plants are fully fertile.

Steps IV. Commercial Maize Grain Production

Commercial maize grain production using the F1seed generated using the male-sterile female inbred parent will take place normally. The F1 hybrid plants grown in the growers' fields are non-transgenic for SPT and therefore, the grain harvested from growers' fields is also non-transgenic for SPT (Figure 3, Step IV).

Figure 3. Schematic of Hybrid Maize Seed Production Using the SPT Process

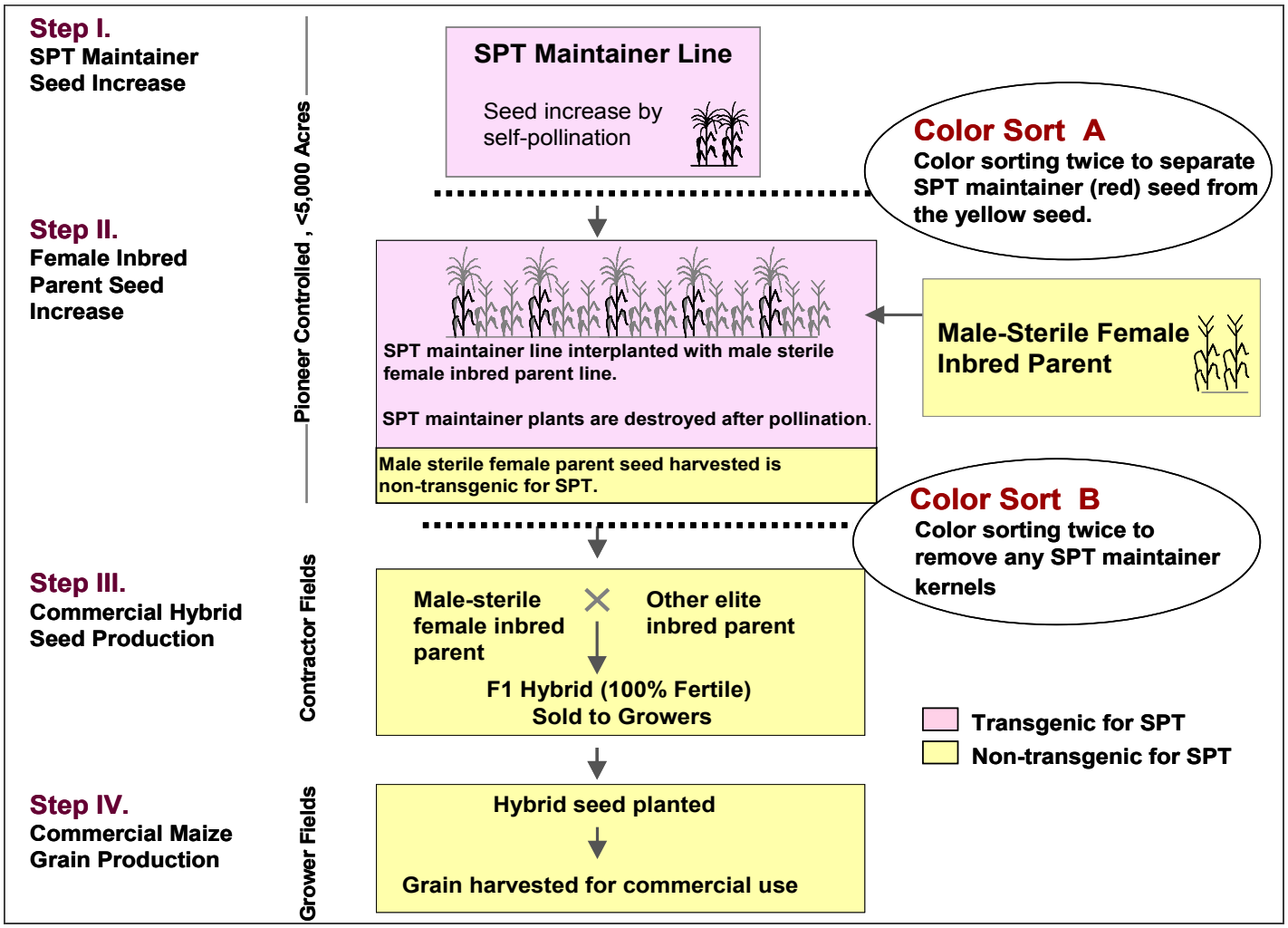


Figure 4. Propagation of 32138 SPT Maintainer (Figure 3, Step I)

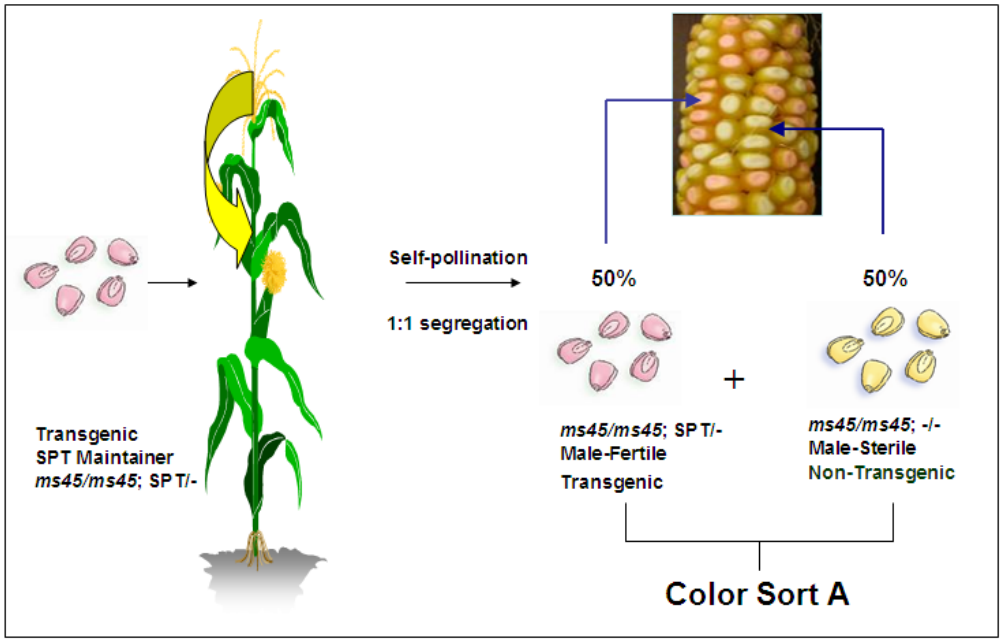
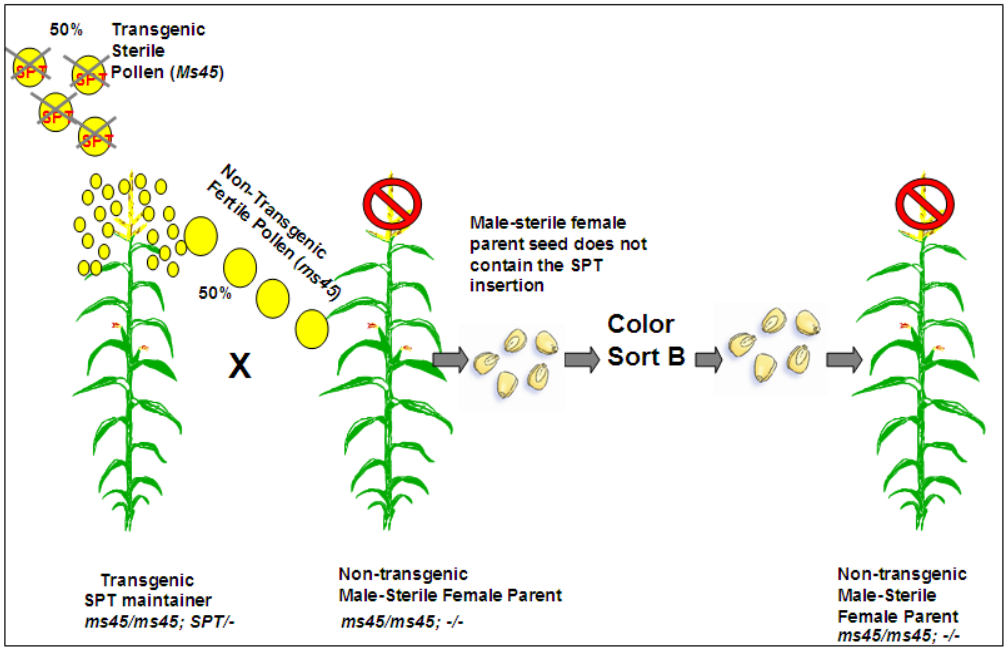


Figure 5. Propagation of Non-Transgenic Male-Sterile Female Inbred Parent Lines (Figure 3, Step II)



I-E. 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. 7701-7772) and the Plant Quarantine Act (7 U.S.C. 151-167), to prevent the introduction or dissemination of plant pests into or within the U.S. Part 340 regulates introduction of organisms altered or produced through genetic engineering which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR §340.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

In order to facilitate the use of the SPT process in hybrid seed production, Pioneer is submitting data on transgenic 32138 SPT maintainer and is requesting APHIS determine that 32138 SPT maintainer and its crosses with non-regulated maize lines no longer be considered regulated articles under 7CFR 340.

Furthermore, in order to address questions regarding the movement of progeny seed and grain in international commerce, Pioneer is requesting that USDA-APHIS make an additional finding that the SPT process, as described and operated by Pioneer, consistently and reliably produces progeny that lack the 32138 SPT insertion and are therefore non-transgenic for SPT.

I-F. Submissions to Other Regulatory Agencies

Small amounts of the transgenic 32138 SPT maintainer may enter into feed channels via signed contractual agreements. Therefore, a New Protein Consultation (NPC) for the DsRed2 protein color marker was submitted to FDA on October 11, 2006. An NPC for the ZM-AA1 protein was submitted to FDA on June 18, 2009.

The 32138 SPT maintainer is not a commercial product. The SPT process generates progeny seed that do not contain the 32138 SPT insertion and hence are non-transgenic for SPT genes. Export approvals are not required for grain harvested from hybrid seed that was generated using the SPT process.

I-G. Generation of the 32138 SPT Maintainer

A non-transgenic male-sterile maize line homozygous recessive (*ms45/ms45*) for *Ms45* locus was transformed with the SPT insertion (Figure 6) to produce the 32138 SPT maintainer. The SPT insertion contains gene cassettes for *Ms45, zm-aa1, and DsRed2(Alt1*) (Table 1). The 32138 SPT insertion was integrated in the genome at a locus that segregates independently from the endogenous *ms45* locus (data not shown). Therefore, the 32138 SPT maintainer is homozygous for the *ms45* recessive allele and hemizygous for the *Ms45* gene from the 32138 SPT insertion.

Figure 6. Schematic Diagram of the 32138 SPT Insertion

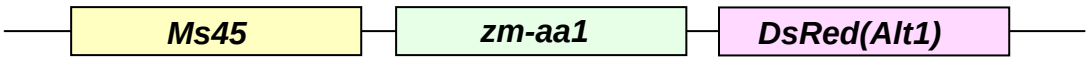


Table 1. Components of the 32138 SPT Insertion

Gene	*Ms45*	*zm-aa1*	*DsRed(Alt1)*
Source	*Zea mays* L.	*Zea mays* L.	*Discosoma sp.*
Promoter	Anther preferred *5126*	Pollen preferred *Pg47*	Endosperm preferred *Ltp2*
Gene Produc	MS45 protein	α-Amylase	DsRed2 fluorescent protein
Function	Restores fertility in *ms45/ms45* background	Makes pollen infertile by depletion of starch	Serves as a screenable marker for identification and separation of transgenic SPT seeds from non-SPT seeds

I-H. Brief Description of Genes

I-H.1. Maize Male-Fertility Gene *Ms45*

The *Ms45* gene is one of the many nuclear fertility genes that have been identified and isolated in maize using activator transposon tagging (Albertsen *et al*., 1993). Mutations in the *Ms45* gene have been shown to cause male-sterility (Albertsen *et al*., 1993). Expression of the *Ms45* gene is localized to anthers and the MS45 protein is produced in the tapetum. Mutations in *Ms45* are typically recessive and homozygous mutants are male-sterile (the mutant *Ms45* allele is designated *ms45)*. A plant that is homozygous for *ms45* (*i.e.*, *ms45/ms45*) is male-sterile and produces no pollen (Figure 7A). In both inbred parent increase and hybrid seed production, it is necessary to maintain the homozygous recessive state of the female inbred parent in order to maintain male-sterility. However, due to the recessive nature of the mutation, any normal maize line carrying the *Ms45* allele, when used as a male inbred parent for hybrid seed production, will restore fertility to the hybrid (Figure 7B). Similarly, male-fertility can be restored by a single copy of the *Ms45* gene transformed into a homozygous recessive plant (*ms45/ms45*). Hence, transformations of the *ms45/ms45* male-sterile plants with the SPT insertion containing a single copy of the *Ms45* gene will restore fertility (Figure 7B).

Anther Morphology

In maize, the anther wall is composed of four cell layers, the epidermis, the endothecium, the middle layer and the innermost layer, the tapetum (Figure 8A). The cell layers of the anther surround the locule that contains the sporogenous cells which undergo meiosis to form a tetrad or quartet of microspores encased in a callose sheath. During anther development, a callose sheath is dissolved by the action of callase. As a result the microspores are released as free floating cells into the locule and formation of the outer cell wall, called the exine takes place. Later, the inner microspore cell wall or intine is produced. The microspores subsequently go through two mitotic divisions, fill with starch, and ultimately become mature pollen (Figure 8B). One of the proposed functions of the tapetum is that it is involved in furnishing precursors during the development of the exine (Bedinger, 1992). Homozygous recessive mutations of the *Ms45* gene result in male-sterility due to a lack of, or incomplete exine formation on the microspores. Consequently, the microspores of *ms45* mutants break down shortly after the early to mid-uninucleate stages of development and pollen is not produced.

Anther-Specific Expression of *Ms45*

The expression of *Ms45* gene has been characterized and is confined to anthers in the developing maize tassels (Cigan *et al*., 2001; Unger *et al*., 2002). Specifically, the MS45 protein is localized to the tapetal cell layers of the anthers and is first detected during the tetrad stage, attains its highest level in the early uninucleate stage and quickly drops off shortly after the start of the mid-uninucleate stage of microsporogenesis (Cigan *et al*., 2001; Unger *et al*., 2002; Figure 8B).

Figure 7. Maize Tassels Showing the Male-Sterile Mutant Phenotype (*ms45/ms45*) and Male-Fertile Restored Phenotype (*Ms45/ms45*)

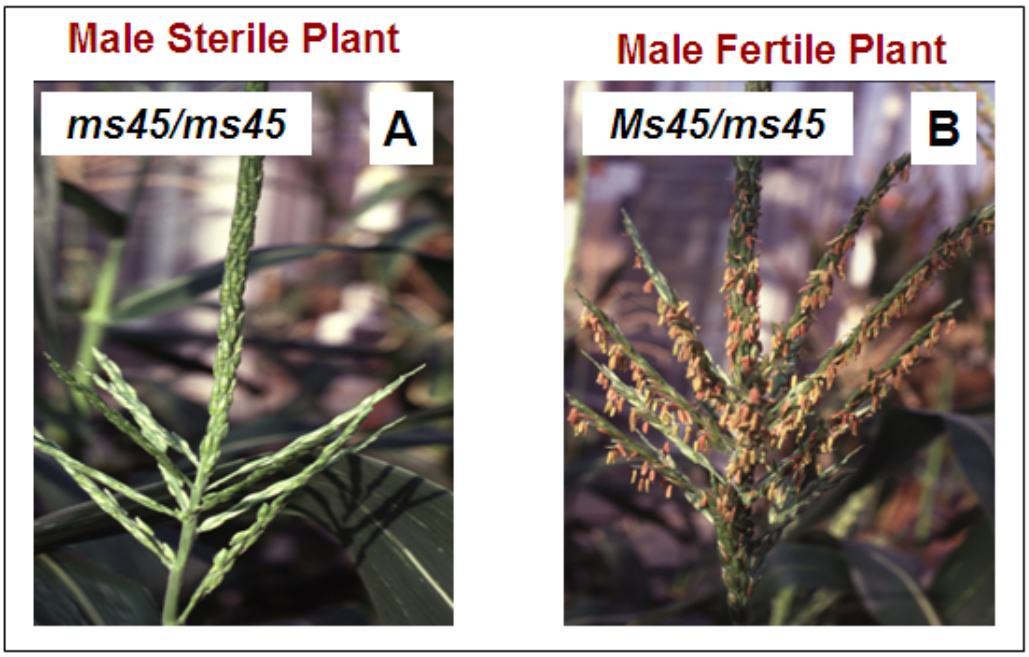
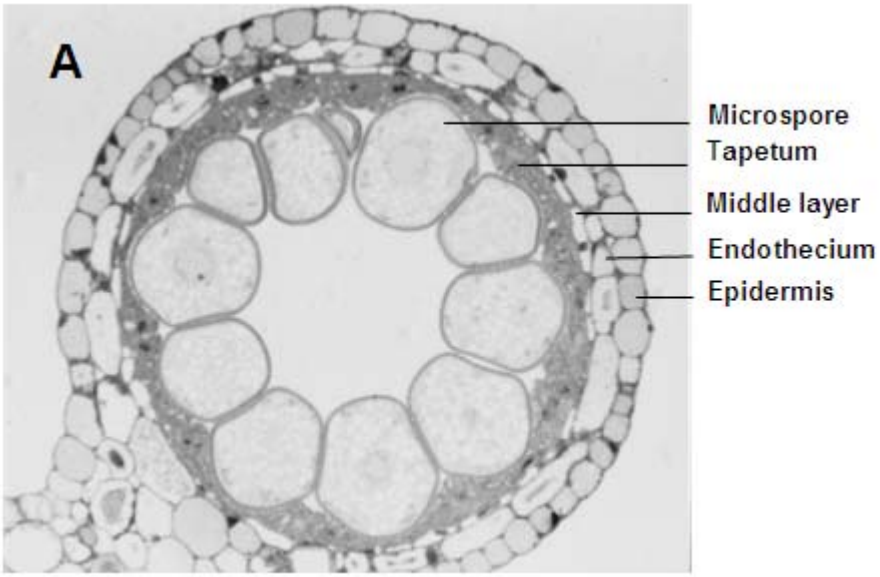
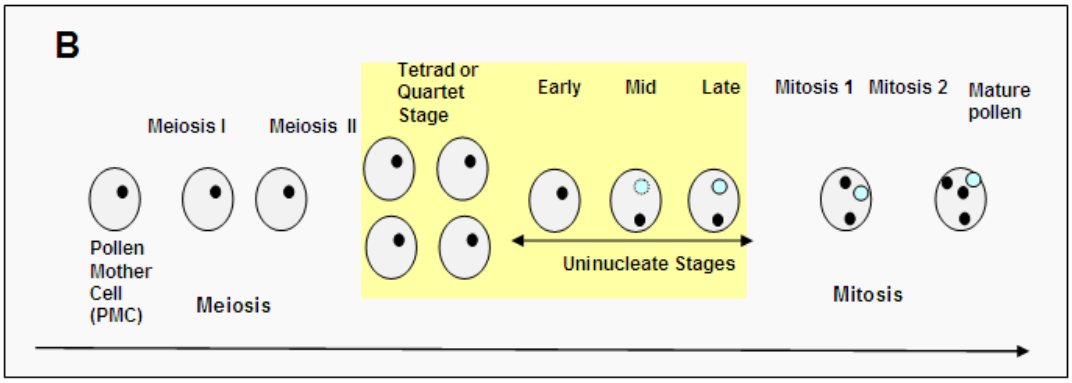


Figure 8. Cross-Section of Maize Anther (A) and Stages in the Pollen Development (B)





Yellow box in Figure 8B identifies the stages for MS45 protein expression (Cigan *et al*., 2001).

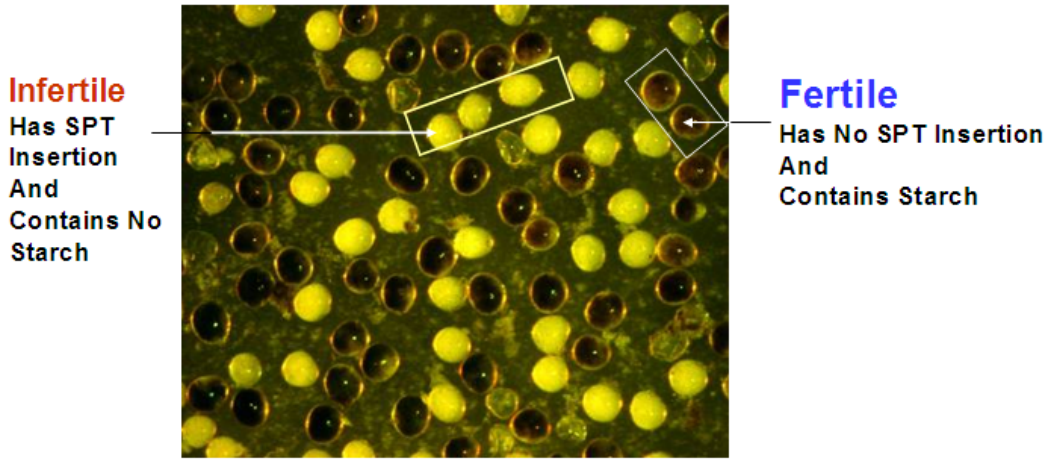
I-H.2. Maize α-Amylase Gene *zm-aa1*

The maize α-amylase *zm-aa1* gene in 32138 SPT maintainer was isolated from a cDNA library of the developing maize embryo and endosperm at ten days after pollination. The endogenous *zm-aa1* gene is predominantly expressed in the scutellum tissues of the geminating seed;; minimal expression of *zm-aa1* (<0.25% of the levels in the scutellum) is observed in endosperm, stalk, leaf and root. Endogenous *zm-aa1* expression was not observed in pollen (internal data not shown.

Alpha-amylases belong to a family of glycosyl-hydrolases catalyzing hydrolysis of (1-4)-α-D-glucosidic linkages in polysaccharide molecules, such as starch. By linking the *zm-aa1* gene to a maize pollen-preferred promoter, *Pg47*, and a transit peptide sequence from the maize *brittle-1* gene that targets the protein to the amyloplast, the ZM-AA1 protein in 32138 SPT maintainer is expressed in the developing pollen grain resulting in the depletion of starch and depriving the pollen of the energy reserves required for successful pollen germination and fertilization. Hence, any pollen containing the SPT insertion will be infertile.

The transgenic 32138 SPT maintainer is homozygous for the *ms45* recessive mutation and hemizygous for the SPT insertion (*i.e.*, *ms45/ms45:SPT/-*). Therefore, 50% of the pollen produced by the 32138 SPT maintainer is transgenic but infertile since it contains the 32138 SPT insertion (*i.e.,* positive for α-amylase) and 50% is non-transgenic and fertile, since it lacks the 32138 SPT insertion (*i.e.,* negative for α-amylase). Figure 9 shows pollen from the 32138 SPT maintainer stained with potassium iodide. There is equal distribution of pollen that contains starch (dark purple) and pollen that lacks starch (unstained).

Figure 9. Potassium Iodide Starch Staining of Pollen Grains from 32138 SPT Maintainer

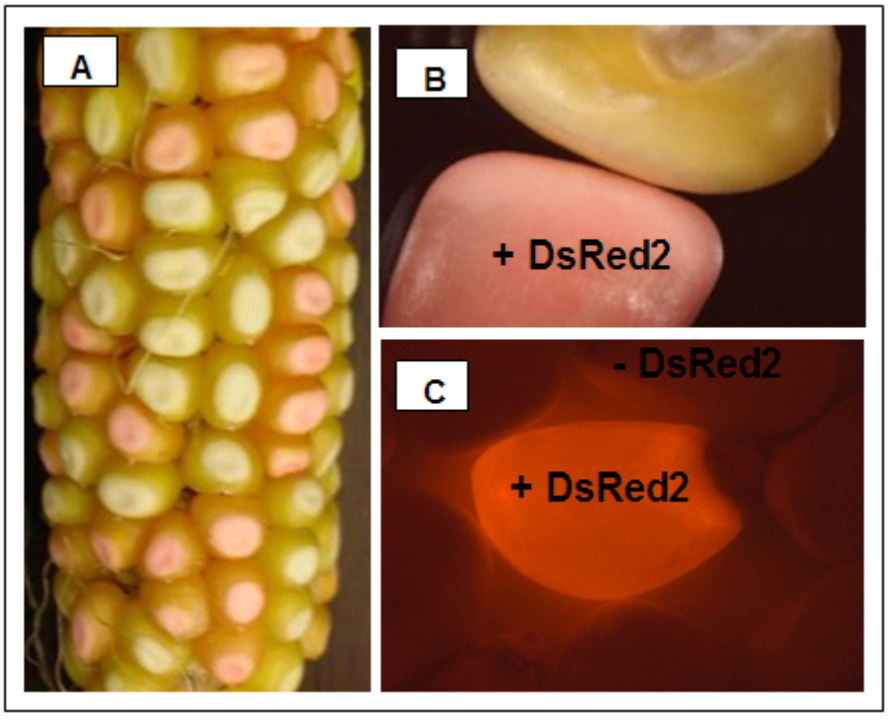


I-H.3 *Discosoma* sp. (coral) Color Marker Gene *DsRed2(Alt1)*

The *DsRed2(Alt1)* gene is the only non-maize gene sequence utilized in the generation of the 32138 SPT maintainer. DsRed2 is a variant of Clontech's (www.clontech.com; Wasson-Blader, 2001) original "living color" red fluorescent protein (DsRed), isolated from a marine coral, *Discosoma sp*. The *Alt1* suffix denotes that this version includes a single base pair substitution, made during cloning, that does not change the protein sequence. The DsRed2 protein belongs to a family of red fluorescent proteins that are used as vital color markers for a variety of applications in biological systems.

The DsRed2 protein expressed from the *DsRed2(Alt1)* gene under the control of the barley *Ltp2* promoter turns seeds containing the SPT insert a pink color that is visible to the naked eye (Figure 10A & B). The DsRed2 protein exhibits a high fluorescent intensity and emits a strong red fluorescence at a wavelength of 583 nm. When seeds expressing the DsRed2 protein are appropriately illuminated (Figure 10C), DsRed2 is efficiently excited and fluoresces a bright red color. Therefore, seeds containing the 32138 SPT insertion can be readily detected with high sensitivity using appropriate illumination and optical filter sets, and separated from non-SPT seed in a highly efficient manner using mechanical color sorting (see Appendix 3 for more details).

Figure 10. Maize Seed Expressing DsRed2 (A & B) and Showing Red Fluorescence (C)



II. The Biology of Maize

Refer to the OECD Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize), 2003, for information pertaining to the following aspects of maize biology:

- general description, including information on use of maize as a crop plant;
- taxonomic status of *Zea;*
- identification methods;
- center of origin / diversity and maize diversity;
- reproductive biology, including sexual and asexual reproduction;
- crosses, including intra- and inter-specific crosses and gene flow; and
- agro-ecology, including information about cultivation, volunteers and weediness, soil ecology, and maize-insect interactions.

III. Method of Development of 32138 SPT Maintainer

III-A. Characterization and Generation of a Male-Sterile (*ms45/ms45*) Recipient Maize

The maize recipient line utilized for transformation and generation of 32138 SPT maintainer is designated as Hi-II(*ms45*). The Hi-II line was converted to male-sterile Hi-II(*ms45*) by backcrossing the *ms45* allele into Hi-II (Cigan *et al*., 2001).

Hi-II maize has been used for maize transformation for a number of years because of its high transformability and good culturability. Hi-II is a derivative of the A188 and B73 maize parent lines which are publicly available from the University of Minnesota and Iowa State University, respectively. Hi-II is approximately 50:50 of A188 and B73 parental lines (Armstrong, *et al*., 1991). The material was developed to have a higher regeneration potential.

In order to generate a male-sterile (*ms45/ms45*) recipient Hi-II maize line, a male-sterile female that was homozygous for an *ms45* mutant Ac excision allele, ms45'-9301 (*ms45*), was pollinated using pollen bulked from Hi-II maize plants (Armstrong, 1994), resulting in the introgression (over four generations) of the *ms45* allele into Hi-II maize germplasm. The resultant source material for transformation consisted of embryos segregating for *ms45*. This allowed for transformation directly into a homozygous *ms45* background (*ms45/ms45*) and provided the opportunity to directly test gene complementation in the T0 generation (fertility of the regenerated plants).

III-B. Description of the Transformation System

Plasmid PHP24597 (containing the *Ms45*, *zm-aa1,* and *DsRed2(Alt1)* gene expression cassettes) was used to generate 32138 SPT maintainer through *Agrobacterium* mediated transformation. Refer to Section IV for the detailed description of the plasmid.

Immature embryos of maize Hi-II(*ms45*), were aseptically removed from the developing caryopsis 9 to 10 days after pollination and infected with *Agrobacterium tumefaciens* strain LBA4404 containing plasmid PHP24597, essentially as described in Zhao *et al*., 2001. After 6 days of embryo and *Agrobacterium* co-cultivation on solid culture medium with no selection, the embryos were transferred to maintenance (no selection) medium that was stimulatory to maize somatic embryogenesis. The maintenance medium contained carbenicillin to kill any remaining *Agrobacterium*.

After approximately two weeks on the maintenance medium, healthy, growing calli that demonstrated red fluorescence were identified, indicating presence of the T-DNA from plasmid PHP24597. The putative transgenic calli were continually transferred to fresh maintenance medium for further growth until the start of the regeneration process.

The embryonic calli were regenerated into whole transgenic plants and transferred to the greenhouse. The regenerated plants (designated T0 plants) were screened for *Ms45* gene complementation and *ms45/ms45* transgenic samples were used for molecular analysis for transgene copy number and the absence of *Agrobacterium* backbone DNA by PCR. Positive plants were further advanced to generate 32138 SPT maintainer. Refer to Figure 11 for a schematic diagram of the development process for 32138 SPT maintainer and Figure 12 for a breeding diagram.

III-C. Selection of Comparators for 32138 SPT Maintainer

To ensure accurate assessment of the impact of transgene insertion on various characteristics of 32138 SPT maintainer, a proper selection of the comparator plants is important. Depending on the study, two types of maize lines were used as comparators: inbred control maize and reference inbred maize (Figure 12).

The inbred control maize plants have a genetic background similar to that of 32138 SPT maintainer but lack the transgenic insert. For most analyses, the near-isoline inbred 705 was used as the control. Hi-II(*ms45*) and Hi-II lines were used as additional negative controls for Southern blot analyses. For the Western blot analysis, male-sterile progeny derived from 32138 SPT maintainer were used as a negative control.

Non-transgenic maize reference inbreds were used to help determine the normal variation seen in inbred maize and to develop the statistical tolerance intervals for the nutrient composition and agronomic assessments. For Western blot analysis, near-isoline inbred 705 was used as a reference.

Figure 11. Schematic of the Development of 32138 SPT Maintainer

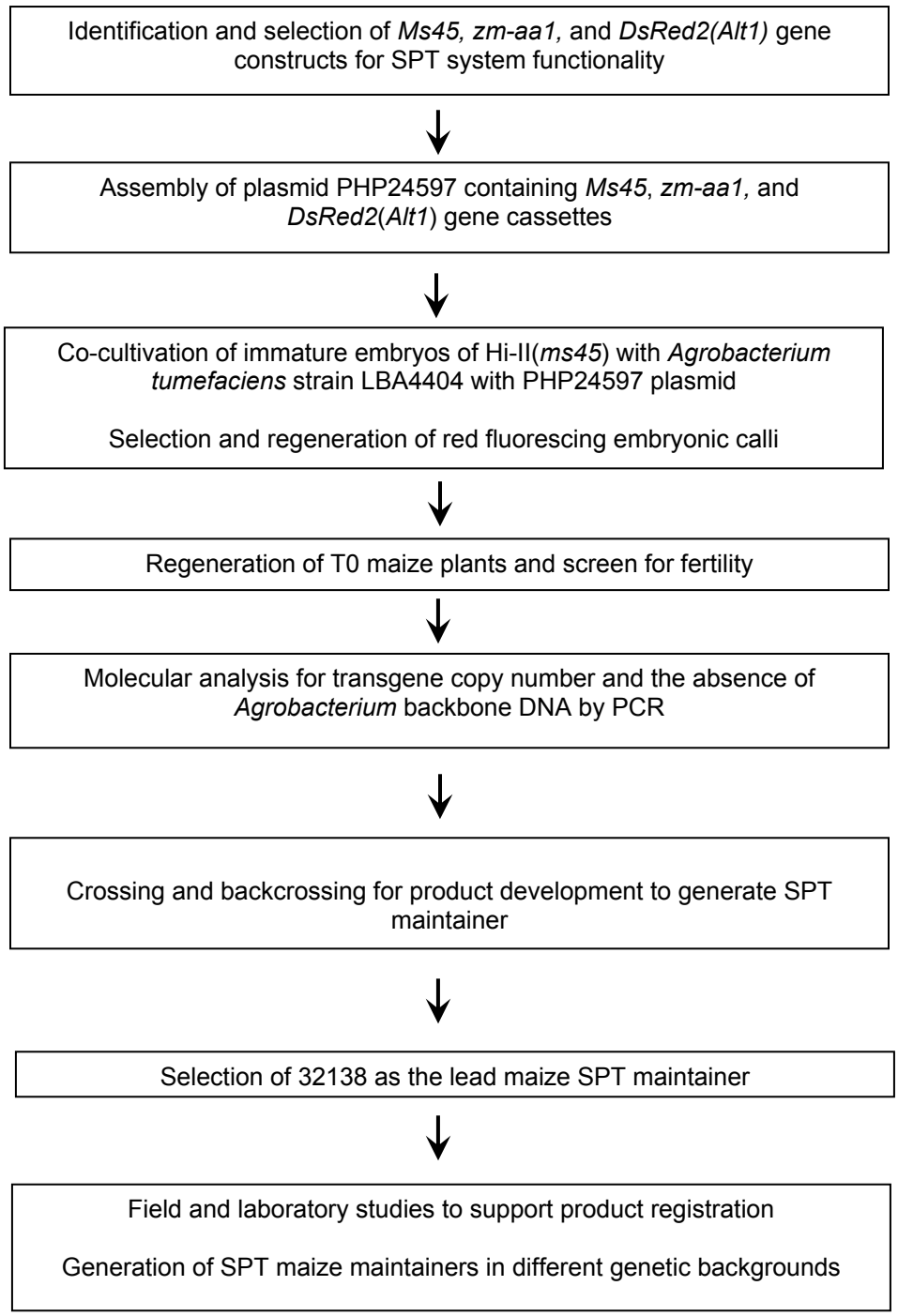
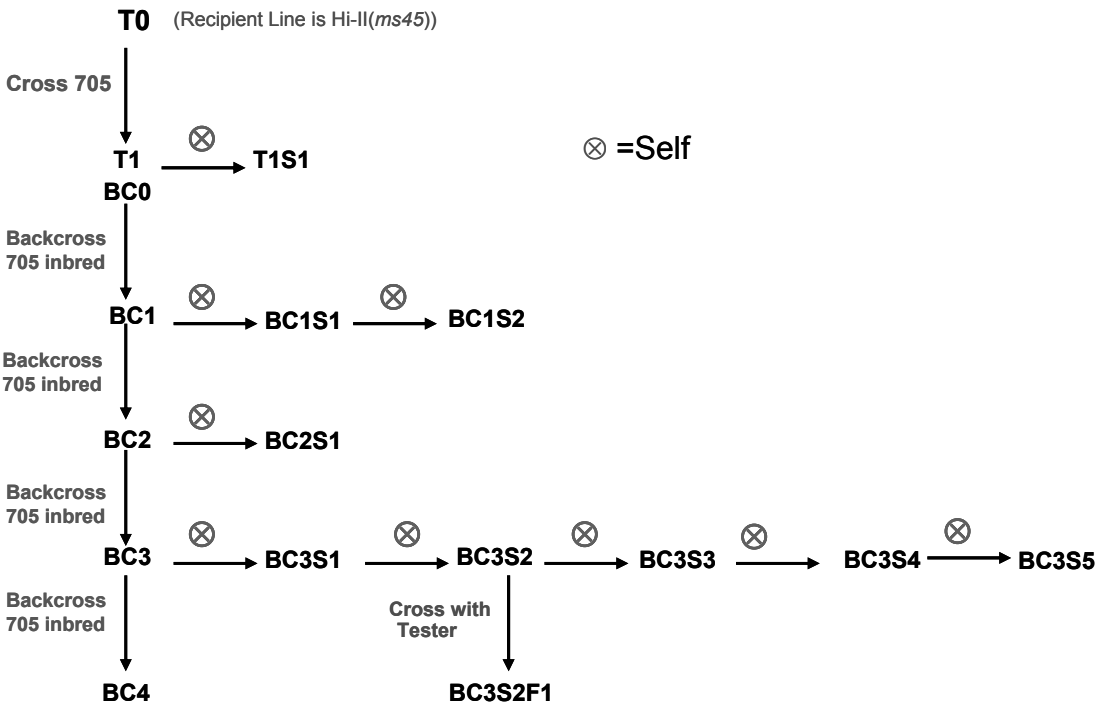


Figure 12. Breeding Diagram for 32138 SPT Maintainer, and Generations Used for Analyses



Analysis	Relevant Section of Petition	32138 SPT Maintainer Generation Used	Control(s) Used
Molecular characterization	V-A, B, C and D	T1S1, BC4, BC3,	Inbred 705, Hi-II, Hi-II(*ms45*)
Mendelian inheritance of the traits	V-E	BC3S2F1, BC3S4, and BC4	N/A
Detection of MS45 by Western blotting	VI-D	BC1S2	Inbred 705 and non-transgenic male-sterile (*ms45/ms45*) progeny inbred derived from transgenic 32138 SPT maintainer
ZM-AA1 and DsRed2 protein concentration	VI-E	BC3S2	Inbred 705
Seed germination / dormancy	VII-A	BC3S5	Inbred 705
Field agronomic characteristics	VII-B	BC3S2	Inbred 705
Nutrient composition assessment	VIII	BC3S2	Inbred 705

IV. Donor Genes and Regulatory Sequences

IV-A. Plasmid PHP24597 Used in Transformation

Maize (*Zea mays* L.) event DP-32138-1 was produced by *Agrobacterium*-mediated transformation with plasmid PHP24597 (Figure 14). The T-DNA region of this plasmid is represented schematically in Figure 13. A summary of the genetic elements and their positions on the T-DNA are described in Table 2.

Figure 13. Schematic Diagram of the T-DNA Region from Plasmid PHP24597

Schematic diagram of the T-DNA indicating the *Ms45* gene region, *zm-aa1* gene, and the *DsRed2(Alt1)* gene along with their respective regulatory elements. The size of the T-DNA is 9950 bp.

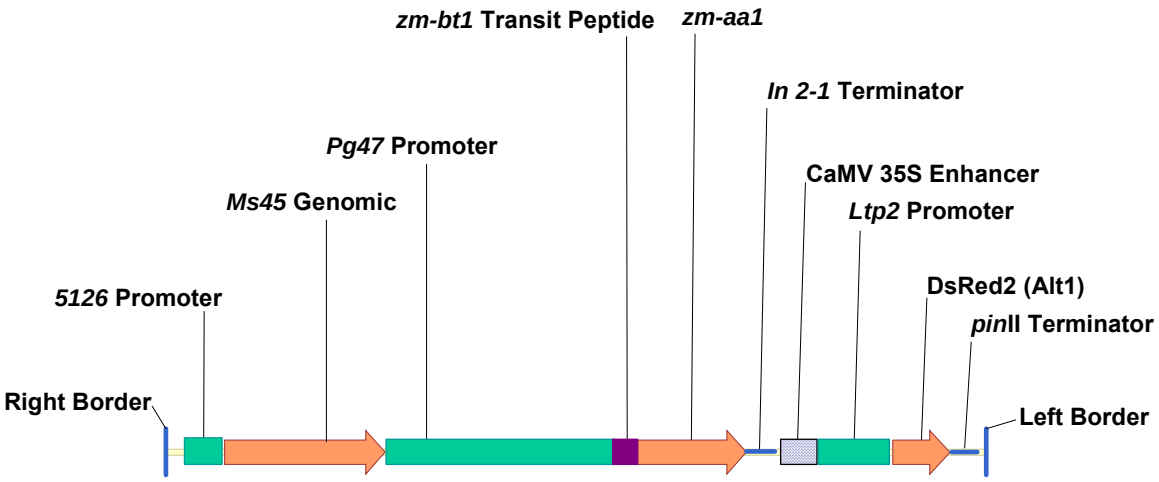


Figure 14. Schematic Diagram of Plasmid PHP24597

Schematic diagram of plasmid PHP24597 with genetic elements indicated.
Plasmid size is 52869 bp.

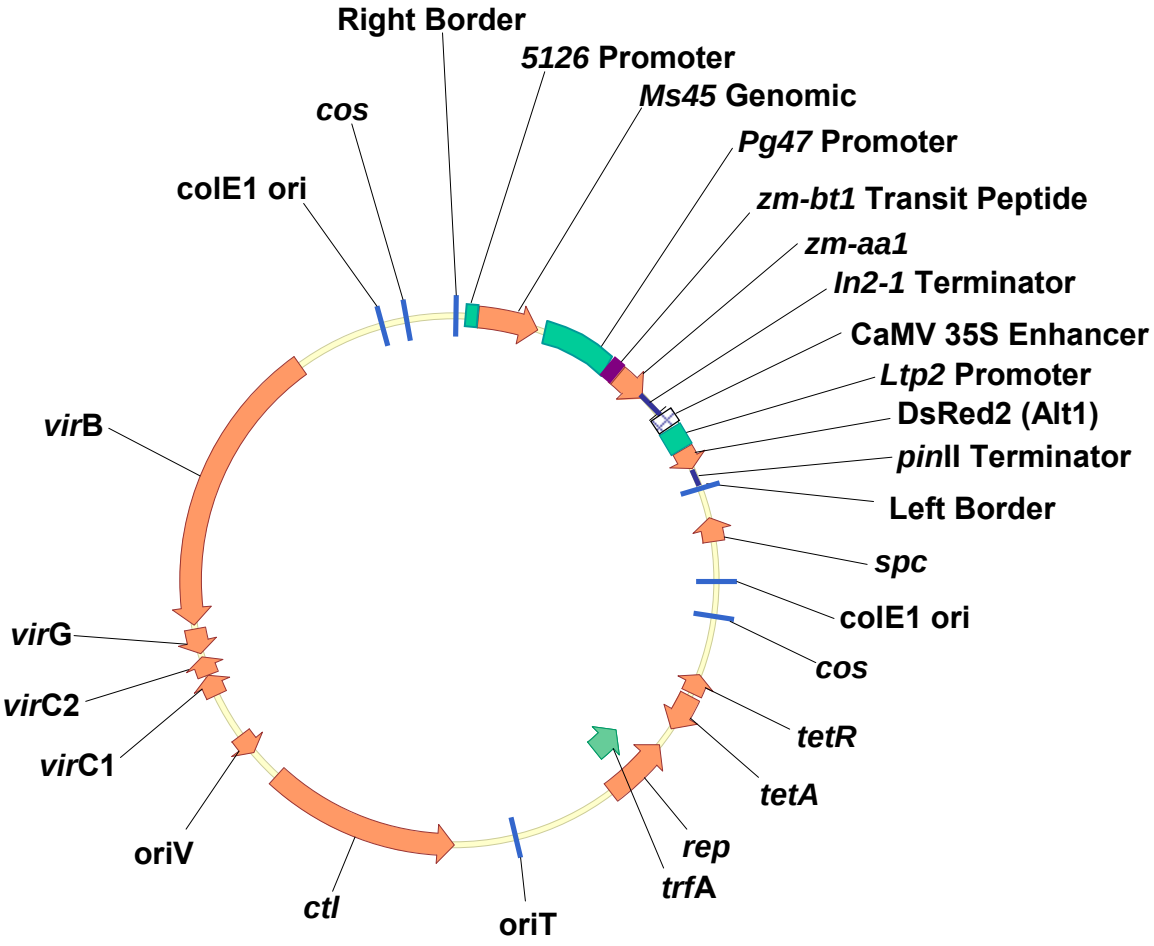


Table 2. Description of Genetic Elements in the T-DNA Region of Plasmid PHP24597

Location on T-DNA (base pair position)	Genetic Element	Size (base pairs)	Description
1 to 25	Right Border	25	T-DNA Right Border region from Ti plasmid of *Agrobacterium tumefaciens*
26 to 177	Ti Plasmid Region	152	Non-functional sequence from Ti plasmid of *A. tumefaciens*
178 to 203	Polylinker Region	26	Region required for cloning genetic elements
204 to 706	*5126* Promoter	503	Maize anther-preferred *5126* promoter (Cigan and Albertsen, 1997)
707 to 2658	*Ms45* Genomic Region	1952	Maize genomic DNA including the *Ms45* coding sequence and associated 3' untranslated region as indicated below: Exon 1 bp 708 to 1086 Intron 1 bp 1087 to 1215 Exon 2 bp 1216 to 1499 Intron 2 bp 1500 to 1596 Exon 3 bp 1597 to 1764 Intron 3 bp 1765 to 1850 Exon 4 bp 1851 to 2258 3' UTR bp 2384 to 2658 (Albertsen *et al.*, 1993; Albertsen *et al.*, 1995)
2659 to 2730	Polylinker Region	72	Region required for cloning genetic elements
2731 to 5466	*Pg47* Promoter	2736	Promoter from the maize, pollen polygalacturonase (*Pg47*) gene (Allen and Lonsdale, 1993)
5467 to 5468	Polylinker Region	2	Region required for cloning genetic elements
5469 to 5693	*zm-bt1* Transit Peptide	225	Amyloplast-targeting transit peptide from the maize *brittle-1* gene (Sullivan *et al*., 1991)
5694 to 6956	*zm-aa1* Gene	1263	Maize α-amylase gene
6957 to 7033	Polylinker Region	77	Region required for cloning genetic elements
7034 to 7377	*In2-1* Terminator	344	Terminator sequence from the maize *In2-1* gene (Hershey and Stoner, 1991)
7378 to 7411	Polylinker Region	34	Region required for cloning genetic elements
7412 to 7849	CaMV 35S Enhancer	438	Enhancer region from the Cauliflower Mosaic Virus genome (Franck *et al.*, 1980; Odell *et al.*, 1985; Odell *et al.,* 1988).

Table 2. Description of Genetic Elements in the T-DNA Region of Plasmid PHP24597 (continued)

Location on T-DNA (base pair position)	Genetic Element	Size (base pairs)	Description
7850 to 7905	Polylinker Region	56	Region required for cloning genetic elements
7906 to 8761	*Ltp2* Promoter	856	Promoter from barley lipid transfer protein (*Ltp2*) gene (Kalla *et al.*, 1994)
8762 to 8809	Polylinker Region	48	Region required for cloning genetic elements
8810 to 9487	*DsRed2(Alt1)* Gene	678	Modified DsRed2 gene (Wasson-Blader T, 2001) with internal *Bst*E II restriction site removed
9488 to 9528	Polylinker Region	41	Region required for cloning genetic elements
9529 to 9839	*pin*II Terminator	311	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (Keil *et al.,* 1986; An *et al.*, 1989).
9840 to 9872	Polylinker Region	33	Region required for cloning genetic elements
9873 to 9925	Ti Plasmid Region	53	Non-functional sequence from Ti plasmid of *A. tumefaciens*
9926 to 9950	Left Border	25	T-DNA Left Border region from Ti plasmid of *Agrobacterium tumefaciens*

IV-B. Identity and Source of Genetic Material in the T-DNA of PHP24597

The T-DNA of plasmid PHP24597 (Figure 13) contains three gene cassettes.

The *Ms45* Cassette

The first cassette contains the *Ms45* Genomic sequence, which is a region of maize genomic DNA comprising the *Ms45* gene and associated 3' untranslated region (Albertsen *et al.*, 1993; Albertsen *et al.*, 1995). The *Ms45* gene includes four exons with three introns that are removed by splicing. Expression of the MS45 protein encoded by the *Ms45* gene in the anther tapetum is required for the production of fertile male pollen by the maize plant (Cigan *et al*., 2001).

The expression of the *Ms45* gene is controlled by the maize anther-specific *5126* promoter (Cigan and Albertsen, 1997). The terminator for the *Ms45* gene is the endogenous *Ms45* 3' untranslated region sequence from the maize genome, which is included in the *Ms45* genomic sequence (Albertsen *et al.*, 1993). The full-length MS45 protein is comprised of 412 amino acids and has a molecular weight of approximately 47 kDa (Figure 41).

The *zm-aa1* Cassette

The second cassette contains a truncated maize α-amylase gene (*zm-aa1*) that encodes the ZM-AA1 protein. The *zm-aa1* gene sequence was truncated to remove the native N-terminal transit peptide sequence and was replaced by sequence from the *zm-bt1* gene *(brittle-1)* encoding the Brittle-1 transit peptide for targeting the ZM-AA1 protein to amyloplasts (Sullivan *et al*., 1991). The ZM-AA1 protein prevents accumulation of starch in the nascent pollen grain, thus preventing the pollen from developing and germinating normally. The complete translation product, including transit peptide, comprises 495 amino acids and has a molecular weight of approximately 54 kDa (Figure 43). Following processing of the Brittle-1 transit peptide the primary ZM-AA1 protein observed is 445 amino acids in length and has a molecular weight of approximately 49 kDa (Figure 43).

The expression of the *zm-aa1* gene and attached transit peptide is controlled by the *Pg47* promoter, which is the 5' regulatory region from the maize polygalacturonase (*Pg47*) gene (Allen and Lonsdale, 1993). The terminator for the *zm-aa1* gene is the 3' terminator sequence from the maize *In2-1* gene (Hershey and Stoner, 1991).

The *DsRed2*(*Alt1*) Cassette

The third cassette contains the *DsRed2*(*Alt1*) gene. The *DsRed2*(*Alt1*) gene is a modified version of the original DsRed2 gene (Wasson-Blader, 2001) in which an internal *Bst*E II restriction site was removed without altering the amino acid sequence of the expressed protein. The expression of the *DsRed2*(*Alt1*) gene is controlled by the barley lipid transfer protein (*Ltp2*) promoter, which provides aleurone-preferred transcription of the gene (Kalla *et al.*, 1994). Located 5' to the *Ltp2* promoter is the enhancer region from the cauliflower mosaic virus (CaMV 35S enhancer) (Franck *et al.*, 1980; Odell *et al.*, 1985 and 1988). The terminator for the *DsRed2*(*Alt1*) gene is the 3' terminator sequence from the proteinase inhibitor II gene of *Solanum tuberosum* (*pin*II terminator) (Keil *et al.,* 1986; An *et al.*, 1989).

The *DsRed2*(*Alt1*) gene encodes the DsRed2 protein. Expression of the DsRed2 protein in the aleurone layer of the maize seed produces a red coloration in seeds containing the DNA insertion, allowing for separation and identification of seed containing the 32138 SPT insertion during seed sorting. The full-length DsRed2 protein has a length of 225 amino acids and a molecular weight of approximately 26 kDa (Figure 45).

V. Genetic Characterization of 32138 SPT Maintainer

V-A. Molecular Analysis Overview

To characterize the DNA insertion in 32138 SPT maintainer, Southern blot analysis was conducted. The 32138 SPT maintainer was generated using *Agrobacterium*-mediated transformation with plasmid PHP24597 (Figure 15). The T-DNA from PHP24597 (Figure 16) has been inserted in 32138 SPT maintainer and contains three gene cassettes. The first cassette is the *Ms45* cassette comprised of the anther-specific *5126* promoter and the *Ms45* gene with associated 3' untranslated region (*Ms45* Genomic). The second is the *zm-aa1* cassette comprised of the *Pg47* promoter, the *zm-bt1* transit peptide, the *zm-aa1* gene, and the *In2-1* terminator. The third cassette is the *DsRed2(Alt1)* cassette containing the CaMV 35S enhancer, the *Ltp2* promoter, the *DsRed2(Alt1)* gene and the *pin*II terminator. Individual plants of the T1S1 generation were analyzed to determine the copy number of each of the genetic elements inserted into 32138 SPT maintainer and to verify that the integrity of the PHP24597 T-DNA was maintained upon integration. The analysis confirmed a single, intact T-DNA of PHP24597 has been inserted into the maize genome to produce 32138 SPT maintainer (Section V-B).

Stability of the 32138 SPT insertion during traditional maize breeding procedures was confirmed using Southern blot analysis. The analysis was conducted on two generations, T1S1 and BC3, and verified that the insertion remained intact and stably integrated as demonstrated by identical hybridization patterns in the two generations. A third generation (BC4) was also analyzed by Southern blot analysis, which confirmed the same stable, event-specific hybridization pattern as exhibited by the T1S1 and BC3 generations. These results confirmed the stability of the insertion in 32138 SPT maintainer across multiple breeding generations (Section V-C).

Both the T1S1 and BC3 generations were analyzed for plasmid sequences from PHP24597 outside of the T-DNA, *i.e.* regions not intended for transformation. The analysis confirmed the absence of backbone sequences in 32138 SPT maintainer (Section V-D).

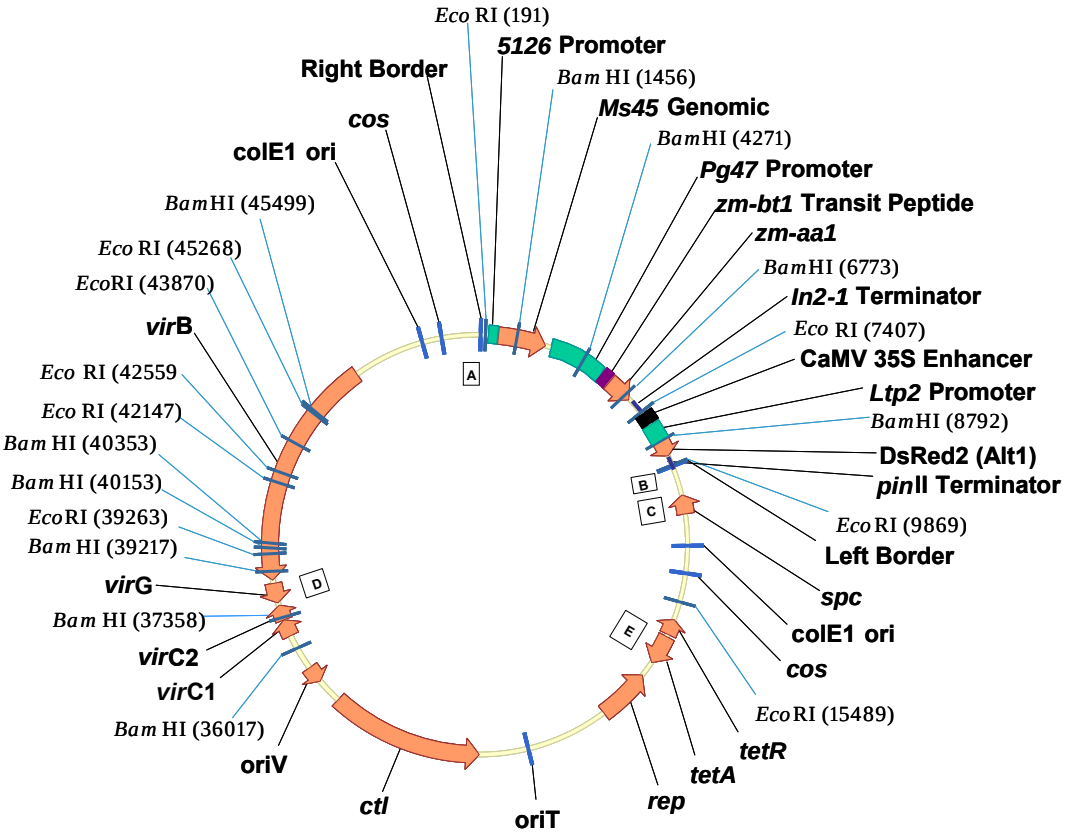
Genomic DNA from leaf material of the Hi-II(*ms45*), Hi-II, and inbred 705 were used as negative controls for all Southern blot analyses to determine which hybridizing bands originated from the endogenous maize genome. Many of the elements in the 32138 SPT insertion are derived from the maize genome and probes would be expected to hybridize to sequences in the endogenous maize genome as well as the 32138 SPT insertion. Plasmid PHP24597 was used as a positive control for probe hybridization and to verify fragment sizes internal to the T-DNA of PHP24597. All probes used for the analysis are indicated on the schematic maps of PHP24597 plasmid and T-DNA (Figures 15 and 16, respectively) and outlined in Table 3.

Based on these analyses, a schematic map of the insertion region in 32138 SPT maintainer was determined and is presented in Figure 17.

Detailed descriptions of the generations analyzed and the methods used for Southern blot analysis are further described in Appendix 8.

Figure 15. Plasmid Map of PHP24597 with Location of Restriction Enzyme Sites and Probes Used for Southern Analyses

Schematic map of plasmid PHP24597 indicating *Eco*R I and *Bam*H I restriction enzyme sites with base pair positions. The Right Border and Left Border regions flank the T-DNA (Figure 16) that is expected to be transferred during *Agrobacterium*-mediated transformation. The location of the probes used for Southern analysis is indicated as lettered boxes inside the plasmid map. **A:** RB probe; **B:** LB probe; **C:** *spc* probe; **D:** *vir*G probe; **E:** *tet* probe.



PHP24597

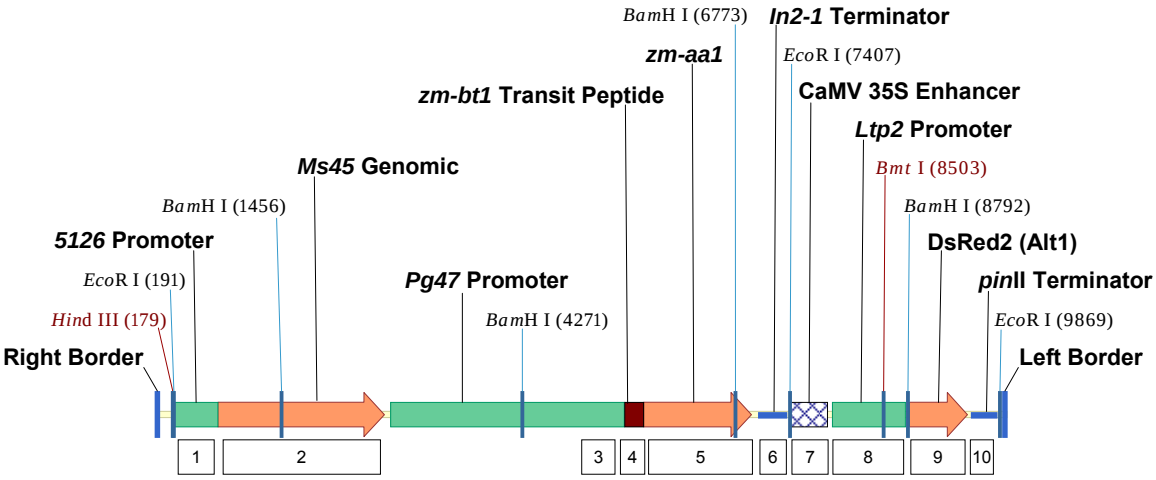
52869 bp

The locations of additional enzymes used for analysis are given below:

Enzyme	Locations (bp)
Bmt I	8503, 36160, 38359, 39502, 45917
Hind III	179, 34837, 45900, 46809, 47930, 49445

Figure 16. Map of T-DNA Region from Plasmid PHP24597 with Location of Restriction Enzyme Sites and Probes Used for Southern Analyses

Schematic map of T-DNA from PHP24597 indicating restriction enzyme sites for *Bam*H I, *Bmt* I, *Eco*R I, and *Hin*d III and the *Ms45, zm-aa1*, and *DsRed2(Alt1)* coding and regulatory regions. T-DNA size is 9950 bp. The locations of the probes used are shown as numbered boxes below the map and are identified below:



Number	Probe Name
1	*5126* promoter
2	*Ms45*
3	*Pg47* promoter
4	*zm-bt1*
5	*zm-aa1*
6	*In2-1* terminator
7	35S enhancer
8	*Ltp2* promoter
9	*DsRed2(Alt1)*
10	*pinII* terminator

Table 3. Description of DNA Probes Used for Southern Hybridization

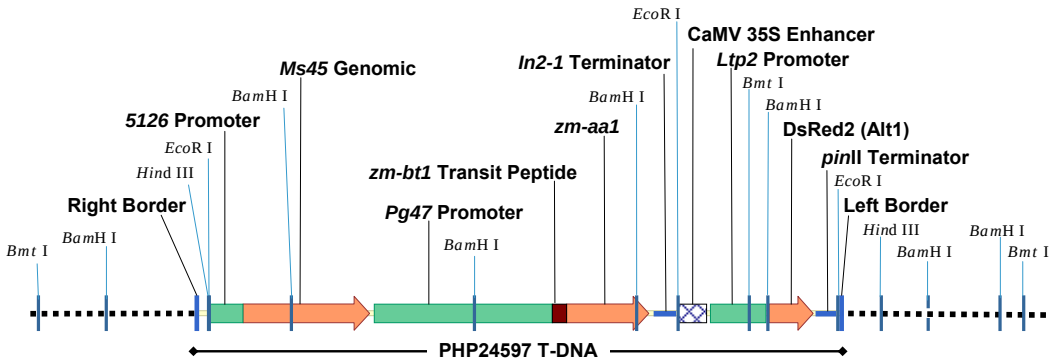
Probe Name	Genetic Element	Figure *Probe*	Position on PHP24597 T-DNA (bp to bp)[1]	Position on PHP24597 Plasmid (bp to bp)[2]	Length (bp)
5126 promoter	*5126* promoter	Figure 16 *probe 1*	304 to 706	304 to 706	403
Ms45[3]	*Ms45* Genomic region	Figure 16 *probe 2*	707 to 1426 1427 to 2258 2263 to 2658	707 to 1426 1427 to 2258 2263 to 2658	720 832 396
Pg47 promoter	*Pg47* promoter (3' region)	Figure 16 *probe 3*	5028 to 5451	5028 to 5451	424
zm-bt1	*zm-bt1* transit peptide	Figure 16 *probe 4*	5469 to 5693	5469 to 5693	225
zm-aa1[4]	*zm-aa1* gene	Figure 16 *probe 5*	5701 to 6333 6334 to 6935	5701 to 6333 6334 to 6935	603 602
In2-1 terminator	*In2-1* terminator	Figure 16 *probe 6*	7049 to 7377	7049 to 7377	329
35S enhancer	CaMV 35S enhancer region	Figure 16 *probe 7*	7427 to 7846	7427 to 7846	420
Ltp2 promoter	*Ltp2* promoter	Figure 16 *probe 8*	7906 to 8759	7906 to 8759	854
DsRed2(Alt1)	*DsRed2(Alt1)* gene	Figure 16 *probe 9*	8810 to 9487	8810 to 9487	678
*pin*II terminator	*pin*II terminator	Figure 16 *probe 10*	9582 to 9815	9582 to 9815	234
RB	Plasmid backbone adjacent to T-DNA Right Border	Figure 15 *probe A*	N/A[5]	52436 to 52825	390
LB	Plasmid backbone adjacent to T-DNA Left Border	Figure 15 *probe B*	N/A	9975 to 10320	346
spc	Spectinomycin resistance gene	Figure 15 *probe C*	N/A	11130 to 11904	775
virG	*virG* gene	Figure 15 *probe D*	N/A	37294 to 38037	744
Tet[6]	Tetracycline resistance gene	Figure 15 *probe E*	N/A	16983 to 17521 17627 to 18084	539 458

Footnotes:

1. The probe position is based on the PHP24597 T-DNA map (Figure 16).
2. The probe position is based on the PHP24597 plasmid map (Figure 15).
3. The *Ms45* probe is comprised of three non-overlapping labeled fragments that are combined in the hybridization solution.
4. The *zm-aa1* probe is comprised of two non-overlapping labeled fragments that are combined in the hybridization solution.
5. Not Applicable as this element is not located in the PHP24597 T-DNA region.
6. The *tet* probe is comprised of two non-overlapping labeled fragments that are combined in the hybridization solution.

Figure 17. Map of the Insertion in 32138 SPT Maintainer

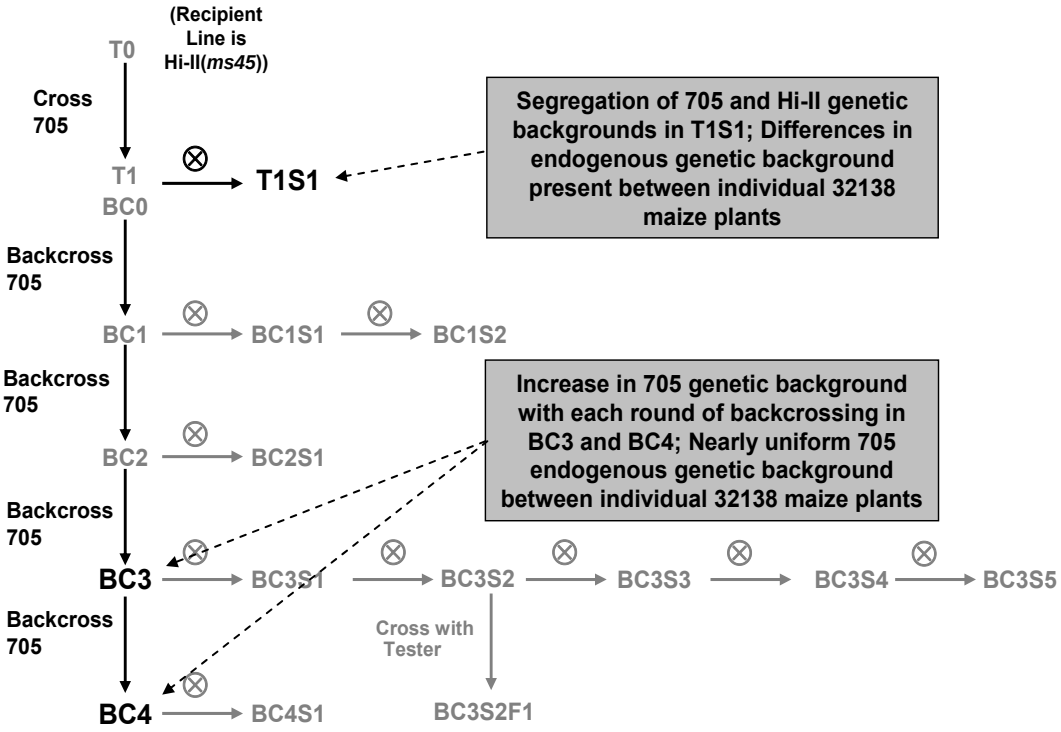
Schematic map of the insertion in 32138 SPT Maintainer based on Southern blot analysis. The flanking maize genome is represented by the horizontal dotted line on either side of the PHP24597 T-DNA insertion. A single, intact copy of the PHP24597 T-DNA integrated into the maize genome. *Bmt* I, *Eco*R I, *Bam*H I, and *Hin*d III restriction enzyme sites are indicated with the observed fragments on Southern blots shown below the map. The dashed vertical line in the *Bam*H I box below(~1500bp) indicates the *Bam*H I restriction enzyme site that appears to be partially blocked from cutting on the Southern blots (Figures 31 and 35). The locations of restriction enzyme sites outside the Right and Left Borders are not shown to scale. **Note:** Complete Southern blot analysis was conducted with *Bam*H I/*Hin*d III and with each of the probes and confirmed the intact regions of the PHP24597 T-DNA (data not shown).



Bmt I	>8600 bp					>8600 bp	
*Eco*R I		7216 bp			2462 bp		
*Bam*H I		~3800 bp	2815 bp	2502 bp	2019 bp	~4600 bp	~1500 bp
*Bam*H I/*Hin*d III		1277 bp	2815 bp	2502 bp	2019 bp	~2600 bp	

Figure 18. Genetic Backgrounds of Generations Used for 32138 SPT Maintainer Southern Analysis

Breeding diagram with the three generations used for Southern analysis shown in black font. The maize genetic backgrounds of each generation are further explained (gray boxes with arrows) to understand the degree of hybridization pattern uniformity between individuals. In several cases, depending on the probes used, differences were observed between individuals of the T1S1 generation because of the segregating genetic backgrounds.



V-B. Characterization of 32138 SPT Maintainer: Copy Number and Insertion Integrity

The integration pattern of the insertion in 32138 SPT maintainer was investigated with *Bmt* I digestion to determine copy number and with *Eco*R I digestion to determine insertion integrity. Southern blots containing samples from the T1S1 generation of 32138 SPT maintainer were hybridized with several probes to confirm copy number and integrity of each genetic element. The *5126* promoter and *Ms45* probes were used to characterize the *Ms45* cassette (Table 3, Figure 16). The *Pg47* promoter, z*m-bt1,* z*m-aa1*, and *In2-1* terminator probes were used to characterize the *zm-aa1* cassette (Table 3, Figure 16). Probes to the 35S enhancer, *Ltp2* promoter, *DsRed2(Alt1)*, and *pin*II terminator elements were used to characterize the *DsRed2(Alt1)* cassette (Table 3, Figure 16).

Some of the probes used to characterize the 32138 SPT insertion are highly homologous to sequences found in the maize genome. The *5126* promoter, *Ms45*, *Pg47* promoter, z*m-bt1, zm-aa1*, and *In2-1* terminator probes all hybridized to sequences in both control and 32138 SPT maintainer genomic DNA in addition to the PHP24597 T-DNA. These bands are identified by their presence in inbred control maize samples and are thus not part of the T-DNA insertion. They are indicated in Tables 4 and 5 by asterisks (*) and gray shading. As expected, based on the breeding diagram presented in Figure 18, the hybridization pattern due to the maize background varied between individual plants of the T1S1 generation of 32138 SPT maintainer depending on the probe used. In addition, differences with some probes were also detected in inbred control maize lines. Therefore, in order to ensure correct identification of bands due to the maize background and not due to the 32138 SPT insertion, all appropriate inbred control maize lines (inbred 705, Hi-II, and Hi-II (*ms45*) were included on these blots). The bands indicated as endogenous in Tables 4 and 5 were identified by their presence in at least one of the maize control plants.

Based on the Southern blot analyses as discussed below, it was determined that a single, intact PHP24597 T-DNA has been inserted into the genome of 32138 SPT maintainer as diagramed on the insertion map (Figure 17).

V-B.1. Copy Number

Copy number of the integrated elements in 32138 SPT maintainer was investigated using *Bmt* I digestion, as there is a single restriction enzyme site in the PHP24597 T-DNA at base pair (bp) position 8503 (Figure 16). Any additional *Bmt* I sites would fall in the flanking genome regions outside the T-DNA confirming the number of copies of the elements. Hybridization with the probes from each cassette, except for the *Ltp2* promoter probe, would indicate the number of copies of each element found in 32138 SPT maintainer based on the number of hybridizing bands (*e.g.,* one hybridizing band indicates one copy of the element). The *Bmt* I site is located within the *Ltp2* promoter element, so two hybridizing bands would be expected with this probe for a single T-DNA insertion. Predicted and observed fragment sizes for 32138 SPT maintainer with *Bmt* I are provided in Table 4. The *Bmt* I Southern blot analysis confirmed a single copy of each of the elements and the expected arrangement of these elements in the 32138 SPT insertion.

Ms45 Cassette:

A single copy of the *Ms45* cassette from PHP24597 was inserted into 32138 SPT maintainer. The *5126* promoter and *Ms45* probes were hybridized to *Bmt* I-digested genomic DNA from individual 32138 SPT maintainer plants of the T1S1 generation (Table 4, Figure 19). Both of the probes hybridized to the same single fragment of greater than 8600 bp (Table 4, Figure 19), indicating a single copy insertion with the expected arrangement of genetic elements on the inserted fragment in 32138 SPT maintainer. The *5126* promoter and *Ms45* probes are

homologous to elements endogenous to the maize genome and therefore each probe also hybridized to bands in inbred control maize samples.

zm-aa1 Cassette:

Likewise, a single copy of the *zm-aa1* cassette was inserted into 32138 SPT maintainer. The four genetic elements comprising this cassette - the *Pg47* promoter, *zm-bt1*, *zm-aa1*, and *In2-1* terminator - were used as probes to determine number of copies inserted. The probes of this cassette are homologous to elements endogenous to the maize genome and therefore each probe also hybridized to bands in inbred control maize samples. In the case of the *Pg47* promoter probe, the 32138-specific band co-migrated with a strong endogenous band and is not visible as a separate band (Figure 20). Each of the *zm-bt1*, *zm-aa1*, and *In2-1* terminator probes hybridized to the same single fragment of greater than 8600 bp (Table 4, Figures 20 and 21) as seen with the *5126* promoter and *Ms45* probes (Table 4, Figure 19), indicating a single copy insertion with the expected arrangement of these genetic elements on the inserted fragment in 32138 SPT maintainer. The *Pg47* promoter element is likely present as a single copy, based on the presence of the same band of greater than 8600 bp for both the *Ms45* and *zm-bt1* elements that flank the *Pg47* promoter in the PHP24597 T-DNA (Table 4, Figures 19 and 20). A single, intact copy of the *Pg47* promoter element with the expected arrangement was confirmed by sequencing of the 32138 SPT insertion. These hybridizations and sequence data confirm that a single copy of the *zm-aa1* cassette was inserted in 32138 SPT maintainer in the expected arrangement on the T-DNA.

DsRed2(Alt1) Cassette:

Similar to the data on the other cassettes, a single copy of the *DsRed2(Alt1)* cassette was inserted into 32138 SPT maintainer. The four elements comprising this cassette – the 35S enhancer, *Ltp2* promoter, *DsRed2(Alt1)* gene and *pin*II terminator - were hybridized to *Bmt* I-digested DNA. The 35S enhancer, *DsRed2(Alt1)* gene and *pin*II terminator probes each hybridized to a single fragment of greater than 8600 bp (Table 4, Figures 22 and 23), indicating a single copy insertion while the *Ltp2* promoter probe hybridized to two fragments, both greater than 8600 bp (Table 4, Figure 22), also confirming a single copy insertion. The presence of two bands hybridizing to the *Ltp2* promoter probe is expected due to the location of the *Bmt* I restriction site within the *Ltp2* promoter element, and also indicates the presence of only one copy of the inserted T-DNA. Comparison of the two bands on the *Ltp2* promoter blot with the other probes of this cassette indicates that the lower band with the *Ltp2* promoter probe is the same band associated with the 35S enhancer element and the upper band is the same associated with the *DsRed2(Alt1)* gene and *pin*II terminator (Figures 22 and 23). These hybridizations confirm the *DsRed2(Alt1)* cassette was inserted as a single copy in the expected arrangement on the T-DNA.

This Southern blot analysis demonstrates that the 32138 SPT insertion contains a single copy of each of the elements and that each of the cassettes inserted as expected based on the map of the PHP24597 T-DNA. A final map of the insertion in 32138 SPT maintainer based on the analysis with *Bmt* I is presented in Figure 17.

Table 4. Predicted and Observed Hybridizing Bands on Southern Blots with Bmt I Restriction Enzyme Digest

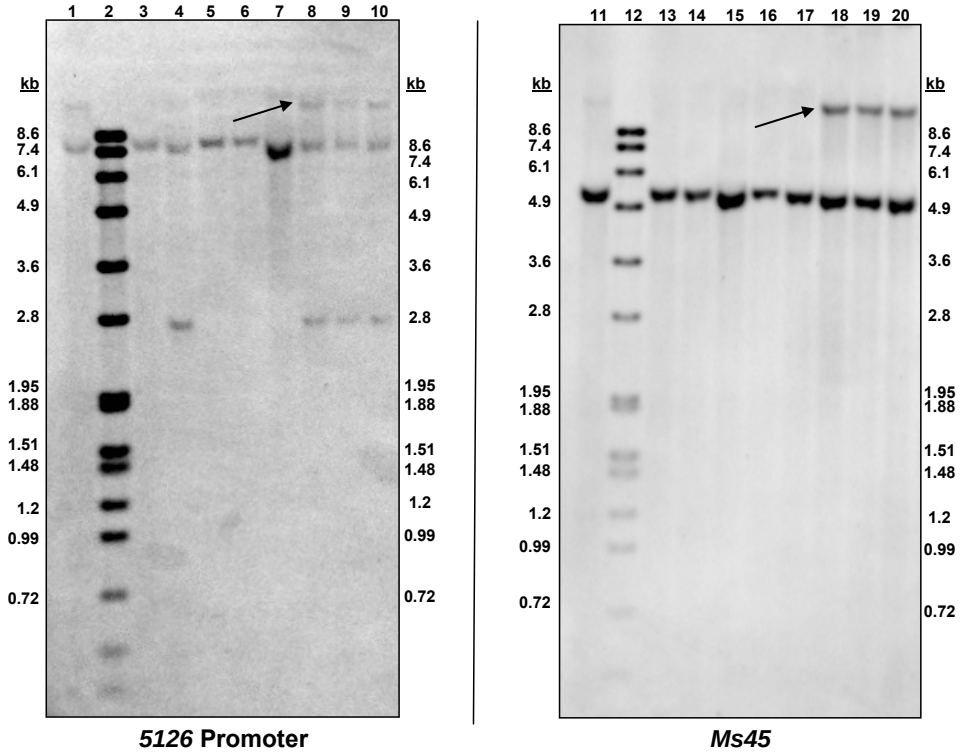
Probe	Restriction Enzyme	Figure	Predicted Fragment Size from PHP24597 T-DNA[1] (bp)	Predicted Fragment Size from Plasmid PHP24597[2] (bp)	Observed Fragment Size in 32138 SPT maintainer [3] (bp)
5126 promoter	*Bmt* I	19	>8500 (border)[4]	15455	>8600 ~7800* ~2700*
Ms45	*Bmt* I	19	>8500 (border)	15455	>8600 ~5200*
Pg47 promoter	*Bmt* I	20	>8500 (border)	15455	Obscured by endogenous bands 3 bands >8600* 2 bands 6100 – 8600* 2 bands 4900 – 6100* 2 bands 3600 – 4900*
zm-bt1	*Bmt* I	20	>8500 (border)	15455	>8600 ~4200*
zm-aa1	*Bmt* I	21	>8500 (border)	15455	>8600 3 bands >8600* 3 bands 6100 – 8600* 2 bands 4900 – 6100* 2 bands 3600 – 4900* ~3000* ~2700* ~2500*[5] ~1500*
In2-1 terminator	*Bmt* I	21	>8500 (border)	15455	>8600 >8600* ~8000* ~4700* ~3200*
35S enhancer	*Bmt* I	22	>8500 (border)	15455	>8600
Ltp2 promoter	*Bmt* I	22	>8500 (border) >1400 (border)	15455 27657	>8600 >8600
DsRed2(Alt1)	*Bmt* I	23	>1400 (border)	27657	>8600
*pin*II terminator	*Bmt* I	23	>1400 (border)	27657	>8600

Note: An asterisk (*) and gray shading indicates the designated band is due to probe hybridization to endogenous maize genome sequences, as determined by the presence of the same band in both 32138 SPT maintainer and inbred control maize lanes. Certain endogenous bands may be difficult to discern on a printed copy but are visible on the original film. Not all endogenous bands are the same in all samples due to genomic differences in varieties used in the breeding process.

Footnotes:

1. Predicted fragment sizes for 32138 SPT maintainer are based on the map of the PHP24597 T-DNA (Figure 16).
2. Predicted fragment sizes for hybridization to samples containing the plasmid positive control are based on the PHP24597 plasmid map as shown in Figure 15.
3. Observed fragment sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight. The sizes of fragments not corresponding directly to plasmid fragments are rounded to the nearest 100 bp.
4. Minimum fragment size predicted based on an intact insertion of the T-DNA from PHP24597 (Figure 16). Fragment size is rounded to the nearest 100 bp. Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion.
5. The ~2500 bp endogenous band is seen in only one of the DP-32138-1 plants and the Hi-II control plants.

Figure 19. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *Bmt* I Digested DNA with *5126* Promoter and *Ms45* Probes



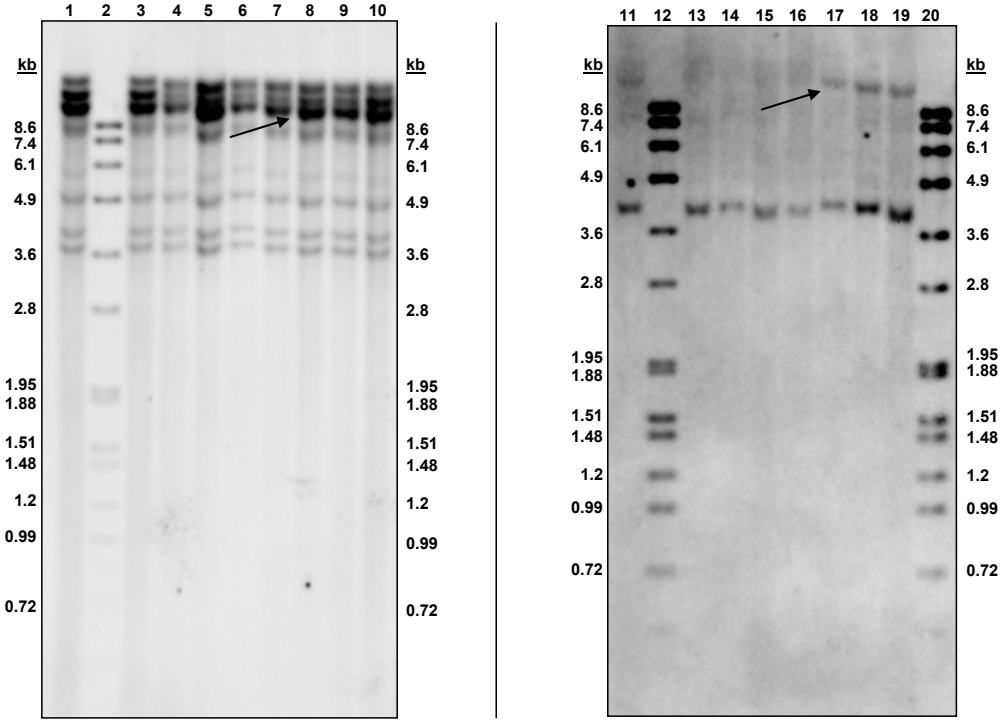
Genomic DNA isolated from leaf tissue from individual plants of 32138 SPT maintainer (T1S1 generation) and of inbred control maize (inbred 705, Hi-II, and Hi-II (*ms45*)) was digested with *Bmt* I and hybridized to the *5126* promoter and *Ms45* probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of inbred control maize (inbred 705) DNA. Sizes of the DIGVII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). An arrow indicates the 32138-specific band.

Note: The hybridization banding pattern is different between the two Hi-II plants with the *5126* promoter probe (Lanes 4 and 5) due to variability of the maize genetic background in this control.

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II)
6	Control (Hi-II(*ms45*))
7	Control (Hi-II(*ms45*))
8	32138 SPT maintainer / plant 4
9	32138 SPT maintainer / plant 5
10	32138 SPT maintainer / plant 6

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II)
16	Control (Hi-II(*ms45*))
17	Control (Hi-II(*ms45*))
18	32138 SPT maintainer / plant 8
19	32138 SPT maintainer / plant 9
20	32138 SPT maintainer / plant 10

Figure 20. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *Bmt* I Digested DNA with *Pg47* Promoter and *zm-bt1* Probes



Pg47* Promoter** ***zm-bt1

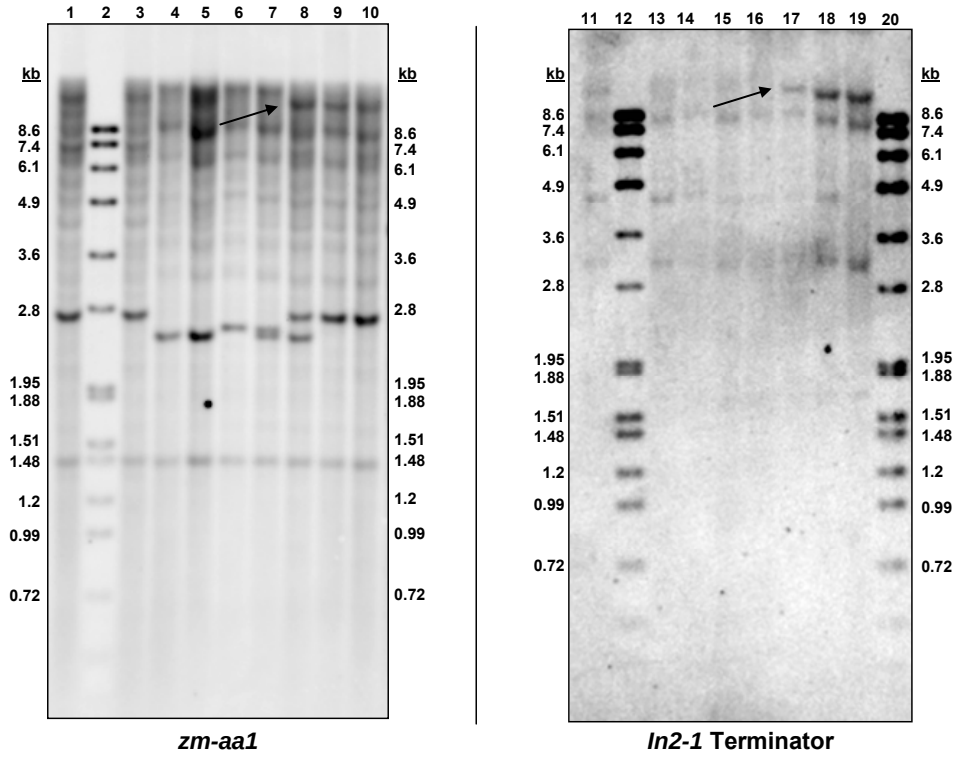
Genomic DNA isolated from leaf tissue from individual plants of 32138 SPT maintainer (T1S1 generation) and of inbred control maize (inbred 705, Hi-II, and Hi-II(*ms45*)) was digested with *Bmt* I and hybridized to the *Pg47* promoter and *zm-bt1* probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of inbred control maize (inbred 705) DNA. Sizes of the DIGVII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). An arrow indicates the 32138-specific band.

Note: The arrow in the *Pg47* promoter panel indicates the putative location of the 32138-specific band based on hybridization with flanking element probes (Figure 19, *Ms45* and Figure 20 *zm-bt1*), but the band itself is obscured by the strong endogenous band of the same size. Sequence data confirmed a single copy of this element as described in Section V-B1.

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II)
6	Control (Hi-II(*ms45*))
7	Control (Hi-II(*ms45*))
8	32138 SPT maintainer / plant 8
9	32138 SPT maintainer / plant 9
10	32138 SPT maintainer / plant 10

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II(*ms45*))
16	Control (Hi-II(*ms45*))
17	32138 SPT maintainer / plant 1
18	32138 SPT maintainer / plant 2
19	32138 SPT maintainer / plant 3
20	DIGVII marker

Figure 21. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *Bmt* I Digested DNA with *zm-aa1* and *In2-1* Terminator Probes



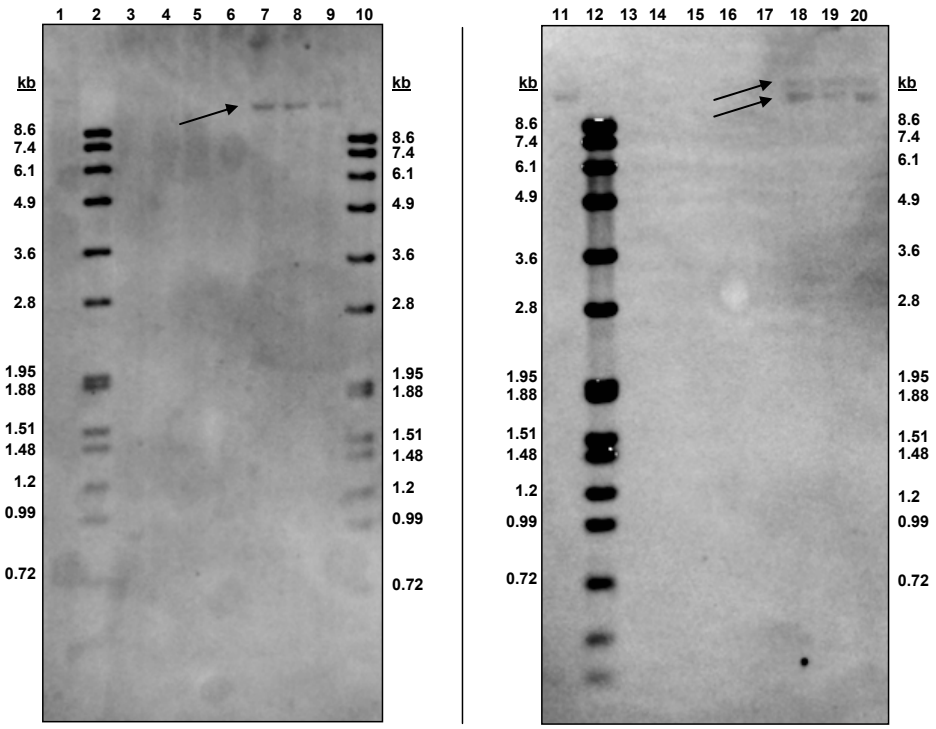
Genomic DNA isolated from leaf tissue from individual plants of 32138 SPT maintainer (T1S1 generation) and of inbred control maize (inbred 705, Hi-II, and Hi-II(*ms45*)) was digested with *Bmt* I and hybridized to the *zm-aa1* and *In2-1* terminator probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of inbred control maize (inbred 705) DNA. Sizes of the DIGVII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). An arrow indicates the 32138-specific band.

Note: The hybridization pattern is different between individuals of the Hi-II(*ms45*) maize (Lanes 6 and 7) and 32138 SPT maintainer (Lanes 8 through 10) with the *zm-aa1* probe due to variability in the maize genetic background.

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II)
6	Control (Hi-II(*ms45*))
7	Control (Hi-II(*ms45*))
8	32138 SPT maintainer / plant 8
9	32138 SPT maintainer / plant 9
10	32138 SPT maintainer / plant 10

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II(*ms45*))
16	Control (Hi-II(*ms45*))
17	32138 SPT maintainer / plant 1
18	32138 SPT maintainer / plant 2
19	32138 SPT maintainer / plant 3
20	DIGVII marker

Figure 22. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *Bmt* I Digested DNA with 35S Enhancer and *Ltp2* Promoter Probes



Genomic DNA isolated from leaf tissue from individual plants of 32138 SPT maintainer (T1S1 generation) and of inbred control maize (inbred 705, Hi-II, and Hi-II(*ms45*)) was digested with *Bmt* I and hybridized to the 35S enhancer and *Ltp2* promoter probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of inbred control maize (inbred 705) DNA. Sizes of the DIGVII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

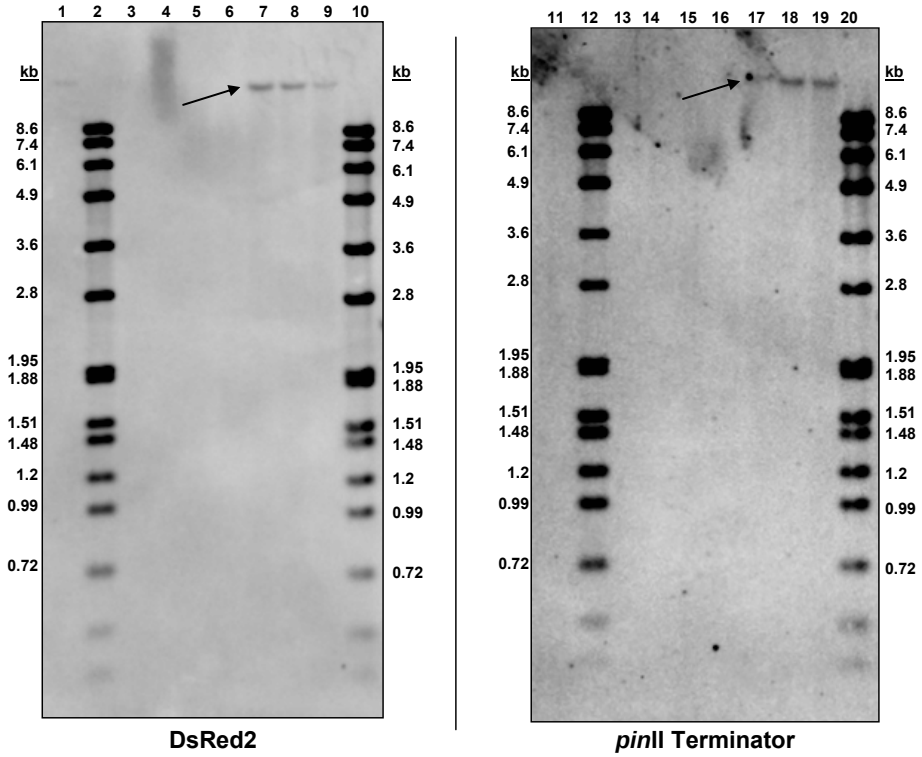
An arrow indicates the 32138-specific band in the 35S Enhancer panel. Two arrows indicate the 32138-specific bands in the *Ltp2* promoter panel.

Note: In Lanes 1 and 11, the PHP24597 plasmid band is faint and migrates above the 8.6 kb marker. This band is observed on the original X-ray film.

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II(*ms45*))
6	Control (Hi-II(*ms45*))
7	32138 SPT maintainer / plant 1
8	32138 SPT maintainer / plant 2
9	32138 SPT maintainer / plant 3
10	DIGVII marker

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II)
16	Control (Hi-II(*ms45*))
17	Control (Hi-II(*ms45*))
18	32138 SPT maintainer / plant 4
19	32138 SPT maintainer / plant 5
20	32138 SPT maintainer / plant 6

Figure 23. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer *Bmt* I Digested DNA with *DsRed2(Alt1)* and *pin*II Terminator Probes



Genomic DNA isolated from leaf tissue from individual plants of 32138 SPT maintainer (T1S1 generation) and of inbred control maize (inbred 705, Hi-II, and Hi-II(*ms45*)) was digested with *Bmt* I and hybridized to the *DsRed2(Alt1)* and *pin*II terminator probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of inbred control maize (inbred 705) DNA. Sizes of the DIGVII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). An arrow indicates the 32138-specific band.

Note: In Lanes 1 and 11, the PHP24597 plasmid band is faint and migrates above the 8.6 kb marker. This band is observed on the original X-ray film.

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II(*ms45*))
6	Control (Hi-II(*ms45*))
7	32138 SPT maintainer / plant 1
8	32138 SPT maintainer / plant 2
9	32138 SPT maintainer / plant 3
10	DIGVII marker

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II(*ms45*))
16	Control (Hi-II(*ms45*))
17	32138 SPT maintainer / plant 1
18	32138 SPT maintainer / plant 2
19	32138 SPT maintainer / plant 3
20	DIGVII marker

V-B.2. Insertion Integrity

*Eco*R I digestion was used to verify that the inserted T-DNA containing the *Ms45, zm-aa1,* and *DsRed2(Alt1)* cassettes was complete and intact in maize 32138 SPT maintainer. There are three *Eco*R I sites in the PHP24597 T-DNA (base pair positions 191, 7407, and 9869; Figure 16). Two internal fragments, one of 7216 bp and one of 2462 bp, would be expected to hybridize to each of the probes and would confirm the integrity of all three cassettes in the insertion. The probes of the *Ms45* and *zm-aa1* cassettes would be expected to hybridize to the 7216 bp band, as these cassettes are contained on this internal fragment. The probes of the *DsRed2(Alt1)* cassette would be expected to hybridize to the internal 2462 bp band containing this cassette. Expected and observed fragment sizes with *Eco*R I are given in Table 5. This analysis described below confirmed that the T-DNA was inserted intact and as expected in 32138 SPT maintainer.

As stated previously, the *5126* promoter, *Ms45*, *Pg47* promoter, z*m-bt1, zm-aa1*, and *In2-1* terminator probes are homologous to elements endogenous to the maize genome and therefore each probe hybridized to bands in inbred control maize samples. These hybridizing bands from the endogenous maize genome are indicated in Table 5 by asterisks (*) and gray shading.

The *5126* promoter and *Ms45* probes for the *Ms45* cassette hybridized to a single insert-derived band of 7216 bp that matched the plasmid control band (Table 5, Figure 24). Similarly, three of the probes for the *zm-aa1* cassette (*Pg47* promoter, *zm-bt1,* and *In2-1* terminator) hybridized to the same internal 7216 bp band (Table 5, Figures 25 and 26). The *zm-aa1* probe hybridized to an endogenous band of a similar size as the 7216 bp internal band, thus obscuring the ability to determine if the 32138-specific band was present on the Southern blot (Figure 26). The *zm-aa1* cassette is likely intact based on the presence of the 7216 bp band for the two probes flanking the *zm-aa1* gene (*zm-bt1* and *In2-1* terminator) (Figures 25 and 26). As further support of the integrity of the *zm-aa1* cassette, an intact copy was confirmed by sequencing of the 32138 SPT insertion (data not shown). The probes for the *DsRed2(Alt1)* cassette (35S enhancer, *Ltp2* promoter, *DsRed2(Alt1)*, and *pin*II terminator) hybridized to the expected internal band of 2462 bp (Table 5, Figures 27 and 28). The size of the band for each probe was confirmed by hybridization to the PHP24597 plasmid fragment corresponding to the T-DNA (Lanes 1 and 11, Figures 24 through 28). In some cases, the hybridization to the plasmid fragment is not clearly visible on a printed copy but can be seen on the film exposure of the Southern blot. Because these probes hybridized to the internal fragments of the predicted sizes, the insertion in 32138 SPT maintainer was determined to be intact and as expected based on the PHP24597 T-DNA map. A schematic map of the 32138 SPT insertion based on this analysis is presented in Figure 17.

Table 5. Predicted and Observed Hybridizing Bands on Southern Blots with *Eco*R I Restriction Enzyme Digest

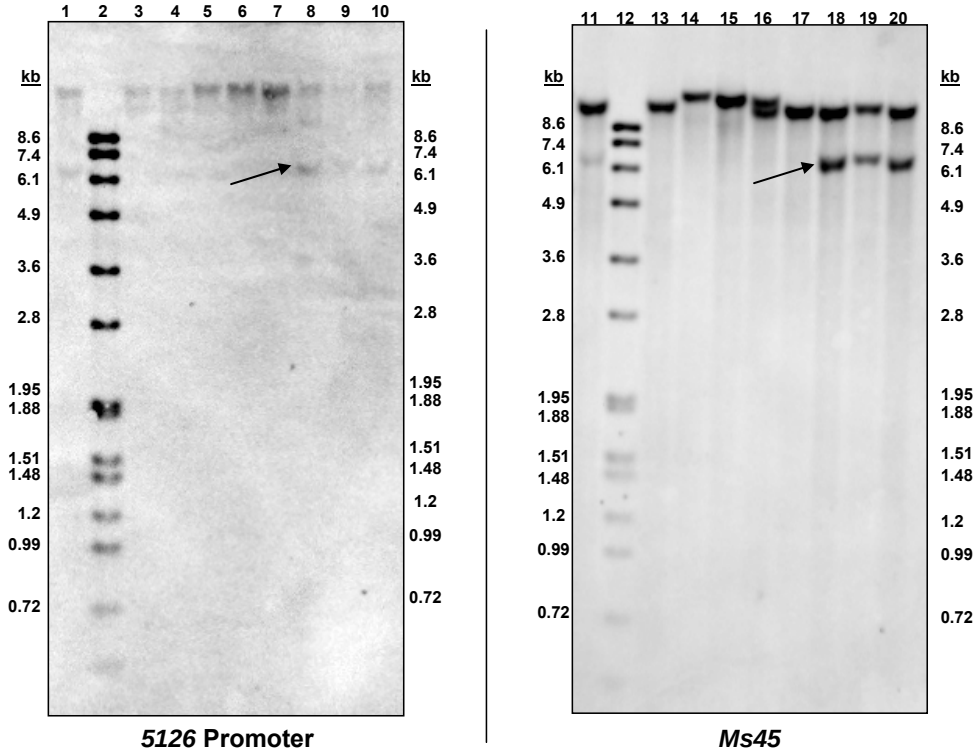
Probe	Restriction Enzyme	Figure	Predicted Fragment Size from PHP24597[1] (bp)	Predicted Fragment Size from PHP24597 T-DNA[2] (bp)	Observed Fragment Size in Maize 32138 SPT Maintainer[3] (bp)
5126 promoter	*Eco*R I	24	7216	7216	7216[4]
					2 bands >8600*
Ms45	*Eco*R I	24	7216	7216	7216[4]
					>8600*
Pg47 promoter	*Eco*R I	25	7216	7216	7216[4]
					3 bands >8600* ~7400* ~5600*
zm-bt1	*Eco*R I	25	7216	7216	7216[4]
					>8600* ~8600* ~3300*
zm-aa1	*Eco*R I	26	7216	7216	Obscured by endogenous bands
					2 bands >8600* 2 bands 4900 – 8600* 4 bands 3600 – 4900* ~4300*[5] 2 bands 2800 – 3600* 4 bands 1950 – 2800* ~1700*
In2-1 terminator	*Eco*R I	26	7216	7216	7216[4]
					2 bands >8600* ~8600* ~7400* ~3400*
35S enhancer	*Eco*R I	26	2462	2462	2462[4]
Ltp2 promoter	*Eco*R I	26	2462	2462	2462[4]
DsRed2(Alt1)	*Eco*R I	28	2462	2462	2462[4]
*pin*II terminator	*Eco*R I	28	2462	2462	2462[4]

Note: An asterisk (*) and gray shading indicates the designated band is due to probe hybridization to endogenous maize genome sequences, as determined by the presence of the same band in both 32138 and inbred control maize lanes. Certain endogenous bands may be difficult to discern on a printed copy but are visible on the original film. Not all endogenous bands are the same in all samples due to genomic differences in varieties used in the breeding process.

Footnotes:

1. Predicted fragment sizes for hybridization to samples containing the plasmid positive control are based on the PHP24597 plasmid map as shown in Figure 15.
2. Predicted fragment sizes for maize 32138 SPT maintainer are based on the map of the PHP24597 T-DNA as shown in Figure 16.
3. Observed fragment sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight. The sizes of fragments not corresponding directly to plasmid fragments are rounded to the nearest 100 bp.
4. Observed fragment size is the same as the predicted fragment size based on equivalent migration on the Southern blots.
5. The ~4300 bp endogenous band is seen in only one of the DP-32138-1 plants and one of the Hi-II control plants.

Figure 24. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *EcoR I* Digested DNA with *5126* Promoter and *Ms45* Probes



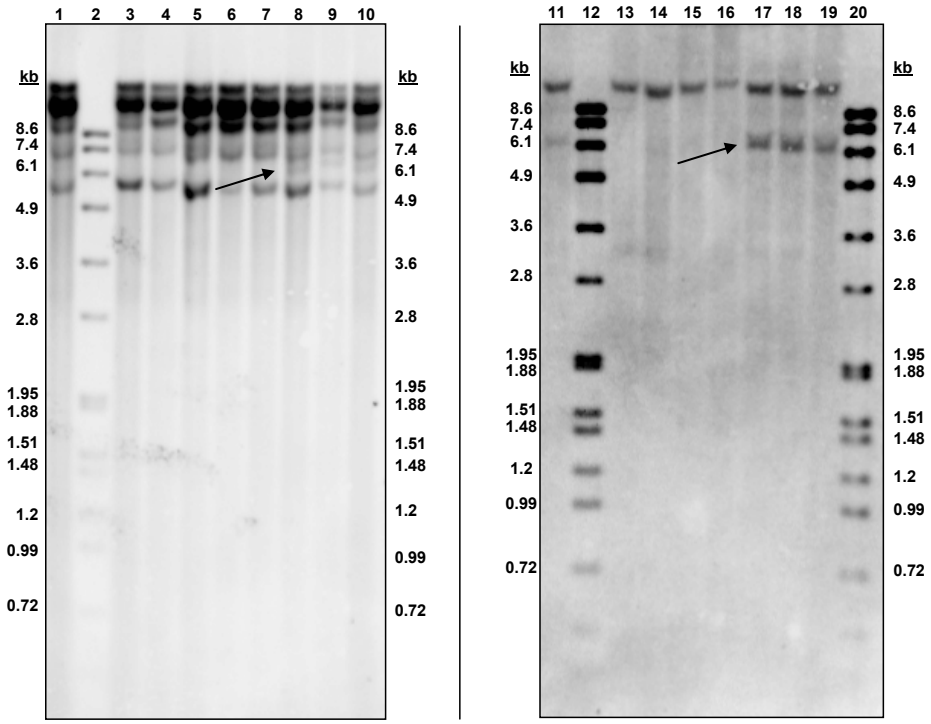
Genomic DNA isolated from leaf tissue from individual plants of maize SPT maintainer (T1S1 generation) and of inbred control maize (inbred 705, Hi-II, and Hi-II(*ms45*)) was digested with *Eco*R I and hybridized to the *5126* promoter and *Ms45* probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of control maize (inbred 705) DNA. Sizes of the DIGVII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). An arrow indicates the 32138-specific band.

Note: The hybridization banding pattern is different between the two Hi-II(*ms45*) plants with the *Ms45* probe (Lanes 16 and 17) due to variability of the maize genetic background in this control.

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II)
6	Control (Hi-II(*ms45*))
7	Control (Hi-II(*ms45*))
8	32138 SPT maintainer / plant 4
9	32138 SPT maintainer / plant 5
10	32138 SPT maintainer / plant 6

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II)
16	Control (Hi-II(*ms45*))
17	Control (Hi-II(*ms45*))
18	32138 SPT maintainer / plant 8
19	32138 SPT maintainer / plant 9
20	32138 SPT maintainer / plant 10

Figure 25. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *Eco*R I Digested DNA with *Pg47* Promoter and *zm-bt1* Probes



Pg47* Promoter** ***zm-bt1

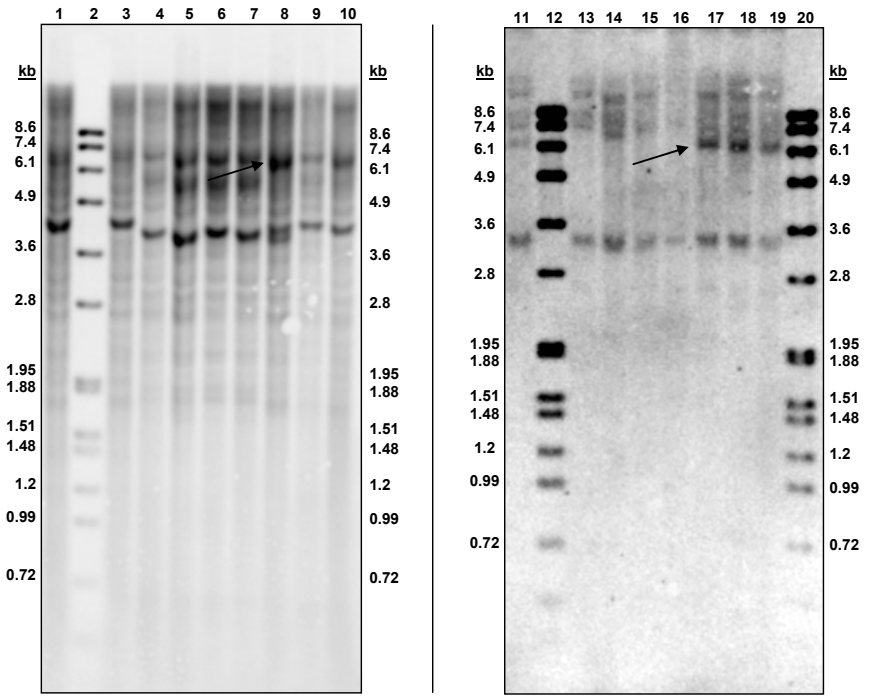
Genomic DNA isolated from leaf tissue from individual plants of 32138 SPT maintainer (T1S1 generation) and of control maize (inbred 705, Hi-II, and Hi-II(*ms45*)) was digested with *Eco*R I and hybridized to the *Pg47* promoter and *zm-bt1* probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of control maize (inbred 705) DNA. Sizes of the DIGVII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). An arrow indicates the 32138-specific band.

Note: In Lane 1 and 11, the PHP24597 plasmid band is faint and migrates between the 6.1 and 7.4 kb markers. This band is observed on the original X-ray film.

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II)
6	Control (Hi-II(*ms45*))
7	Control (Hi-II(*ms45*))
8	32138 SPT maintainer / plant 8
9	32138 SPT maintainer / plant 9
10	32138 SPT maintainer / plant 10

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II(*ms45*))
16	Control (Hi-II(*ms45*))
17	32138 SPT maintainer / plant 1
18	32138 SPT maintainer / plant 2
19	32138 SPT maintainer / plant 3
20	DIGVII marker

Figure 26. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *Eco*R I Digested DNA with *zm-aa1* and *In2-1* Terminator Probes



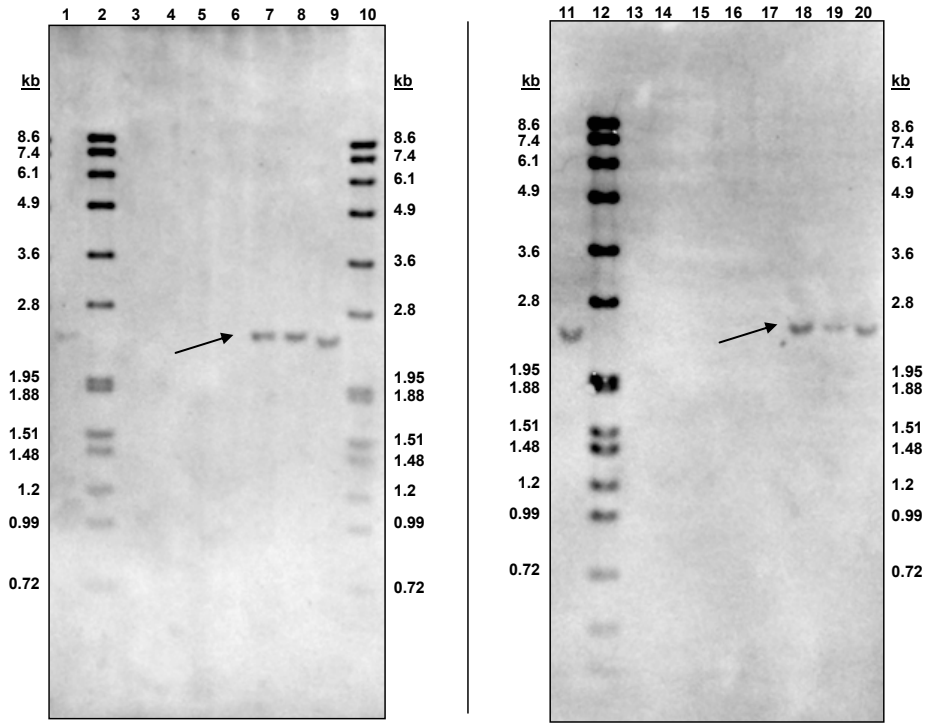
Genomic DNA isolated from leaf tissue from individual plants of 32138 maize (T1S1 generation) and of control maize (705, Hi-II, and Hi-II(*ms45*)) was digested with *Eco*R I and hybridized to the *zm-aa1* and *In2-1* terminator probes. Probes used are indicated below each control lanes included plasmid PHP24597 at approximately one gene copy and 4 µg of control maize (705) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). An arrow indicates the 32138-specific band.

Note: The arrow in the *zm-aa1* probe panel indicates the putative location of the 7216 bp internal band based on hybridization with flanking element probes (Figure 25, *zm-bt1* and Figure 26, *In2-1* terminator), but the band itself is obscured by strong endogenous bands of the same size. Sequence data confirmed an intact copy of the *zm-aa1* cassette as described in Section V-B2. The hybridization pattern between the 32138 plants with the *zm-aa1* probe is different due to variability in the maize genetic background (Lanes 8 through 10).

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II)
6	Control (Hi-II(*ms45*))
7	Control (Hi-II(*ms45*))
8	32138 SPT maintainer / plant 8
9	32138 SPT maintainer / plant 9
10	32138 SPT maintainer / plant 10

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II(*ms45*))
16	Control (Hi-II(*ms45*))
17	32138 SPT maintainer / plant 1
18	32138 SPT maintainer / plant 2
19	32138 SPT maintainer / plant 3
20	DIG VII marker

Figure 27. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *Eco*R I Digested DNA with 35S Enhancer and *Ltp2* Promoter Probes.



35S Enhancer **_Ltp2_ Promoter**

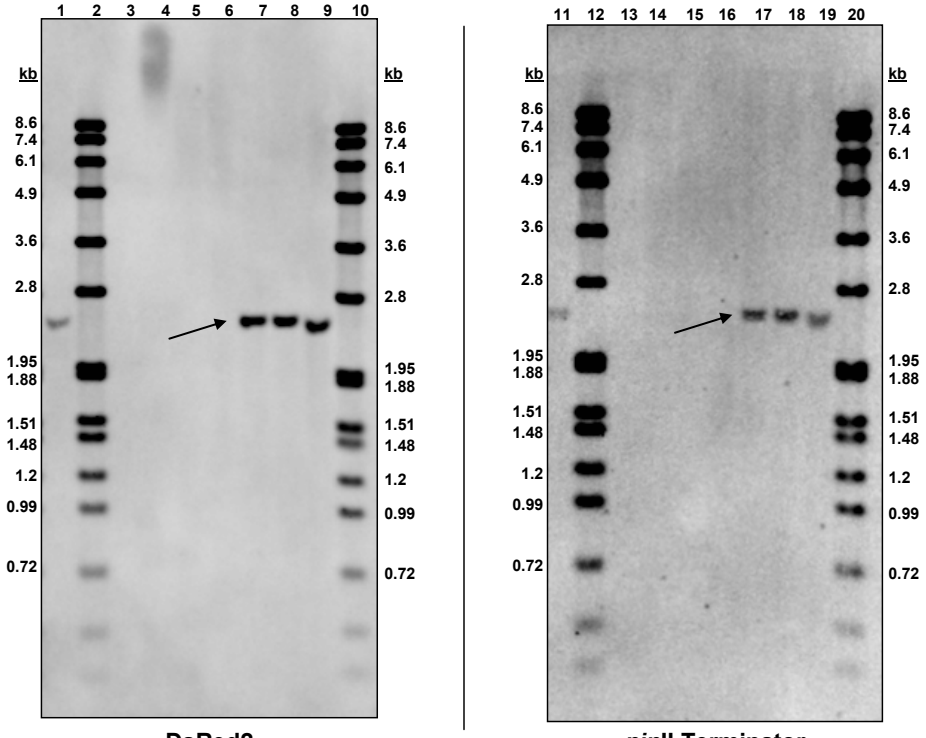
Genomic DNA isolated from leaf tissue from individual plants of 32138 maize (T1S1 generation) and of control maize (705, Hi-II, and Hi-II(*ms45*)) was digested with *Eco*R I and hybridized to the 35S enhancer and *Ltp2* promoter probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of control maize (705) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). An arrow indicates the 32138-specific band.

Note: In Lane 1, the PHP24597 plasmid band is faint and migrates between the 1.95 and 2.8 kb markers. This band is observed on the original X-ray film.

Lane	Sample
1	control (705) + 1 copy of PHP24597
2	DIGVII marker
3	control (705)
4	control (Hi-II)
5	control (Hi-II(*ms45*))
6	control (Hi-II(*ms45*))
7	32138 maize / plant 1
8	32138 maize / plant 2
9	32138 maize / plant 3
10	DIG VII marker

Lane	Sample
11	control (705) + 1 copy of PHP24597
12	DIGVII marker
13	control (705)
14	control (Hi-II)
15	control (Hi-II)
16	control (Hi-II(*ms45*))
17	control (Hi-II(*ms45*))
18	32138 maize / plant 4
19	32138 maize / plant 5
20	32138 maize / plant 6

Figure 28. Southern Blot Analysis of the T1S1 Generation of 32138 SPT Maintainer, *Eco*R I Digested DNA with *DsRed2(Alt1)* and *pin*II Terminator Probes



Genomic DNA isolated from leaf tissue from individual plants of 32138 SPT maintainer (T1S1 generation) and of control maize (inbred 705, Hi-II, and Hi-II(*ms45*)) was digested with *Eco*R I and hybridized to the *DsRed2(Alt1)* and *pin*II terminator probes. Probes used are indicated below each panel. Approximately 4 μg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24597 at approximately one gene copy and 4 μg of control maize (inbred 705) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

An arrow indicates the 32138-specific band.

Lane	Sample
1	Control (inbred 705) + 1 copy of PHP24597
2	DIGVII marker
3	Control (inbred 705)
4	Control (Hi-II)
5	Control (Hi-II(*ms45*))
6	Control (Hi-II(*ms45*))
7	32138 SPT maintainer / plant 1
8	32138 SPT maintainer / plant 2
9	32138 SPT maintainer / plant 3
10	DIG VII marker

Lane	Sample
11	Control (inbred 705) + 1 copy of PHP24597
12	DIGVII marker
13	Control (inbred 705)
14	Control (Hi-II)
15	Control (Hi-II(*ms45*))
16	Control (Hi-II(*ms45*))
17	32138 SPT maintainer / plant 1
18	32138 SPT maintainer / plant 2
19	32138 SPT maintainer / plant 3
20	DIG VII marker

V-C. Stability of the Insertion across Generations

Stability of the 32138 SPT insertion was confirmed by using Southern blot analysis on three generations: T1S1, BC3, and a segregating BC4 generation. *Bam*H I restriction digestion was selected for characterization of these generations; the sites are indicated on the maps of the PHP24597 plasmid and T-DNA (Figures 15 and 16, respectively). Four *Bam*H I sites are located within the T-DNA of PHP24597 at base pair positions 1456, 4271, 6773, and 8792 (Figure 16). This digestion provides a unique Southern hybridization pattern at the Right and Left Borders to confirm the stability of the insertion between the T1S1, BC3, and BC4 generations.

These hybridizations, as discussed below, confirmed the stability of the insertion in 32138 SPT maintainer across these three generations. The Southern blot analysis for *Bam*H I, described below, is presented in a schematic map of the 32138 SPT insertion (Figure 17).

T1S1 and BC3 Generation Analysis

*Bam*H I analysis was conducted on individual plants of the T1S1 and BC3 generations of 32138 SPT maintainer and from the control maize lines. The *Ms45*, *zm-aa1*, and *DsRed2(Alt1)* gene probes were used to confirm the genetic stability across the two generations. Both the *Ms45* and *zm-aa1* probes are highly identical or similar to the genes found in the endogenous maize genome, and therefore hybridized to a number of bands of varying sizes in the control plant lines. These hybridizing endogenous bands were identified in 32138 SPT maintainer samples based on their presence in the control maize lines and are identified in Table 6 by an asterisk (*) and gray shading.

A band of greater than 1500 bp and one of 2815 bp would be expected at the Right Border of the 32138 SPT insertion with the *Ms45* probe and would confirm the stability of the Right Border flanking region (Table 6). All T1S1 and BC3 samples of 32138 SPT maintainer were consistent with the plasmid controls and showed the two bands attributed to the insertion at approximately 3800 bp and 2815 bp as expected (Figure 29). The 2815 bp band migrated equivalently with the plasmid control (Figure 29). This hybridization result confirms the stability of the Right Border flanking region of the 32138 SPT insertion across the two generations.

The *zm-aa1* probe would be expected to hybridize with two internal bands of 2019 bp and 2502 bp, confirming sites internal to the T-DNA (Table 6). As expected, bands of 2019 bp and 2502 bp were observed and migrated equivalently with the plasmid control (Figure 30). These observed bands from the 32138 SPT insertion were consistent between the T1S1 and BC3 generations (Figure 30), confirming the stability of the T-DNA insertion.

At the Left Border of the 32138 SPT insertion, a band of greater than 1200 bp would be expected with the *DsRed2(Alt1)* probe and would confirm the stability of the Left Border flanking region (Table 6). One band of approximately 4600 bp and a second faint band at approximately 6100 bp were visible in all samples of the 32138 SPT maintainer plants on the Southern blot (Figure 31). The second faint 6100 bp band was determined to be from partially blocked digestion at the *Bam*H I site in the genomic DNA flanking the insertion, which may be due to methylation or secondary structure of DNA surrounding this site. In order to clarify the partial digestion of the *Bam*H I site, a *Bam*H I/*Hin*d III double digestion was conducted and was hybridized with the *DsRed2(Alt1)* probe (Figure 32). A single band of approximately 2600 bp was observed in all T1S1 and BC3 samples of 32138 SPT maintainer. The presence of only one band in the double digestion (Figure 32) confirms the previous conclusions of a single insertion of the *DsRed2(Alt1)* gene in 32138 SPT maintainer. The *Bam*H I and *Bam*H I/*Hin*d III Southern blots both showed consistent banding patterns for all samples of 32138 SPT maintainer from both generations, further confirming the conservation

of the Left Border of the T-DNA insertion and the equivalence of the insertion between the two generations analyzed.

BC4 Generation Analysis

Similar hybridization patterns to the T1S1 and BC3 generations were also observed for the segregating BC4 generation. The BC4 generation was also analyzed using *Bam*H I digestion and hybridization with the three gene probes, *Ms45*, *zm-aa1*, and *DsRed2(Alt1)*. For those individual maize 32138 SPT maintainer plants that contained the 32138 SPT insertion, the *Ms45* probe hybridized to a band of approximately 3800 bp and an internal band of 2815 bp due to the 32138 SPT insertion (Table 6, Figure 33) similar to the T1S1 and BC3 generations discussed above (Figure 29). As with the T1S1 and BC3 generations, the *zm-aa1* probe also hybridized to the two expected internal bands of 2019 bp and 2502 bp due to the 32138 SPT insertion (Table 6, Figure 34). The *DsRed2(Alt1)* probe also hybridized to two bands, one at 4600 bp and a faint one at 6100 bp (Table 6, Figure 35), which were also observed in the T1S1 and BC3 generations (Figure 31). These results indicated that the 32138 SPT insertion remained stable during the typical maize breeding process.

Table 6. Predicted and Observed Hybridizing Bands on Southern Blots with *Bam*H I and Bam*H I*/Hin*d III* Restriction Enzyme Digests

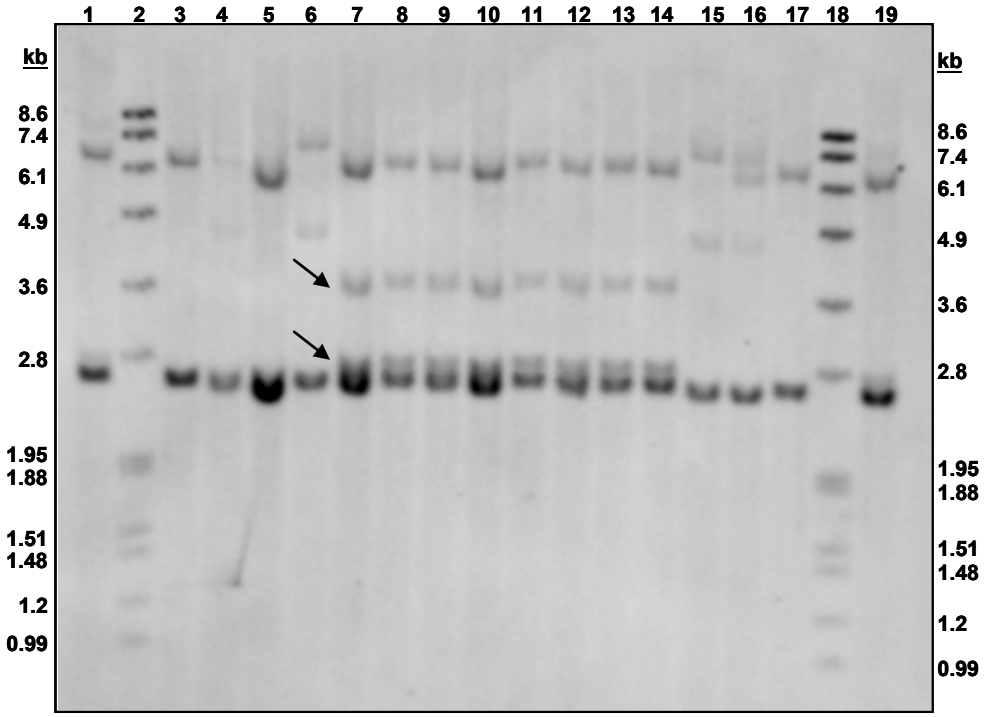
Probe	Restriction Enzyme(s)	Figure	Predicted Fragment Size from PHP24597 T-DNA[1] (bp)	Predicted Fragment Size from Plasmid PHP24597[2] (bp)	Observed Fragment Size[3] in 32138 SPT maintainer (bp)
Ms45	*Bam*H I	29, 33	>1500 (border)[4] 2815	2815 8826	~3800 2815[5]
					~7000* ~2700*
zm-aa1	*Bam*H I	30, 33	2019 2502	2019 2502	2502[5] 2019[5]
					multiple bands >2800*[6] 1-2 bands 1950-2800*[6] 3 bands <1950*[6]
DsRed2(Alt1)	*Bam*H I	30, 34	>1200 (border)	27225	~6100 (faint) ~4600
DsRed2(Alt1)	*Bam*H I/ *Hin*d III	32	>1200 (border)	26045	~2600

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences. These bands were identified in the maize control lines that were analyzed and are not due to the 32138 SPT insertion.

Footnotes:

1. Predicted size for hybridization in genomic DNA samples is based on the map of the T-DNA from PHP24597 (Figure 16). Border fragment sizes are rounded to the nearest 100 bp.
2. Predicted size is based on the plasmid map of PHP24597 (Figure 15).
3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion. Border fragment sizes are rounded to the nearest 100 bp.
5. Observed size is same as expected size due to equivalent migration to the plasmid band.
6. Not all bands present across T1S1, BC3, and BC4 generations. These bands represent a summary of observations in these generations.

Figure 29. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Bam*H I Digest and *Ms45* Probe

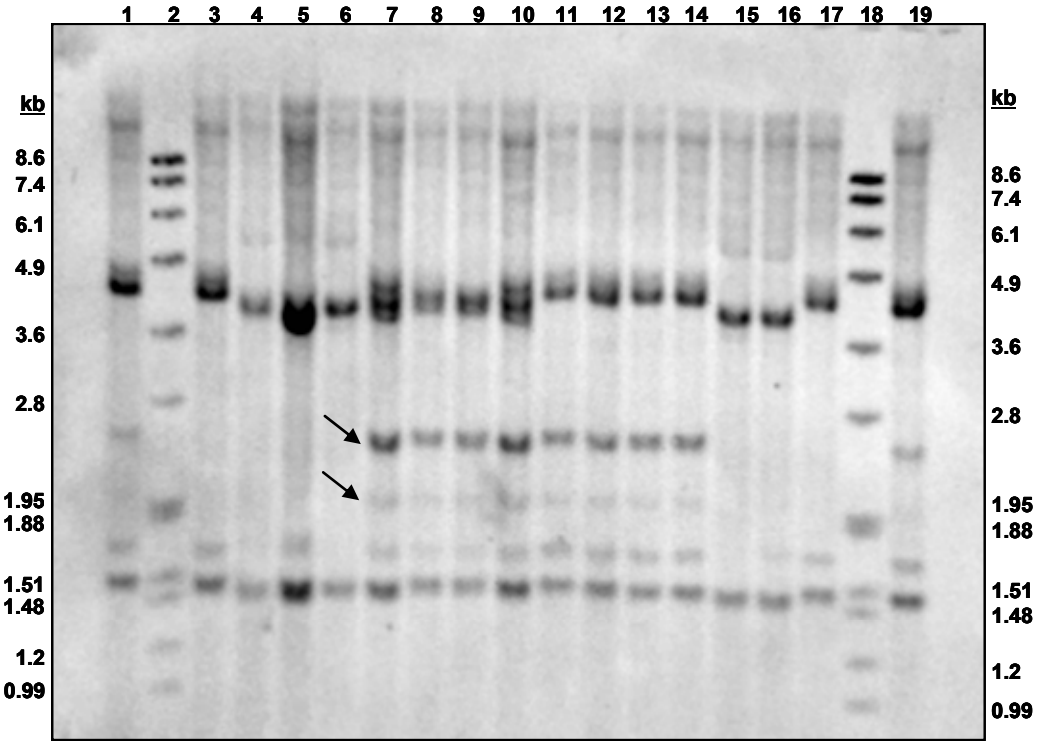


Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer and control maize was digested with *Bam*H I and hybridized to the *Ms45* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 4 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). Arrows indicate the 32138-specific bands.

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	DIGVII marker
3	Inbred 705 control
4	Hi-II(*ms45*) control
5	Hi-II(*ms45*) control
6	Hi-II control
7	32138 SPT maintainer T1S1 (plant 3-Red)
8	32138 SPT maintainer T1S1 (plant 4-Red)
9	32138 SPT maintainer T1S1 (plant 6-Red)
10	32138 SPT maintainer T1S1 (plant 7-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (plant 21-Red)
12	32138 SPT maintainer BC3 (plant 22-Red)
13	32138 SPT maintainer BC3 (plant 23-Red)
14	32138 SPT maintainer BC3 (plant 24-Red)
15	Hi-II control
16	Hi-II(*ms45*) control
17	Inbred 705 control
18	DIGVII marker
19	3 copy PHP24597 + Inbred 705 control

Figure 30. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Bam*H I Digest and *zm-aa1* Probe



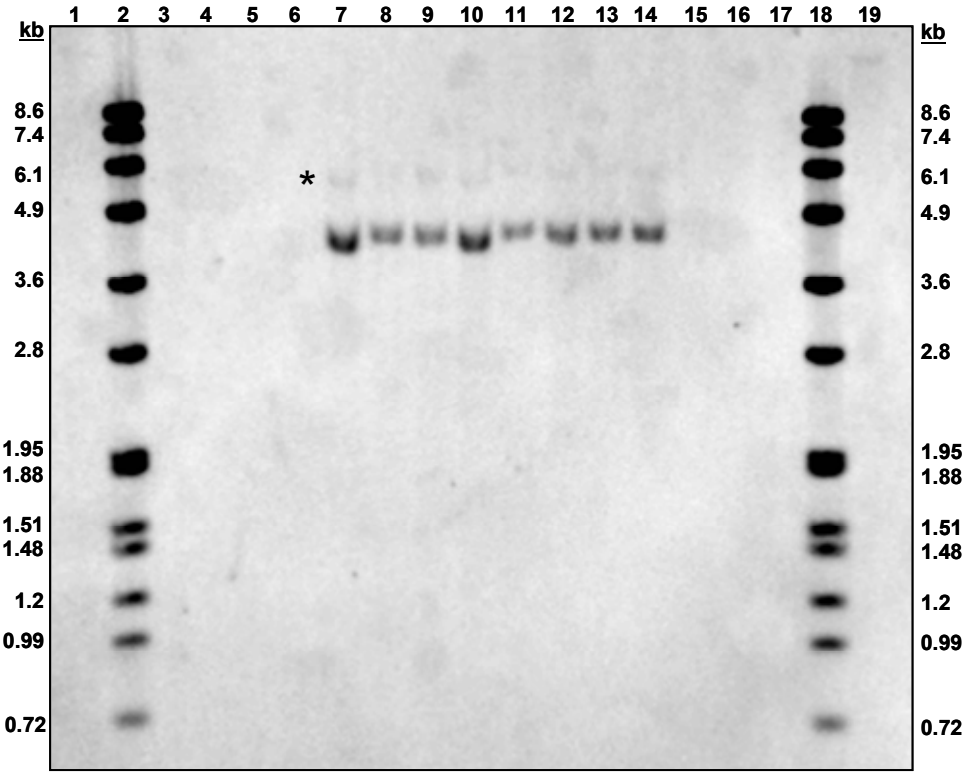
Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer and control maize was digested with *Bam*H I and hybridized to the *zm-aa1* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 4 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). Arrows indicate the 32138-specific bands.

Note: The 2019 bp band expected in both plasmid control and 32138 SPT maintainer samples is faint, but is observed on the original X-ray film.

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	DIGVII marker
3	Inbred 705 control
4	Hi-II(*ms45*) control
5	Hi-II(*ms45*) control
6	Hi-II control
7	32138 SPT maintainer T1S1 (plant 3-Red)
8	32138 SPT maintainer T1S1 (plant 4-Red)
9	32138 SPT maintainer T1S1 (plant 6-Red)
10	32138 SPT maintainer T1S1 (plant 7-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (plant 21-Red)
12	32138 SPT maintainer BC3 (plant 22-Red)
13	32138 SPT maintainer BC3 (plant 23-Red)
14	32138 SPT maintainer BC3 (plant 24-Red)
15	Hi-II control
16	Hi-II(*ms45*) control
17	Inbred 705 control
18	DIGVII marker
19	3 copy PHP24597 + Inbred 705 control

Figure 31. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Bam*H I Digest and *DsRed2(Alt1)* Probe



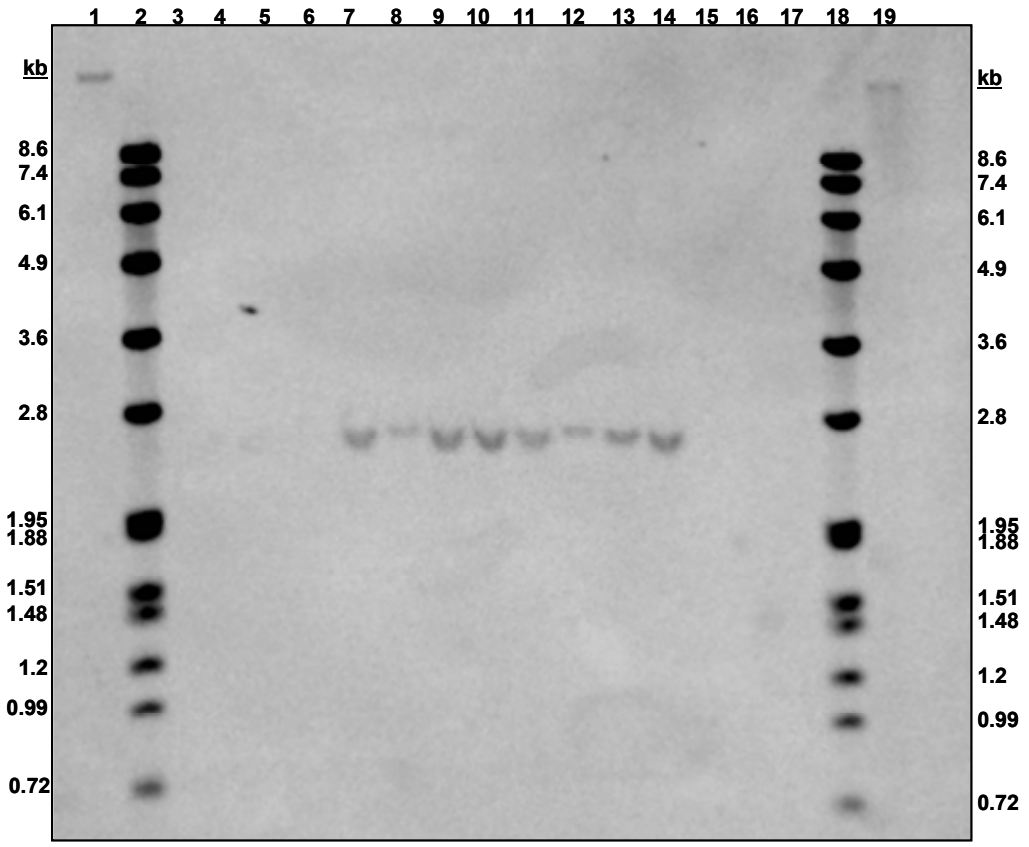
Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer and control maize was digested with *Bam*H I and hybridized to the *DsRed2(Alt1)* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 4 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Note: In lanes 1 and 19, the PHP24597 plasmid band is faint and migrates above the 8.6 kb marker. This band is observed on the original X-ray film. An asterisk indicates the faint band observed at ~6100 bp.

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	DIGVII marker
3	Inbred 705 control
4	Hi-II(*ms45*) control
5	Hi-II(*ms45*) control
6	Hi-II control
7	32138 SPT maintainer T1S1 (plant 3-Red)
8	32138 SPT maintainer T1S1 (plant 4-Red)
9	32138 SPT maintainer T1S1 (plant 5-Red)
10	32138 SPT maintainer T1S1 (plant 6-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (plant 21-Red)
12	32138 SPT maintainer BC3 (T22-Red)
13	32138 SPT maintainer BC3 (T23-Red)
14	32138 SPT maintainer BC3 (T24-Red)
15	Hi-II control
16	Hi-II(*ms45*) control
17	Inbred 705 control
18	DIGVII marker
19	3 copy PHP24597 + Inbred 705 control

Figure 32. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Bam*H I/*Hin*d III Digest and *DsRed2(Alt1)* Probe

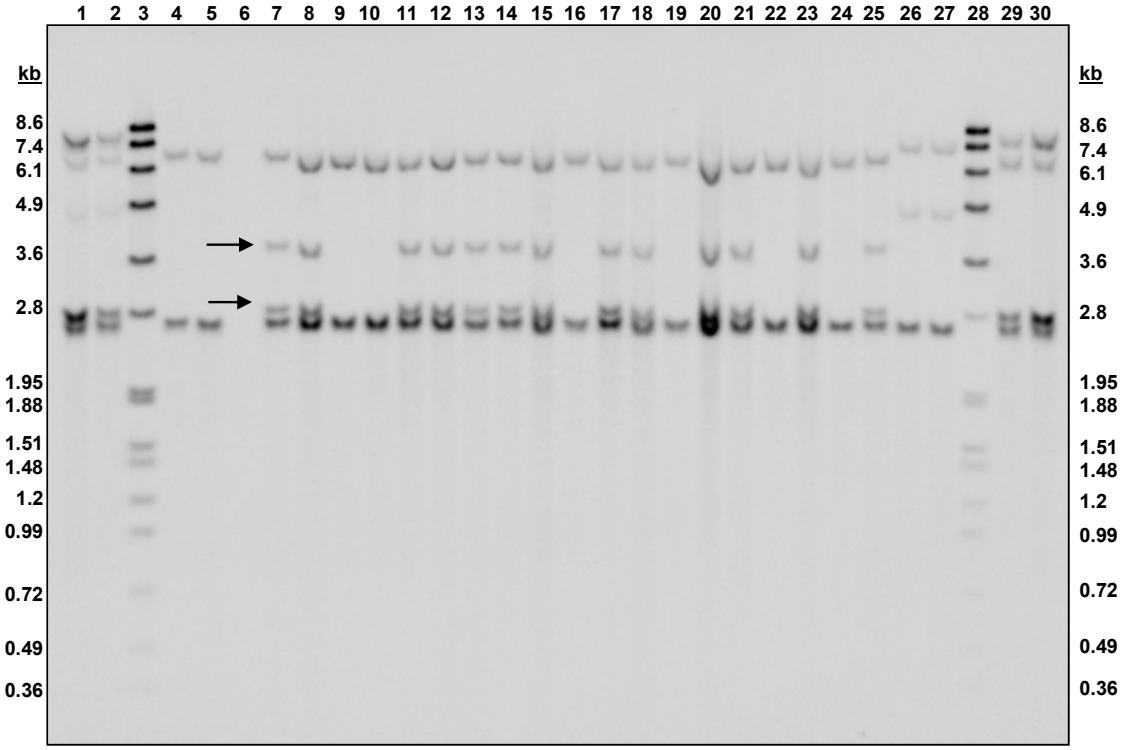


Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer and control maize was digested with *Bam*H I/*Hin*d III and hybridized to the *DsRed2(Alt1)* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 4 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	DIGVII marker
3	Inbred 705 control
4	Hi-II(*ms45*) control
5	Hi-II(*ms45*) control
6	Hi-II control
7	32138 SPT maintainer T1S1 (T3-Red)
8	32138 SPT maintainer T1S1 (T4-Red)
9	32138 SPT maintainer T1S1 (T5-Red)
10	32138 SPT maintainer T1S1 (T6-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (T21-Red)
12	32138 SPT maintainer BC3 (T22-Red)
13	32138 SPT maintainer BC3 (T23-Red)
14	32138 SPT maintainer BC3 (T24-Red)
15	Hi-II control
16	Hi-II(*ms45*) control
17	Inbred 705 control
18	DIGVII marker
19	3 copy PHP24597 + Inbred 705 control

Figure 33. Southern Blot Analysis of the BC4 Generation of 32138 SPT Maintainer: *Bam*H I Digest and *Ms45* Probe

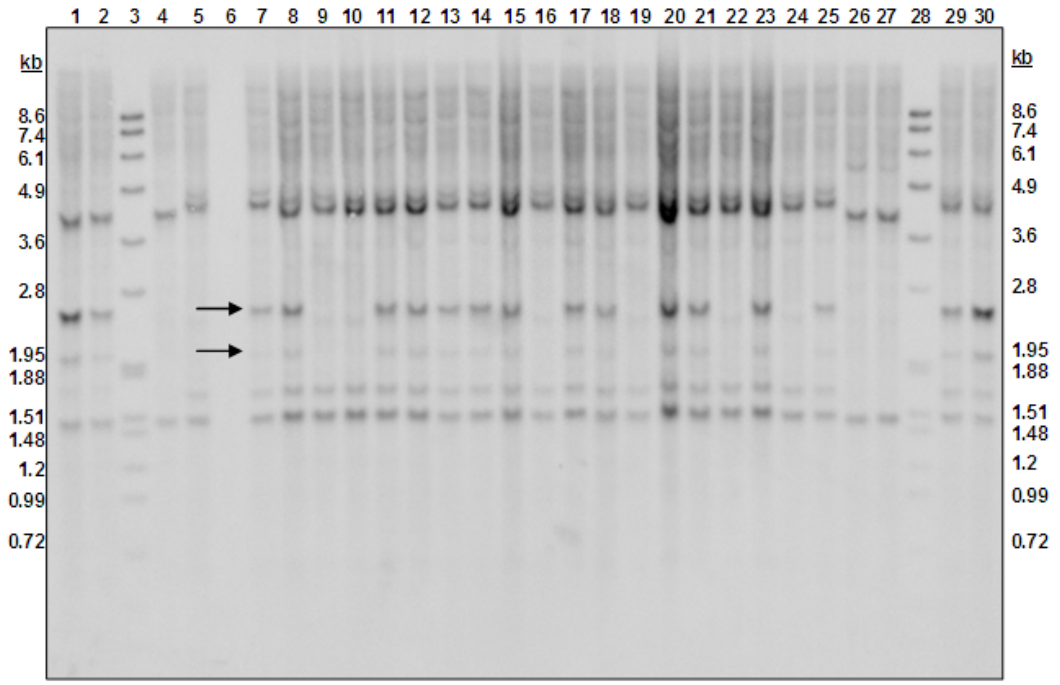


Genomic DNA isolated from leaf tissue of individual plants of the BC4 generation of 32138 SPT maintainer and of the controls was digested with *Bam*H I and hybridized to the *Ms45* probe. The BC4 generation was segregating for the 32138 SPT insertion. Approximately 5 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 5 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). Arrows indicate the 32138-specific bands.

Lane	Sample
1	3 copy PHP24597 + Hi-II(*ms45*) control
2	1 copy PHP24597 + Hi-II(*ms45*) control
3	DIGVII marker
4	Hi-II(*ms45*) control
5	Inbred 705 control
6	blank
7	32138 SPT maintainer BC4 / plant 61
8	32138 SPT maintainer BC4 / plant 62
9	32138 SPT maintainer BC4 / plant 63
10	32138 SPT maintainer BC4 / plant 65
11	32138 SPT maintainer BC4 / plant 66
12	32138 SPT maintainer BC4 / plant 67
13	32138 SPT maintainer BC4 / plant 68
14	32138 SPT maintainer BC4 / plant 69
15	32138 SPT maintainer BC4 / plant 70

Lane	Sample
16	32138 SPT maintainer BC4 / plant 71
17	32138 SPT maintainer BC4 / plant 72
18	32138 SPT maintainer BC4 / plant 73
19	32138 SPT maintainer BC4 / plant 74
20	32138 SPT maintainer BC4 / plant 75
21	32138 SPT maintainer BC4 / plant 76
22	32138 SPT maintainer BC4 / plant 77
23	32138 SPT maintainer BC4 / plant 78
24	32138 SPT maintainer BC4 / plant 79
25	32138 SPT maintainer BC4 / plant 80
26	Hi-II control
27	Hi-II control
28	DIGVII marker
29	1 copy PHP24597 + Inbred 705 control
30	3 copy PHP24597 + Inbred 705 control

Figure 34. Southern Blot Analysis of the BC4 Generation of 32138 SPT Maintainer: *BamH I* Digest and *zm-aa1* Probe



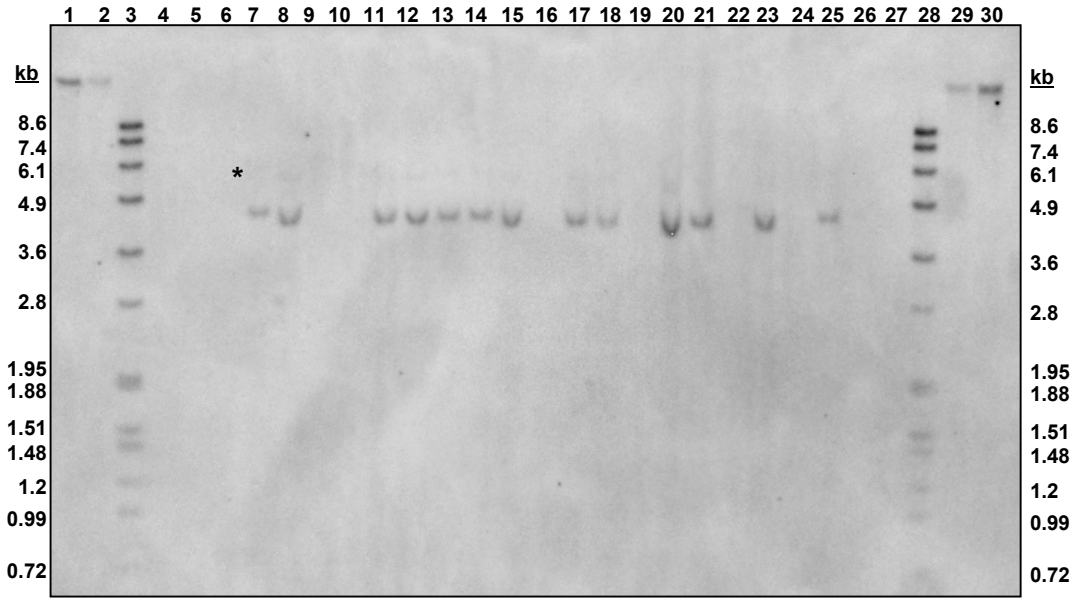
Genomic DNA isolated from leaf tissue of individual plants of the BC4 generation of 32138 SPT maintainer and of the controls was digested with *Bam*H I and hybridized to the *zm-aa1* probe. The BC4 generation was segregating for the 32138 SPT insertion. Approximately 5 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 5 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). Arrows indicate the 32138-specific bands.

Note: The 2019 bp band expected in both plasmid control and 32138 SPT maintainer samples is faint in this figure, but was observed on the original X-ray film.

Lane	Sample
1	3 copy PHP24597 + Hi-II(*ms45*) control
2	1 copy PHP24597 + Hi-II(*ms45*) control
3	DIGVII marker
4	Hi-II(*ms45*) control
5	Inbred 705 control
6	Blank
7	32138 SPT maintainer BC4 / plant 61
8	32138 SPT maintainer BC4 / plant 62
9	32138 SPT maintainer BC4 / plant 63
10	32138 SPT maintainer BC4 / plant 65
11	32138 SPT maintainer BC4 / plant 66
12	32138 SPT maintainer BC4 / plant 67
13	32138 SPT maintainer BC4 / plant 68
14	32138 SPT maintainer BC4 / plant 69
15	32138 SPT maintainer BC4 / plant 70

Lane	Sample
16	32138 SPT maintainer BC4 / plant 71
17	32138 SPT maintainer BC4 / plant 72
18	32138 SPT maintainer BC4 / plant 73
19	32138 SPT maintainer BC4 / plant 74
20	32138 SPT maintainer BC4 / plant 75
21	32138 SPT maintainer BC4 / plant 76
22	32138 SPT maintainer BC4 / plant 77
23	32138 SPT maintainer BC4 / plant 78
24	32138 SPT maintainer BC4 / plant 79
25	32138 SPT maintainer BC4 / plant 80
26	Hi-II control
27	Hi-II control
28	DIGVII marker
29	1 copy PHP24597 + Inbred 705 control
30	3 copy PHP24597 + Inbred 705 control

Figure 35. Southern Blot Analysis of the BC4 Generation of 32138 SPT Maintainer: *Bam*H I Digest and *DsRed2(Alt1)* Probe



Genomic DNA isolated from leaf tissue of individual plants of the BC4 generation of 32138 SPT maintainer and of the controls was digested with *Bam*H I and hybridized to the *DsRed2(Alt1)* probe. The BC4 generation was segregating for the 32138 SPT insertion. Approximately 5 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 5 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Note: An asterisk indicates the faint band observed at ~6100 bp.

Lane	Sample
1	3 copy PHP24597 + Hi-II(*ms45*) control
2	1 copy PHP24597 + Hi-II(*ms45*) control
3	DIGVII marker
4	Hi-II(*ms45*) control
5	Inbred 705 control
6	Blank
7	32138 SPT maintainer BC4 / plant 61
8	32138 SPT maintainer BC4 / plant 62
9	32138 SPT maintainer BC4 / plant 63
10	32138 SPT maintainer BC4 / plant 65
11	32138 SPT maintainer BC4 / plant 66
12	32138 SPT maintainer BC4 / plant 67
13	32138 SPT maintainer BC4 / plant 68
14	32138 SPT maintainer BC4 / plant 69
15	32138 SPT maintainer BC4 / plant 70

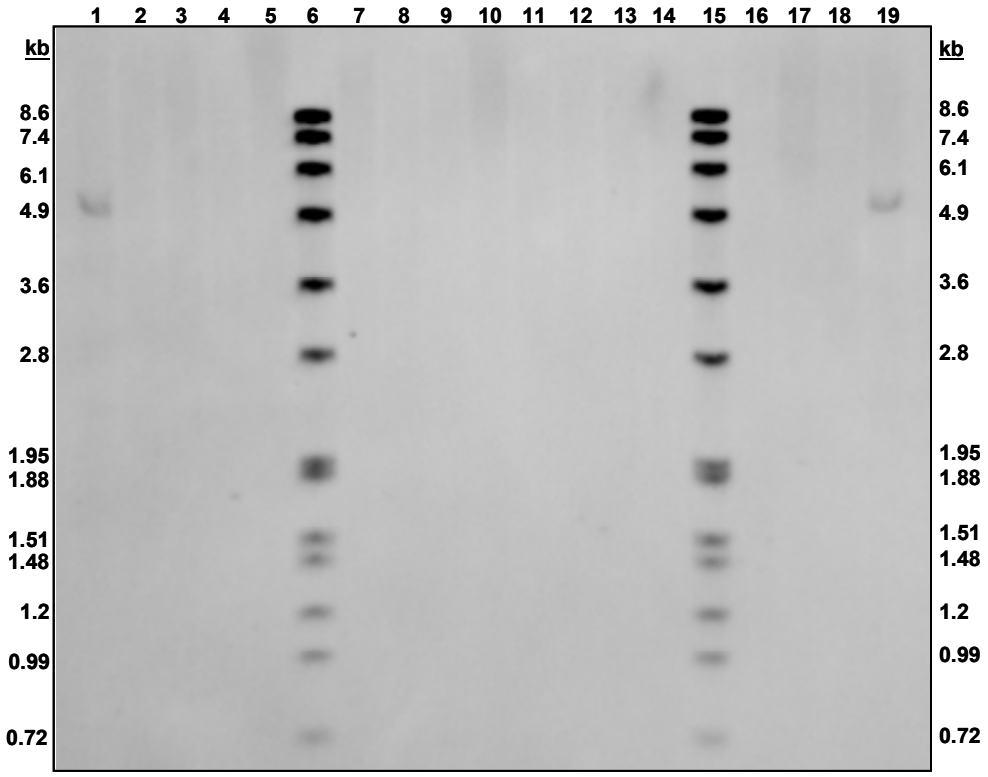
Lane	Sample
16	32138 SPT maintainer BC4 / plant 71
	32138 SPT maintainer BC4 / plant 72
18	32138 SPT maintainer BC4 / plant 73
19	32138 SPT maintainer BC4 / plant 74
20	32138 SPT maintainer BC4 / plant 75
21	32138 SPT maintainer BC4 / plant 76
22	32138 SPT maintainer BC4 / plant 77
23	32138 SPT maintainer BC4 / plant 78
24	32138 SPT maintainer BC4 / plant 79
25	32138 SPT maintainer BC4 / plant 80
26	Hi-II control
27	Hi-II control
28	DIGVII marker
29	1 copy PHP24597 + Inbred 705 control
30	3 copy PHP24597 + Inbred 705 control

V-D. Plasmid Backbone DNA Analysis

Both the T1S1 and BC3 generations of 32138 SPT maintainer were analyzed by Southern blot analysis for plasmid sequences from the PHP24597 plasmid backbone. Probes for genes and regions on the PHP24597 plasmid backbone, *i.e.* outside of the T-DNA region were used to determine if any plasmid backbone was inserted into 32138 SPT maintainer as a result of transformation. The *spc*, *tet,* and *virG* probes hybridize to the spectinomycin resistance, tetracycline resistance, and *virG* genes, respectively (Table 3, Figure 15). The LB probe hybridizes to the region outside the Left T-DNA Border, and the RB probe hybridizes to the region outside the Right Border of the T-DNA of PHP24597 (Table 3, Figure 15). These probes would confirm if any region of the backbone outside the T-DNA was transferred into 32138 SPT maintainer. Based on the analysis as described below, 32138 SPT maintainer does not contain plasmid backbone sequences from PHP24597.

Genomic DNA from individuals of the T1S1 and BC3 generations was digested with *Eco*R I and hybridized to the backbone probes described above. For each of the probes, no hybridization to any of the 32138 SPT maintainer individuals was observed (Figures 36 through 38). In each case, the plasmid positive control hybridized to the probe (Figures 36 through 40, Lanes 1 and 19), indicating successful hybridization of the probes. The lack of hybridization in 32138 SPT maintainer confirms that the backbone of plasmid PHP24597 was not inserted during transformation.

Figure 36. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Eco*R I Digest and *spc* Probe

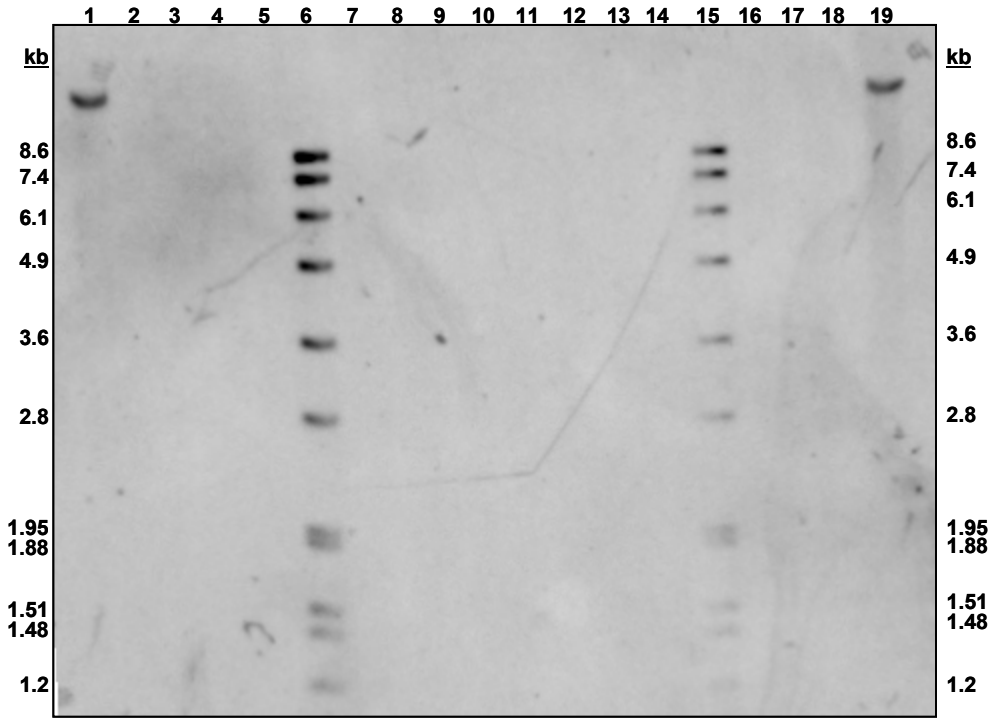


Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer and control maize lines was digested with *Eco*R I and hybridized to the *spc* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 4 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	Inbred 705 control
3	Hi-II(*ms45*) control
4	Hi-II(*ms45*) control
5	Hi-II control
6	DIGVII marker
7	32138 SPT maintainer T1S1 (T3-Red)
8	32138 SPT maintainer T1S1 (T4-Red)
9	32138 SPT maintainer T1S1 (T6-Red)
10	32138 SPT maintainer T1S1 (T7-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (T21-Red)
12	32138 SPT maintainer BC3 (T22-Red)
13	32138 SPT maintainer BC3 (T23-Red)
14	32138 SPT maintainer BC3 (T24-Red)
15	DIGVII marker
16	Hi-II control
17	Hi-II(*ms45*) control
18	Inbred 705 control
19	3 copy PHP24597 + Inbred 705 control

Figure 37. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Eco*R I Digest and *tet* Probe



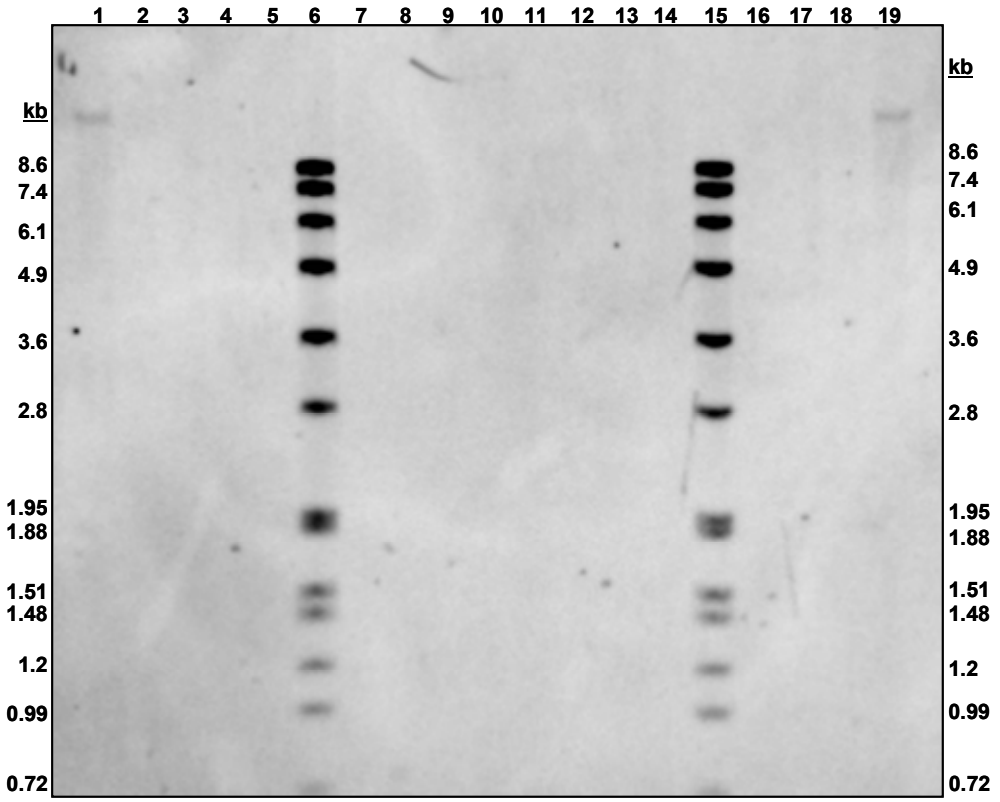
Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer and control maize was digested with *Eco*R I and hybridized to the *tet* probe. Approximately 3 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 3 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Note: The lines across Lanes 4 through 6 and Lanes 7 through 14 are due to scratches on the blot membrane.

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	Inbred 705 control
3	Hi-II(*ms45*) control
4	Hi-II(*ms45*) control
5	Hi-II control
6	DIGVII marker
7	32138 SPT maintainer T1S1 (plant 3-Red)
8	32138 SPT maintainer T1S1 (plant 4-Red)
9	32138 SPT maintainer T1S1 (plant 5-Red)
10	32138 SPT maintainer T1S1 (plant 6-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (plant 21-Red)
12	32138 SPT maintainer BC3 (plant 22-Red)
13	32138 SPT maintainer BC3 (plant 23-Red)
14	32138 SPT maintainer BC3 (plant 24-Red)
15	DIGVII marker
16	Hi-II control
17	Hi-II(*ms45*) control
18	Inbred 705 control
19	3 copy PHP24597 + Inbred 705 control

Figure 38. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Eco*R I Digest and *virG* Probe

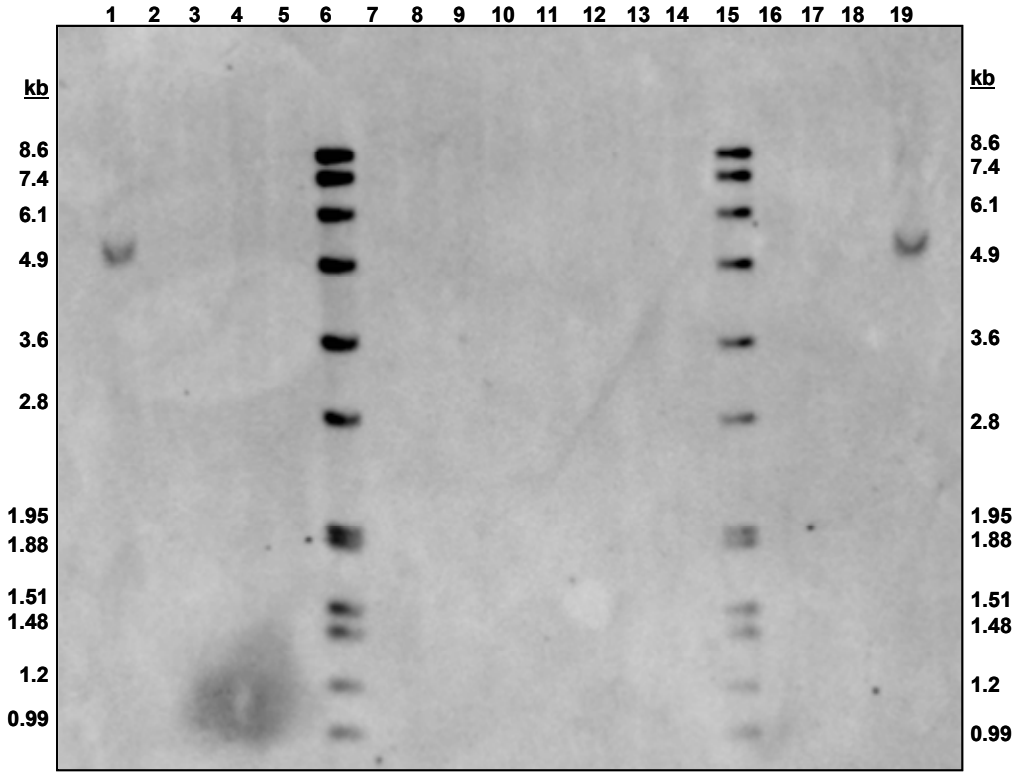


Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer and control maize was digested with *Eco*R I and hybridized to the *virG* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 4 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	Inbred 705 control
3	Hi-II(*ms45*) control
4	Hi-II(*ms45*) control
5	Hi-II control
6	DIGVII marker
7	32138 SPT maintainer T1S1 (plant 3-Red)
8	32138 SPT maintainer T1S1 (plant 4-Red)
9	32138 SPT maintainer T1S1 (plant 6-Red)
10	32138 SPT maintainer T1S1 (plant 7-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (plant 21-Red)
12	32138 SPT maintainer BC3 (plant 22-Red)
13	32138 SPT maintainer BC3 (plant 23-Red)
14	32138 SPT maintainer BC3 (plant 24-Red)
15	DIGVII marker
16	Hi-II control
17	Hi-II(*ms45*) control
18	Inbred 705 control
19	3 copy PHP24597 + Inbred 705 control

Figure 39. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Eco*R I Digest and LB Probe



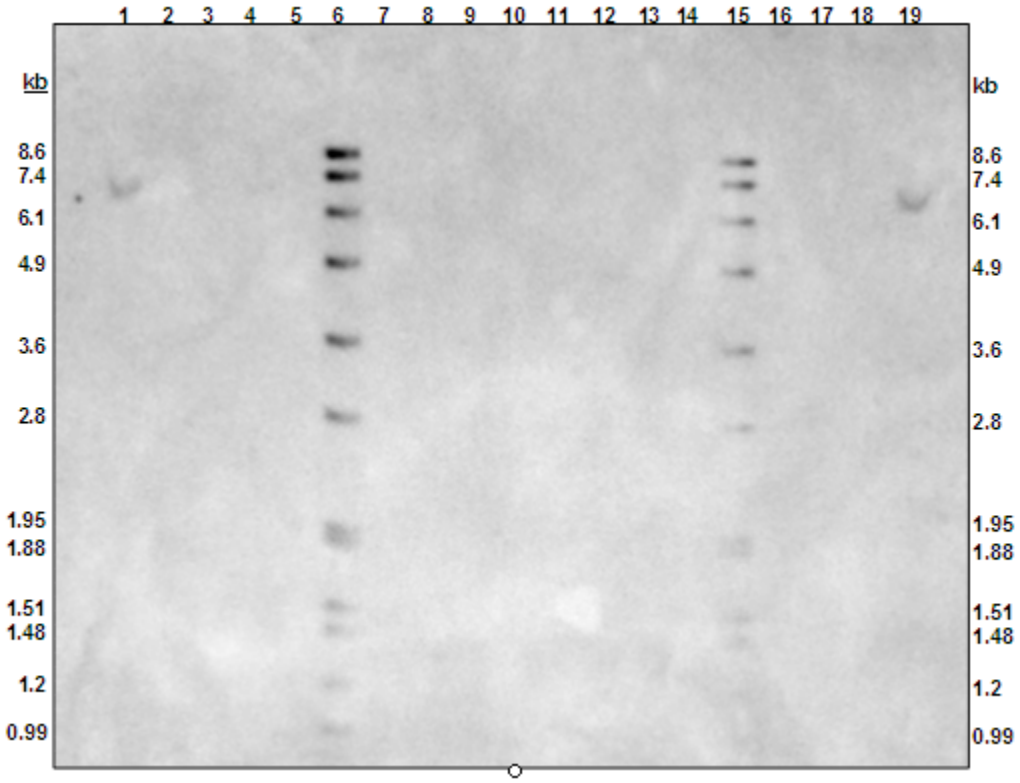
Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer control maize lines was digested with *Eco*R I and hybridized to the LB probe. Approximately 3 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 3 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Note: A dark spot across Lanes 3 through 5 is an artifact of the blot hybridization and not due to specific hybridization.

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	Inbred 705 control
3	Hi-II(*ms45*) control
4	Hi-II(*ms45*) control
5	Hi-II control
6	DIGVII marker
7	32138 SPT maintainer T1S1 (plant 3-Red)
8	32138 SPT maintainer T1S1 (plant 4-Red)
9	32138 SPT maintainer T1S1 (plant 5-Red)
10	32138 SPT maintainer T1S1 (plant 6-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (plant 21-Red)
12	32138 SPT maintainer BC3 (plant 22-Red)
13	32138 SPT maintainer BC3 (plant 23-Red)
14	32138 SPT maintainer BC3 (plant 24-Red)
15	DIGVII marker
16	Hi-II control
17	Hi-II(*ms45*) control
18	Inbred 705 control
19	3 copy PHP24597 + Inbred 705 control

Figure 40. Southern Blot Analysis of the T1S1 and BC3 Generations of 32138 SPT Maintainer: *Eco*R I Digest and RB Probe



Genomic DNA isolated from leaf tissue from individual plants grown from pinkish red seed from two generations of 32138 SPT maintainer and control maize was digested with *Eco*R I and hybridized to the RB probe. Approximately 3 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP24597 at the indicated approximate gene copy numbers and 3 µg of control maize DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Lane	Sample
1	3 copy PHP24597 + Inbred 705 control
2	Inbred 705 control
3	Hi-II(*ms45*) control
4	Hi-II(*ms45*) control
5	Hi-II control
6	DIGVII marker
7	32138 SPT maintainer T1S1 (plant 3-Red)
8	32138 SPT maintainer T1S1 (plant 4-Red)
9	32138 SPT maintainer T1S1 (plant 5-Red)
10	32138 SPT maintainer T1S1 (plant 6-Red)

Lane	Sample
11	32138 SPT maintainer BC3 (plant 21-Red)
12	32138 SPT maintainer BC3 (plant 22-Red)
13	32138 SPT maintainer BC3 (plant 23-Red)
14	32138 SPT maintainer BC3 (plant 24-Red)
15	DIGVII marker
16	Hi-II control
17	Hi-II(*ms45*) control
18	Inbred 705 control
19	3 copy PHP24597 + Inbred 705 control

V-E. Inheritance of the Traits in 32138 SPT Maintainer

Chi-square analysis of trait inheritance data from three different generations (BC3S2F1, BC3S4, and BC4) was performed to determine the heritability and stability of the insertion in 32138 SPT maintainer. The breeding history of the three generations evaluated for Mendelian inheritance is described in Figure 12. The BC3S2F1, BC3S4, and BC4 generations of 32138 SPT maintainer were expected to segregate 1:1 for the trait. Stability and inheritance of the insertion in 32138 SPT maintainer was evaluated using phenotypic and genotypic analyses.

Seed from BC3S2F1, BC3S4, and BC4 generations were visually sorted for kernel color (red versus yellow) phenotype using a Leica[3] KL 2500 LCD halogen cold light source. The red kernel phenotype is imparted due to the expression of *DsRed2*(*Alt1)* gene and the corresponding protein DsRed2. Yellow kernel color confirms the absence of the *DsRed2*(*Alt1)* gene expression. As described in Sections V-B and V-C, Southern blot analysis confirmed the integrity and stability of the T-DNA insertion in 32138 SPT maintainer, and thus the evaluation of the *DsRed2(Alt1)* gene expression also correlates to the presence of the intact T-DNA.

Color sorted seed were planted separately and all the plants that emerged were analyzed for the presence of the 32138 SPT insertion (scored as positive) or absence of the insertion (scored as negative) using event-specific Polymerase Chain Reaction (PCR). Results of phenotypic and genotypic analyses are summarized in Tables 7a and 7b, respectively. Upon comparison of phenotypic and genotypic analysis, the results of the phenotypic analysis for each individual plant corresponded to the event-specific PCR results with one exception. One event-specific PCR negative plant from the BC3S2F1 generation was scored as a red kernel (Table 7b). This is ascribed to an error in the color assignment during the manual color sorting process as the negative PCR result was confirmed by three replicate samples.

To confirm that the insertion in 32138 SPT maintainer segregated according to Mendel's laws of genetics, chi-square analysis was performed on the observed and the expected trait segregation ratios based on the results from the phenotypic and genotypic evaluation (Tables 7a and 7b). All P-values were greater than 0.05, indicating no statistically significant differences between the observed and the expected frequencies for inheritance of the trait in three generations of 32138 SPT maintainer, confirming Mendelian inheritance of the introduced trait. Details of the statistical methodology can be found in Appendix 11.

In summary, the Mendelian inheritance and the stability of the insertion have been demonstrated in three generations of self- and cross- pollinated 32138 SPT maintainer. These results are consistent with the Southern blot analysis (Sections V-B and V-C) that confirmed insertion of a single intact copy of the T-DNA at a single locus in 32138 SPT maintainer.

[3] Registered trademark of Schott Corporation

Table 7a. Comparison of Observed and Expected Segregation Ratios for 32138 SPT Maintainer Based on Phenotypic Analysis (Seed Color)

Generation	# of Seeds Analyzed	Seed Color				Chi-Square
		Observed		Expected		
		Red	Yellow	Red	Yellow	P-Value
BC3S2F1	260	127	133	130	130	0.7098
BC3S4	275	136	139	137.5	137.5	0.8564
BC4	100	52	48	50	50	0.6892

Table 7b. Comparison of Observed and Expected Segregation Ratios for 32138 SPT Maintainer Based on Genotypic Analysis (Event-Specific PCR)

Generation	# of Plants Emerged[1]		Event-Specific PCR Results				Chi-Square
	Pinkish Red Seed	Yellow Seed	Observed		Expected		
			Positive	Negative	Positive	Negative	P-Value
BC3S2F1	126	130	125[2]	131[2]	128	128	0.7077
BC3S4	127	135	127	135	131	131	0.6211
BC4	52	48	52	48	50	50	0.6892

Footnotes:

1. All red and yellow seed (from Table 7a) were planted. The numbers of plants in this column represent actual numbers of red and yellow plants that emerged.
2. Numbers do not correlate with the number of plants that emerged. This is ascribed to an error in the color assignment during the visual color sorting process as the negative PCR result was confirmed by three replicate samples.

V-F. Molecular Characterization of 32138 SPT Maintainer: Summary and Conclusions

Southern blot analysis was conducted to characterize the DNA insertion in 32138 SPT maintainer. The analysis confirmed that a single, intact PHP24597 T-DNA was inserted into the maize genome to produce 32138 SPT maintainer. A single copy of each of the *Ms45*, *zm-aa1*, and *DsRed2(Alt1)* expression cassettes was present and the integrity of the PHP24597 T-DNA was maintained. The analysis confirmed the stability of the insertion in 32138 SPT maintainer across the T1S1, BC3, and BC4 generations, thus confirming stability of inheritance during traditional breeding procedures. In addition, Southern blot analysis verified the absence of plasmid backbone sequences in 32138 SPT maintainer.

Chi-square analysis of trait inheritance data from three different generations (BC3S2F1, BC3S4, and BC4) was performed to determine the heritability and stability of the insertion in 32138 SPT maintainer. This analysis confirmed the Mendelian inheritance of the 32138 SPT insertion.

VI. Characterization of the Introduced Proteins

VI-A. The MS45 Protein

VI-A.1. Identity of the MS45 Protein

The *Ms45* gene, one of the many nuclear fertility genes identified in maize that contribute to the male-fertility phenotype, and the gene has been isolated using an activator-transposon tagging approach (Albertsen *et al*., 1993). *Ms45* is an endogenous maize gene, tightly expressed at particular developmental stages in anthers. Expression of the MS45 protein encoded by the *Ms45* gene in the anther tapetum is required for the production of fertile pollen by the maize plant (Cigan *et al*., 2001). The full-length MS45 protein is comprised of 412 amino acids and has a molecular weight of approximately 47 kDa (Figure 41).

Figure 41. Deduced Amino Acid Sequence of the Full-Length MS45 Protein

```
  1     MEKRNLQWRR GRDGIVQYPH LFFAALALAL LVADPFGLSP LAEVDYRPVK
 51     HELAPYGEVM GSWPRDNASR LRRGRLEFVG EVFGPESIEF DLQGRGPYAG
101     LADGRVVRWM GEEAGWETFA VMNPDWSEEV CANGVNSTTR KQHEKEEFCG
151     RPLGLRFHGE TGELYVADAY YGLMVVGQSG GVASSVAREA DGDPIRFAND
201     LDVHRNGSVF FTDTSMRYSR KDHLNILLEG EGTGRLLRYD PETSGVHVVL
251     KGLVFPNGVQ ISEDHQFLLF SETTNCRIMR YWLEGPRAGE VEVFANLPGF
301     PDNVRSNGRG QFWVAIDCCR TPAQEVFAKR PWLRTLYFKF PLSLKVLTWK
351     AARRMHTVLA LLDGEGRVVE VLEDRGHEVM KLVSEVREVG RKLWIGTVAH
401     NHIATIPYPL ED
```

VI-A.2. Stage-Specific Expression of MS45 Protein during Pollen Development

In order to evaluate and further confirm the anther-specific and stage-specific expression of the MS45 protein, anther samples from conventional maize inbred 705 were collected at targeted stages of development between the Pollen Mother Cell (PMC) and binucleate pollen and analyzed by western blot analysis (Figure 42A). As expected, the maximum expression of MS45 protein was observed during quartet (tetrad) to uninucleate stages (Figure 42A, lanes 5-10). A bacterially produced non-glycosylated form of MS45 protein was used as a reference standard protein (Figure 42A, lanes 2-4) and migrated at a slightly lower molecular weight on SDS-PAGE gels than the endogenous maize MS45 protein. The shift in the expected molecular weight (47 kDa) of the endogenous MS45 protein in anther tissues was confirmed to be due to glycosylation as shown in Figure 42B.

VI-A.3. Intended Function of *Ms45* Gene in the 32138 SPT Maintainer

The *Ms45* gene from the 32138 SPT insertion complements the *ms45* male-sterile allele and restores fertility in the transgenic 32138 SPT maintainer. Male-sterile female inbred plants are homozygous for *ms45* (*i.e., ms45/ms45*) and produce no pollen (Figure 7A). Male-fertility can be restored by a single copy of the *Ms45* gene transformed into a homozygous recessive male-sterile plant (*ms45/ms45*). Hence, transformation of the *ms45/ms45* genotype with the 32138 SPT insertion containing a single copy of the *Ms45* gene restores fertility (Figure 7B) in the transgenic 32138 SPT maintainer.

Figure 42A. Western Blot Showing Anther and Stage-Specific Expression of MS45 Protein during Pollen Development. Anther samples from maize inbred line 705 were collected and analyzed as described in Appendix 9.2-9.5. MWM=Molecular weight marker.

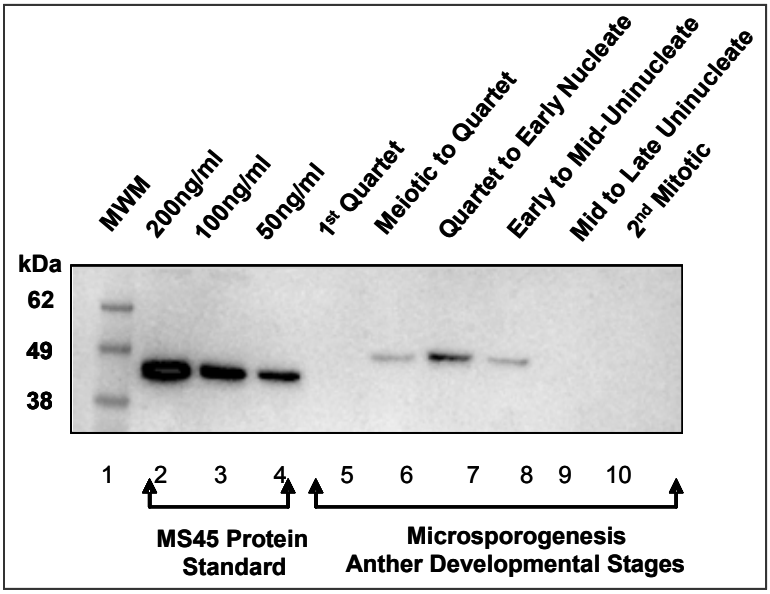
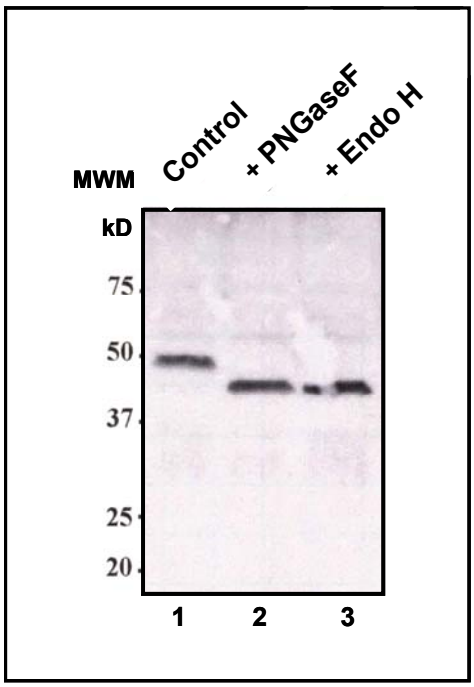


Figure 42B. Western Blot Showing Endogenous Glycosylation of MS45 Protein in Anthers. Approximately 18 anthers were extracted in 0.2 ml buffer containing 0.5% SDS and 1% ß-mercaptoethanol. The extract was centrifuged and the protein extract was incubated with either the extraction buffer (Control), or 500 units each of N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) (New England Biolabs, Inc.) at 37°C for 2 h. A shift in the mobility due to the endogenous glycosylation of MS45 protein was examined by Western blotting using an MS45 polyclonal antibody R3976. MWM=Molecular Weight Marker



VI-B. The ZM-AA1 Protein

VI-B.1. Identity of the ZM-AA1 Protein

The endogenous *zm-aa1* gene that encodes ZM-AA1 maize α-amylase protein (EC 3.2.1.1) is expressed predominantly in the scutellum of germinating seed and minimal expression is observed in the developing endosperm. No expression of the endogenous *zm-aa1* gene is detected in pollen (internal data not shown). The *zm-aa1* sequence in plasmid PHP24597 was isolated from a maize cDNA library prepared from combined tissue of the developing embryo and endosperm at ten days after pollination. The *zm-aa1* gene sequence was truncated to remove the native N-terminal transit peptide sequence and was replaced by sequence from the *zm-bt1* gene *(brittle-1)* encoding the Brittle-1 transit peptide for targeting the ZM-AA1 protein to amyloplasts (Sullivan *et al*., 1991). Figure 43 shows the deduced amino acid sequence from translation of the *zm-bt1* transit peptide sequence linked to the *zm-aa1* gene region from plasmid PHP24597. The complete translation product, including the transit peptide (underlined region) is 495 amino acids in length and has a molecular weight of approximately 54 kDa. Following processing of the Brittle-1 transit peptide the primary ZM-AA1 protein observed is 445 amino acids in length and has a molecular weight of approximately 49 kDa. The observed 445 amino acid ZM-AA1 protein in 32138 SPT maintainer is comprised of maize endogenous protein sequences from the Brittle-1 transit peptide and the ZM-AA1 protein.

Figure 43. Deduced Amino Acid Sequence of the ZM-AA1 Protein

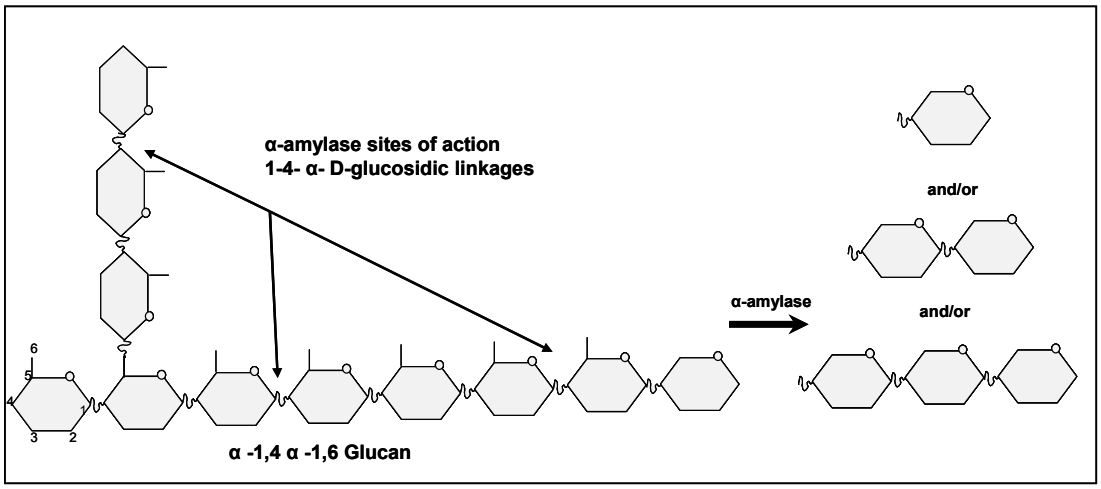
```
  1   MAATMAVTTM VTRSKESWSS LQVPAVAFPW KPRGGKTGGL EFPRRAMFAS↓
 51   VGLNVCPGVP AGRDPREPDP KVVRAACGLV QAQVLFQGFN WESCKQQGGW
101   YNRLKAQVDD IAKAGVTHVW LPPPSHSVSP QGYMPGRLYD LDASKYGTAA
151   ELKSLIAAFH GRGVQCVADI VINHRCAEKK DARGVYCIFE GGTPDDRLDW
201   GPGMICSDDT QYSDGTGHRD TGEGFAAAPD IDHLNPRVQR ELSAWLNWLR
251   SDAVGFDGWR LDFAKGYSPA VARMYVESTG PPSFVVAEIW NSLSYSGDGK
301   PAPNQDQCRQ ELLDWTRAVG GPAMAFDFPT KGLLQAGVQG ELWRLRDSSG
351   NAAGLIGWAP EKAVTFVDNH DTGSTQKLWP FPSDKVMQGY AYILTHPGVP
401   CIFYDHMFDW NLKQEISTLS AIRARNGIRA GSKLRILVAD ADAYVAVVDE
451   KVMVKIGTRY GVSSVVPSDF HPAAHGKDYC VWEKASLRVP AGRHL
```

The underlined region represents the Brittle-1 transit peptide (75 amino acids). The arrow (↓) following the serine residue at position 50 indicates the primary transit peptide processing site observed *in vivo* in 32138 SPT maintainer.

VI-B.2. Intended Function of ZM-AA1 in the 32138 SPT Maintainer

Starch molecules are glucose polymers linked together by α -1,4 and α -1,6 glucosidic bonds (Figure 44). α -amylases belong to a family of glycosyl-hydrolases (1,4-α-D-Glucan glucanohydrolase; EC 3.2.1.1) catalyzing hydrolysis of (1-4)-α-D-glucosidic linkages in polysaccharide molecules, such as starch. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose, maltose and/or, glucose. Expression of ZM-AA1 in the developing pollen of 32138 SPT maintainer results in the depletion of starch and deprivation of the energy reserves required for pollen germination and fertilization, thereby resulting in pollen infertility.

Figure 44. Illustration of the Mode of Action of α-Amylase



VI-C. The DsRed2 Protein

VI-C.1. Identity of DsRed2 Protein

Red fluorescent protein, known commercially as DsRed, was originally isolated from the coral-like anemone *Discosoma* species by Matz *et al.* (1999), who referred to the protein as drFP583. DsRed2 is a variant of the original red fluorescent protein (DsRed), modified with six point mutations (http://www.clontech.com/products/detail.asp?product_id=157257&tabno=2). These mutations improve the solubility of DsRed2 by reducing its tendency to form aggregates. The DsRed2 protein is encoded by the*DsRed2(Alt1)* gene, a modified version of the original *DsRed2* gene (Wasson-Blader, 2001) in which an internal *Bst*E II restriction enzyme recognition site was removed without altering the amino acid sequence of the expressed protein. Figure 45 below shows the deduced amino acid sequence from translation of the *DsRed2(Alt1)* gene from plasmid PHP24597. The DsRed2 protein is 225 amino acids in length with an approximate molecular weight of 26 kDa.

Figure 45. Deduced Amino Acid Sequence of the DsRed2 Protein

```
  1     MASSENVITE FMRFKVRMEG TVNGHEFEIE GEGEGRPYEG HNTVKLKVTK
 51     GGPLPFAWDI LSPQFQYGSK VYVKHPADIP DYKKLSFPEG FKWERVMNFE
101     DGGVATVTQD SSLQDGCFIY KVKFIGVNFP SDGPVMQKKT MGWEASTERL
151     YPRDGVLKGE THKALKLKDG GHYLVEFKSI YMAKKPVQLP GYYYVDAKLD
201     ITSHNEDYTI VEQYERTEGR HHLFL
```

VI-C.2. Intended Function of DsRed2 Protein in the 32138 SPT Maintainer

The DsRed2 protein belongs to a family of red fluorescent proteins which are members of a larger group of proteins in several *Anthozoa* species (this class includes corals, anemones and sea pens). The unique feature of fluorescent proteins is their ability to enter an excited state and emit light of a certain wavelength (*i.e.* fluoresce) upon absorption of ultraviolet or visible light. Application of the DsRed protein as a visual selection marker for high-throughput seed sorting has been previously demonstrated in *Arabidopsis* (Stuitje *et al.*, 2003). The DsRed2 protein provides a very effective means of tracking transgenic maize seed expressing this protein and is used as a quality assurance color marker that allows monitoring of seeds to ensure seed purity.

VI-D. Expression of MS45 in 32138 SPT Maintainer

The 32138 SPT maintainer contains the *Ms45, zm-aa1* and *DsRed2(Alt1)* genes encoding the MS45, ZM-AA1 and DsRed2 proteins, respectively. The transcription of the *Ms45* gene is under the control of the maize anther-specific *5126* promoter (Cigan and Albertsen, 1997). A quantitative enzyme linked immunosorbent assay (ELISA) for the MS45 protein was not developed due to marginal solubility of MS45 in the compatible ELISA buffers. Hence, the MS45 protein was analyzed using Western blot analysis.

In order to facilitate collection of anther samples at the targeted developmental stages (Chang and Neuffer, 1989), greenhouse tissue samples from BC1S2 (Figure 12) generation were collected and used for Western blot analysis of the MS45 protein. A non-transgenic male-sterile (*ms45/ms45*) female inbred derived from transgenic 32138 SPT maintainer was used as a negative control, and a male-fertile inbred 705 was used as a 'reference' (positive control) for MS45 detection. Developing anther samples were collected at the following developmental stages: pollen mother cell, meiosis, quartet to early uninucleate, mid uninucleate, mid to late uninucleate, late uninucleate, binucleate (2^{nd} mitosis) and starch filling (see Figure 8B). The developmental stage of the anthers was determined based on the staging scheme of Chang and Neuffer (1989). Western blot analysis of the MS45 protein utilized two samples of four anthers each for each stage.

As expected, the MS45 protein was only detected in anthers and not in the other tissues analyzed (Figure 46). The MS45 protein was detected in both 32138 SPT maintainer (test) and the male-fertile inbred 705 (reference) during the late meiosis-quartet stage to the early uninucleate stage of microspore development. The anther-expressed MS45 migrated at a higher molecular weight than the microbially expressed protein, due to the observed glycosylation of the endogenous MS45 protein (Figure 42B). MS45 protein was undetectable in any of the negative control male-sterile (*ms45/ms45*) female inbred samples. Some non-specific cross reactivity of MS45 antibodies with other plant proteins at lower molecular weights (~30kDa) was observed in the male-sterile (*ms45/ms45*) female inbred negative control and male-fertile inbred 705 positive control samples. The MS45 protein was not consistently observed in all of the 32138 SPT maintainer and male-fertile 705 materials sampled due to a narrow window of expression for the MS45 protein and also due to the rapid progress of the microspore development from one stage to the next (within hours) making it difficult to obtain a single exact stage of development.

Based on the Western blot analysis, detectable levels of the MS45 protein in both 32138 SPT maintainer and inbred 705 reference line are limited to the anthers.

VI-E. Expression of ZM-AA1 and DsRed2 in 32138 SPT Maintainer

Concentrations of the ZM-AA1 and DsRed2 proteins were measured in tissue samples collected from a replicated field study grown at six field locations in the United States in 2007. Three replicated samples per tissue per location were collected for 32138 SPT maintainer and one sample per tissue per location was collected for the male-fertile inbred 705 control maize. Growth stages indicated are as described by Ritchie *et al.* (1993). Tissue samples from 32138 SPT maintainer and inbred controls were analyzed from the following tissues and growth stages: leaf (V9, R1, R4, R6), whole plant (V9, R1, R6), pollen (R1), forage (R4), and seed (R6). Forage refers to all above ground material of the plant, *i.e.* ears and all vegetative material except the roots. The concentrations of ZM-AA1 and DsRed2 in different tissues of 32138 SPT maintainer were quantified using ELISA and fluorometric assays, respectively (see Appendix 9 for the details of the methodology).

Results of the ZM-AA1 protein concentration measurements are presented in Table 8. The endogenous ZM-AA1 protein was not detected in any of the inbred 705 control tissues (data not presented). In the 32138 SPT maintainer, as expected the ZM-AA1 protein was highest in the pollen (Table 8), with values 16 to 400 fold higher as compared to the other tissues. Low levels of ZM-AA1 protein concentration was detected in the leaf, whole plant, pollen, forage and seed tissues. The tissue expression pattern may be due to the influence of the CaMV35S enhancer (Odell *et al.*, 1988) located immediately downstream of the *zm-aa1* expression cassette (See Figures 14 and 15). Of the different tissues analyzed, the lowest concentrations of ZM-AA1 protein were found in the seed tissue (Table 8), and were approximately 400 fold less than the protein levels measured in pollen. In leaf and whole plant tissue samples, the ZM-AA1 protein concentrations decreased as the plants matured and were below the limit of quantitation by the R6 stage of development.

Results of the DsRed2 protein concentration measurements are presented in Table 9. As expected, the DsRed2 protein concentrations were found to be highest in the 32138 SPT maintainer seed. The DsRed2 was also detected in leaf, whole plant and forage tissues. The DsRed2 protein was below the limit of quantitation in the pollen tissue. This expression pattern may be due to the influence of CaMV35S enhancer located upstream of the *Ltp2* promoter in the *DsRed2(Alt1)* expression cassette (Figures 14 and 15). As expected, the protein concentration of DsRed2 was below the limit of quantitation for all inbred 705 control tissues (data not shown).

These results demonstrate that, in the 32138 SPT maintainer, the MS45 protein is confined to anthers. In addition, the ZM-AA1 protein is detected at the highest concentration in the pollen, and the DsRed2 protein is detected at the highest concentration in the seed tissue.

Figure 46. Western Blot Analysis of the MS45 Protein in Developing Anthers (Panel A & B) and in Different Tissues (Panel C) of the 32138 SPT Maintainer
The expression of MS45 was evaluated in multiple tissues from: a) 32138 SPT maintainer (test), b) non-transgenic male-sterile (*ms45/ms45*) female inbred negative control and c) non-transgenic male-fertile (*Ms45/Ms45)* reference inbred 705. No sample was loaded in the lane marked "Blank" in panel C. See Appendix 9.1-9.5 for details on the sample extraction, SDS-PAGE, and Western Blot analysis.

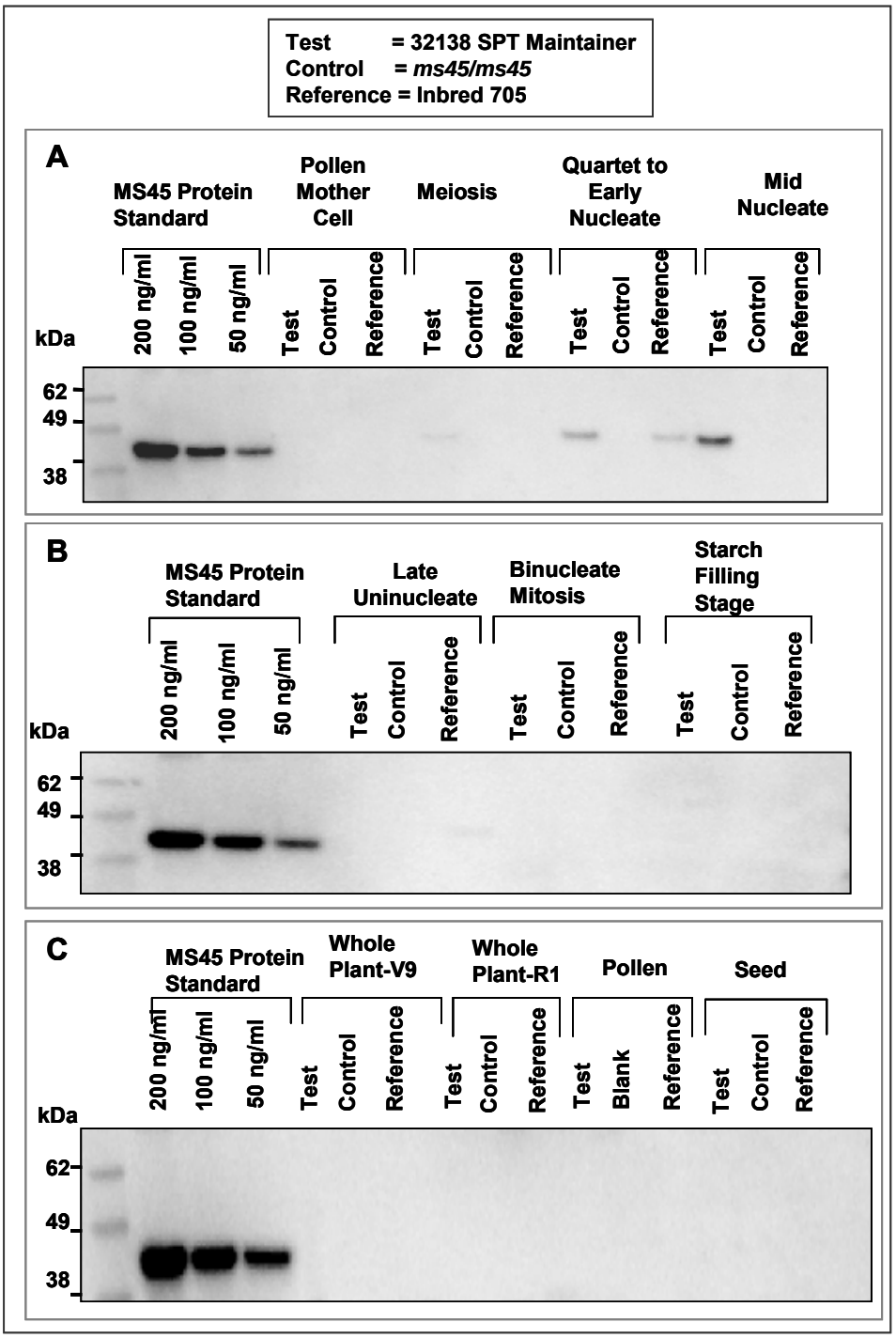


Table 8. Concentration of ZM-AA1 Protein Measured in 32138 SPT Maintainer

Tissue	Growth Stage[1]	ng/mg Tissue Dry Weight			Number of Samples <LLOQ/ Number of samples Analyzed
		LLOQ[2]	Expression in 32138 SPT Maintainer (Mean ± SD)	Range of Expression[3]	
Leaf	V9	0.28	7.3 ± 3.0	2.6 -12.0	0/18
	R1		6.3 ± 2.1	3.0 – 10.0	0/18
	R4		2.8 ± 1.8	<0.28 – 6.6	1/18
	R6		NA[4]	NA[4]	18/18
Whole Plant	V9	0.092	11.0± 3.2	3.4 – 15.0	0/18
	R1		3.8 ± 0.89	2.0- 5.8	0/18
	R6		NA[4]	NA[4]	18/18
Pollen	R1	0.55	180.0 ± 52.0	110.0 - 260.0	0 /13
Forage	R4	0.092	1.7 ± 0.73	0.12 – 3.0	0/18
Seed	R6	0.14	0.44 ± 0.23	0.14 – 0.84	0/18

Footnotes:

1. Ritchie *et al*., 1993
2. Lower Limit of Quantitation (LLOQ) for the assay and tissue type
3. Range denotes the lowest and highest individual value across sites
4. NA=not applicable; all samples below LLOQ

Table 9. Concentration of DsRed2 Protein Measured in 32138 SPT Maintainer

Tissue	Growth Stage[1]	ng/mg Tissue Dry Weight			Number of Samples <LLOQ/ Number of Samples Analyzed
		LLOQ[2]	Expression in 32138 SPT Maintainer (Mean ± SD)	Range of Expression[3]	
Leaf	V9	72	87 ± 15	<72 - 120	2/18
	R1		160 ± 28	110 - 220	0/18
	R4		210 ± 75	<72 - 360	2/18
	R6		NA[4]	NA[4]	18/18
Whole Plant	V9	36	80 ± 9.1	60 - 96	0/18
	R1		77 ± 7.5	64 - 92	0/18
	R6		40 ± 5.7	<36 - 52	8/18
Pollen	R1	72	NA[4]	NA[4]	13/13
Forage	R4	36	60 ± 8.1	44 - 76	0/18
Seed	R6	36	410 ± 110	300 - 770	0/18

Footnotes:

1. Ritchie *et al*., 1993
2. Lower Limit of Quantitation (LLOQ) for the assay and tissue type
3. Range denotes the lowest and highest individual value across sites
4. NA=not applicable; all samples below LLOQ

VI-F. Safety Assessment of the MS45 Protein

The *Ms45* gene is a native maize fertility gene that encodes the MS45 protein. The MS45 protein is normally present in maize in developing anthers (Cigan *et al*., 2001). Expression of MS45 protein in the 32138 SPT maintainer is targeted to the developing anthers where it complements the naturally occurring male-sterile *ms45* mutation in the endogenous *Ms45* gene. As compared to the male-fertile inbred 705, the levels of MS45 protein in 32138 SPT maintainer appear slightly elevated on Western blots (Figure 46). However, neither the endogenous nor the recombinant MS45 protein is expressed in the mature pollen, seed, or any other tissues (Section VI-D). Therefore there is no increase in exposure to the MS45 protein from 32138 SPT maintainer.

Bioinformatic analysis was conducted to evaluate the potential allergenicity of the MS45 protein. The MS45 protein sequence was compared to a database of 1313 known and putative allergen sequences comprising the FARRP8 dataset at the University of Nebraska (updated 1/2008). Potential identities between the MS45 protein sequence and proteins in the allergen database were evaluated with the FASTA34 sequence alignment tool using the default parameters. A 35% or greater identity threshold over any 80 or greater amino acid length sequence between the query protein and an allergen was used to indicate the potential for cross-reactivity (FAO/WHO, 2001; Codex, 2003). No identity matches of ≥ 35% over ≥ 80 residues were observed. In addition, protein sequences were evaluated for any 8 or greater contiguous identical amino acid matches to the allergens contained in the database noted above. There were no 8 or greater contiguous identical amino acid matches observed.

The native MS45 protein was found to be glycosylated (Figure 42B). Based on the similar mobility of MS45 protein from 32138 SPT maintainer and the reference line (Figure 46), it is likely that the MS45 protein in 32138 SPT maintainer is also glycosylated. Based on its glycosylation status the MS45 protein in the 32138 SPT maintainer is no more likely to be an allergen than the native MS45, and there is no risk of increased allergenic potential.

To search for potential similarity to known toxins, the MS45 sequence was queried using the BLASTP 2.2.13 algorithm against Release 165.0 (4/15/08) of the Genpept “nr” dataset, which incorporates non-redundant entries from all Genbank nucleotide translations along with protein sequences from SWISS-PROT (http://www.expasy.org/sprot/), PIR (http://pir.georgetown.edu/), PRF (http://www4.prf.or.jp/en/), and PDB (http://www.wwpdb.org/). Because of the large number of automated conceptual translations derived from genomic sequences, protein sequences from the RefSeq collection (http://www.ncbi.nlm.nih.gov/RefSeq/) were excluded from the search using the filtering statement "protein_all [filter] NOT srcdb_refseq [prop]” to delimit the results returned. A cutoff expectation (*E*) value of 1.0 was used to generate biological meaningful similarity between the proteins of interest and proteins in the NCBI datasets. Although a statistically significant sequence similarity generally requires a match with an expectation value less than 0.01, a cutoff of $E < 1.0$ insures that proteins with even limited similarity will not be overlooked in the search (Pearson, 2000). The scoring matrix used was the default (BLOSUM62), and the number of alignments returned was set to the maximum value (2000).

The MS45 BLASTP search returned 509 protein sequences with an *E* score of less than 1.0. Five of the accessions returned by the searches displayed complete significance ($E = 0$) and represent identical or very closely related male-fertility protein sequences from maize, rice, and wheat. Two hundred and sixty of the remaining 504 accessions represented bacterial, plant, and animal derived sequences described as strictosidine synthases, gluconolactonases, or hemomucins, and 194 of the accessions returned were described as hypothetical, predicted, putative, unknown, or unnamed proteins. The remaining 50 sequence alignments represent five small groups classified as adipocyte plasma membrane-

associated proteins, ABC transporters, diisopropyl fluorophosphatases, senescence marker proteins, or NHL repeat proteins. The functionalities attributed to the aligning accessions were often based upon possession of one or more conserved domains within the aligned sequence. None of the protein sequences returned by the BLASTP search identified safety concerns that might arise from the expression of MS45 in genetically modified plants.

Based on this information, Pioneer determined that the MS45 protein is unlikely to cause an allergic reaction in humans or be toxic in humans or animals.

VI-G. Safety Assessment of the ZM-AA1 Protein

The α-*amylase* expressed in 32138 maintainer is derived from two endogenous maize gene sequences that encode the ZM-AA1 protein, and the Brittle-1 transit peptide required for directing the ZM-AA1 protein into the amyloplasts. Endogenous *zm-aa1* gene is predominantly expressed in the scutellum tissues of the germinating seed and minimally expressed (<0.25% of the levels in the scutellum) in endosperm, stalk, leaf and root (internal data not shown). Expression of the endogenous *zm-aa1* in the scutellum tissues corresponds well with the literature evidence that α-amylases are required during cereal seed germination are required for the hydrolysis of endosperm starch into metabolizable sugars, which provide the energy for the growth of roots and shoots (Akazawa and Hara-Mishimura, 1985; Beck and Ziegler, 1989).

Germinating maize seeds, known as sprouts, are extensively used in Latin America in the production of alcoholic beverages, for example, Chicha (maize beer), Sora, Napú, Fubá, Champuz, Acupe, and Tesgüino (Lorence-Quiñones *et al.*, 1999). Chicha is the most important traditional fermented beverage in Latin America and is made by germinating maize seed to produce amylase required for starch conversion. Chicha has been consumed by the Andean Indians for centuries. Sprouted maize seed has been increasingly used in bread and tortilla products (see, for example, http://www.foodforlife.com) and has been explored as a feed supplement (Naga, 1986). In addition, livestock feeding of maize grain that has sprouted from moisture exposure is an accepted practice (Heiniger, 1999). Therefore, there is a previous history of exposure and safe use of ZM-AA1 α-amylase endogenous to the germinating maize seed.

The α-amylases are ubiquitous enzymes present in many organisms including plants and insects (Campos *et al*., 1989; Raimbaud *et al*., 1989; Silva *et al*., 1999; Cristofoletti *et al.*, 2001). The α-amylases from multiple sources including plants, fungi and bacteria have a long history of safe consumption in foods. Purified microbial α-amylases are commonly used in the modern food processing industry (Pariza and Johnson, 2001) and these enzymes have a long history of safe use. Additionally their safety has been verified by an array of laboratory studies (see, for example, Landry *et al*., 2003).

In plants, α-amylases aid in the hydrolysis of transient starch in the leaves, which occurs during the dark cycle of photosynthesis, and are also required for the hydrolysis of storage starch during seed germination or tuber sprouting. Multiple forms of α-amylases exist in maize, and are expressed in the endosperm and other tissues (Chao and Scandalios, 1971; James *et al.*, 2007). Amylases are reported to occur endogenously in the pollen of numerous plants (Castro and Clement, 2007; Bhadula and Sawhney, 1989), including maize, where starch hydrolysis is associated with pollen germination (Brewbaker, 1971; Agarwala *et al*., 1981; Wakhle *et al*., 1983). A recent work of Castro and Clement (2007) demonstrated that the amylase activity in *Lilium* pollen is attributed, in part, to α-amylase protein.

Maize has a long history of safe food and feed use for humans and animals. Maize is not considered a common allergenic food (Hefle *et al*., 1996; Moneret-Vautrin *et al.,* 1998),

although in a few case studies, allergenic reactions were reported and maize allergens identified (Pastorello *et al.,* 2000, 2003; Pasini *et al.,* 2002; Weichel *et al*., 2006b). The ZM-AA1 protein, however, was not identified as an allergenic protein in maize (Weichel et al., 2006a).

There is only one α-amylase protein currently included in the peer-reviewed FARRP8 database from the Food Allergy Research and Resource Program (FARRP) at the Department of Food Science and Technology at the University of Nebraska at Lincoln (Release 8 - January 2008). It is derived from *Aspergillus oryzae* and has been reported to be one of the most often described causes of allergenic Baker's asthma (Blanco *et al.*, 1991; Houba *et al*., 1998). The ZM-AA1 α-amylase did not show significant similarity to the *Aspergillus* α-amylase in the bioinformatics screen noted below. The US FDA has granted GRAS status to cereal and bacterial-derived α-amylase-containing preparations and permits the use of flour containing α-amylase from *Aspergillus oryzae*.

There has been one report suggesting an association of barley amylase with allergic reactions to flour (Sandiford *et al*., 1994). However, there are insufficient data to prove a cause and effect relationship, so the finding has been judged as inconclusive. For this reason, barley and other cereal α-amylases are not included in the AllergenOnline database.

Bioinformatic analysis was conducted to evaluate the potential allergenicity of the ZM-AA1 protein sequence. The ZM-AA1 complete translation product, including the Brittle-1 transit peptide is 495 amino acids in length (420 amino acids of the ZM-AA1 protein itself plus 75 additional amino acids (residues 1-75) contributed by the maize Brittle-1 transit peptide used to target the protein to amyloplasts) and was compared to a database of 1313 known and putative allergen sequences comprising the FARRP8 database. Potential identities between the ZM-AA1 protein sequence and proteins in the allergen database were evaluated with the FASTA34 sequence alignment tool using the default parameters. A 35% or greater identity threshold over any 80 or greater amino acid length sequence between the query protein and an allergen was used to indicate the potential for cross-reactivity (FAO/WHO, 2001; Codex, 2003). No identity matches of ≥ 35% over ≥ 80 residues were observed. In addition, protein sequences were evaluated for any 8 or greater contiguous identical amino acid matches to the allergens contained in the database noted above. There were no 8 or greater contiguous identical amino acid matches observed.

Based on this information, Pioneer determines that ZM-AA1 protein is unlikely to cause an allergic reaction in humans or to be toxins in humans or animals.

To search for potential similarity to known toxins, α-amylase sequence was queried using the BLASTP 2.2.13 algorithm against Release 165.0 (4/15/08) of the Genpept "nr" dataset, which incorporates non-redundant entries from all Genbank nucleotide translations along with protein sequences from SWISS-PROT (http://www.expasy.org/sprot/), PIR (http://pir.georgetown.edu/), PRF (http://www4.prf.or.jp/en/), and PDB (http://www.wwpdb.org/). Because of the large number of automated conceptual translations derived from genomic sequences, protein sequences from the RefSeq collection (http://www.ncbi.nlm.nih.gov/RefSeq/) were excluded from the search using the filtering statement "protein_all [filter] NOT srcdb_refseq [prop]" to delimit the results returned. A cutoff expectation (*E*) value of 1.0 was used to generate biological meaningful similarity between the proteins of interest and proteins in the NCBI datasets. Although a statistically significant sequence similarity generally requires a match with an expectation value less than 0.01, a cutoff of $E < 1.0$ insures that proteins with even limited similarity will not be overlooked in the search (Pearson, 2000). The scoring matrix used was the default (BLOSUM62), and the number of alignments returned was set to the maximum value (2000).

The ZM-AA1 BLASTP search returned 1893 protein sequences with an *E* score of less than 1.0. Seventeen of the accessions returned by the searches displayed complete significance (*E* = 0) and represent very closely related α-amylases from rice, maize, and banana. A total of 741 additional protein sequences were identified as α-amylases from various bacterial, archaebacterial, and eukaryotic species. Five of the sequences returned aligned primarily with the Brittle-1 transit peptide portion of the α-amylase sequence. The remaining 1130 sequences represented a variety of protein sequences that are functionally related to α-amylases and contain one or more well-characterized glycosyl hydrolase domains. None of the protein sequences returned by the BLASTP search identified safety concerns that might arise from the expression of ZM-AA1 in genetically modified plants.

We conclude from our assessment that the presence of *zm-aa1* encoded α-amylase in maize 32138 SPT maintainer does not represent any safety concerns

VI-H. Characterization of the DsRed2 Protein Expressed in 32138 SPT Maintainer

Protein safety assessment studies were conducted on DsRed2 protein as it is the only protein not derived from a maize gene sequence incorporated into 32138 SPT maintainer. In order to obtain sufficient protein to conduct safety assessment studies, DsRed2 protein was expressed and purified by using an *E.coli* protein expression system. Characterization of the protein was achieved through the determination of purity, concentration, and identity. Purity was determined by utilizing a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method. Concentration was determined by using a modified Bradford assay and the identity was determined by utilizing Western blot analysis, electrospray mass spectroscopy, tryptic peptide analysis by liquid chromatography/mass spectrometry (LC/MS), N-terminal amino acid sequencing, and florescence activity assay.

Small quantities of DsRed2 protein were also purified from 32138 SPT maintainer in order to evaluate and establish equivalency of the DsRed2 protein derived from the *E.coli* expression system with DsRed2 protein derived from 32138 SPT maintainter.

Equivalence of the microbially expressed and plant-derived DsRed2 protein was achieved using the following analyses:

1) SDS-PAGE to confirm equivalent molecular weight;
2) Western blot analysis to confirm equivalent molecular weight and immunoreactivity;
3) N-terminal amino acid sequence analysis to determine the identity of the proteins;
4) Identification of tryptic peptides by Liquid chromatography/mass spectroscopy (LC/MS) to confirm the identity of the proteins;
5) Glycoprotein staining to determine potential post-translational modification (glycosylation).

A detailed description of the methods used in the equivalency studies and the resulting data are included in Appendix 10.

Based on the results from the above analyses, the equivalency of the DsRed2 protein expressed in *E. coli* to the DsRed2 protein expressed *in planta* in 32138 SPT maintainer was demonstrated. Therefore, the DsRed2 protein derived from the microbial expression system was appropriate for utilization in safety assessment studies as a substitute for DsRed2 protein expressed in the 32138 SPT maintainer. Microbially expressed DsRed2 was used subsequently for *in vitro* and *in vivo* safety assessment studies summarized in Section VI-I.

VI-I. Summary of the Food and Feed Safety Assessment for the DsRed2 Protein

A food and feed safety assessment was conducted to assess the allergenicity and toxicity potential of the DsRed2 protein. The data and assessment form the basis of the conclusion that the DsRed2 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals, and therefore is safe for human and animal consumption. A detailed assessment of animal and human safety of the DsRed2 protein was submitted to FDA on October 11, 2006 as part of New Protein Consultation 004. The conclusions of the safety assessment are summarized below.

Toxicological Safety Assessment

1) The DsRed2 protein belongs to a family of red fluorescent proteins (RFP) which are members of a group of fluorescent proteins identified in several *Anthozoa* species. DsRed2 is a modified variant of the original RFP isolated from a coral-like anemone *Discosoma* sp. Although no evidence could be found regarding the specific use of the DsRed2 protein in the food industry, a DsRed variant has been successfully used as a vital marker in mice. There were no observed detrimental effects (Long *et al*., 2005), or signs of toxicity (Figueiredo *et al*., 2008), when DsRed variants were over expressed in mice.

2) Plants stably expressing DsRed variants did not show any abnormalities, indicating that the protein did not interfere with plant growth, development, fertility, germination or morphogenesis (Jach *et al*., 2001; Dietrich and Maiss, 2002; Wenck *et al*., 2003; Mirabella *et al*., 2004; Stuitje *et al*., 2003).

3) No biologically relevant amino acid sequence identities were observed between known protein toxins and the DsRed2 protein sequence. The DsRed2 protein sequence was queried using the BLASTP 2.2.13 algorithm against Release 165.0 (4/15/08) of the Genpept “nr” dataset. Bioinformatic analyses revealed the DsRed2 to be similar to other colored fluorescent proteins. Additionally, no evidence of toxicity has been observed in studies that have been conducted using RFPs or related green fluorescent proteins in transgenic plants and animals (Richards *et al.,* 2003). These data support the conclusion that the DsRed2 protein is unlikely to be a toxin.

4) There was no evidence of acute toxicity in mice at a target dose of 2000 mg (equivalent to approximately 1860 mg of DsRed2 protein) protein preparation per kg of body weight. To translate this type of mouse acute toxicity dose to human exposure, based on expression levels of the DsRed2 protein in 32138 SPT maintainer seed, a child weighing 10 kg would have to consume approximately 45 kg of 32138 SPT maintainer and an adult weighing approximately 60 kg would have to consume about 270 kg of 32138 SPT maintainer seed. Based on these simplistic calculations, it is clear there is a wide margin of safety for the DsRed2 protein, especially considering other factors such as the low likelihood of exposure to this protein due to its use as part of an internal seed production process.

Allergenicity Assessment

5) The DsRed2 protein was evaluated for its allergenicity and toxicity potential using FDA's published guidance for the early food safety evaluation of new proteins in new plant varieties (FDA, 2006). The allergenic potential of DsRed2 was assessed by: 1) bioinformatic comparison of the amino acid sequence of the DsRed2 protein with known or putative protein allergen sequences; 2) evaluation of the stability of the DsRed2 protein using an *in vitro* gastric digestion model; and 3) assessment of the DsRed2 gene source and history of use or exposure.

6) The DsRed2 amino acid sequence was compared to a database of 1313 known and putative allergen sequences comprising the FARRP8 dataset at the University of Nebraska (updated 1/2008). Potential identities between the protein sequences and proteins in the allergen database were evaluated with the FASTA34 sequence alignment tool using the default parameters. A 35% or greater identity threshold over any 80 or greater amino acid length sequence between the query protein and an allergen was used to indicate the potential for cross-reactivity (FAO/WHO, 2001, and Codex, 2003). No identity matches of ≥ 35% over ≥ 80 residues were observed for the DsRed2 protein sequences. In addition, the DsRed2 protein sequence was evaluated for any 8 or greater contiguous identical amino acid matches to the allergens contained in the database noted above. There were no 8 or greater contiguous identical amino acid matches observed. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the DsRed2 protein and known or putative allergens.

7) The DsRed2 parent protein was rapidly hydrolyzed (less than 30 seconds) in simulated gastric fluid containing pepsin at pH 1.2 as demonstrated by SDS-PAGE analysis. A faint band was visible near the dye front around the 3kDa molecular weight marker and was visible through 60 minutes. This band is likely a mix of breakdown products from the DsRed2 protein.

8) Finally, the DsRed2 protein is not glycosylated (refer to Appendix 10).

Exposure Assessment

9) The DsRed2 protein will be used only as a screenable marker that will enable efficient detection and removal of maize seed that express the protein. The fluorescent protein will therefore be contained within an internal process and is not intended to be present in a commercial seed product. *De minimis* amounts of 32138 SPT maintainer seed and vegetative material may be used for feed. Any unused 32138 SPT maintainer inbred seed or discarded seed from color sorting (Figure 3, Stage II) will be disposed of using established procedures for the destruction of proprietary inbred materials. Discarded inbred parent seed and seed by-products (*e.g.*, husks and cobs) will not enter into the commodity grain supply. Pioneer procedures for disposal of unwanted material include devitalization followed by landfills, composting, and/or incineration. Discard maize seed and by-products can also be fed directly to animals, in which case, the feed supplier must sign a purchase agreement that the materials will be utilized for farm use only and cannot be delivered or sold commercially. Outdated 32138 SPT maintainer seed inventory (*i.e.*, lines that are no longer used as parents in commercial hybrid seed products) will be disposed of using the discard procedures described above. Therefore, exposure to the DsRed2 protein is extremely low. Results of the safety assessment indicate that the DsRed2 protein is safe for animal and human consumption. Based on the data and information provided in this submission, we have determined that the DsRed2 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals.

VII. Agronomic Performance and Ecological Observations

Agronomic evaluations were conducted to assess the agronomic comparability of 32138 SPT maintainer to conventional inbred maize. This evaluation also included observed responses to insect and disease stressors and formed the basis for Pioneer's conclusion that 32138 SPT maintainer is comparable to conventional inbred maize and plant pest risk is no more than conventional inbred maize.

The agronomic evaluations were based on both laboratory experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of maize. For each assessment, 32138 SPT maintainer was compared to null segregant near-isoline inbred that did not contain the 32138 SPT insertion.

To evaluate the agronomic characteristics of 32138 SPT maintainer, data were collected on representative characteristics that influence reproduction and survival of the crop.

VII-A. Germination and Dormancy Evaluations

Seed is considered germinated with the uptake of water (*i.e.,* when there is visible elongation of embryonic axis and visible penetration of the structures surrounding the embryo by the radicle. Seed dormancy is regarded as the failure of an intact viable seed to complete germination under favorable conditions (Bewley, 1997). In maize, dormancy is extremely rare. Standardized germination tests are routinely used to assess any potential seed dormancy, which is considered an important weediness trait (Basu *et al*., 2004; Anderson, 1996; Lingenfelter and Hartwig, 2007).

In order to test the germination and potential dormancy of the 32138 SPT maintainer, seeds from the BC3S5 generation (see Figure 12) were tested under 3 different germination conditions. The Rules for Testing Seeds published by the Association of Official Seed Analysts (AOSA) in 2007 were used as general guidelines for the germination method and interpretation of results.

a. **Warm Germination Test**: This test is conducted under optimal growing conditions, 25^{o}C (78^{o}F) at 90% relative humidity for 10 days. The AOSA recommends a temperature range of 20-30^{o}C as optimal for germination of corn (AOSA, 2007).

b. **Cold Germination Test**: This test is conducted at 10^{o}C (50^{o}F) at 90% relative humidity for 10 days. The cold test is a stress test that simulates early spring planting conditions in the Midwestern US.

c. **Diurnal Test**: Germination and potential dormancy were also evaluated at alternating temperatures of 25^{o}C and 10^{o}C (78^{o}F and 50^{o}F) at 90% relative humidity for 10 days. This test simulates daily weather conditions in the major maize growing regions in the U.S. The lower temperature was maintained for 16 hours and the higher temperature for 8 hours.

All three tests above included four replicates of 100 seeds of each entry: i) maize 32138 SPT maintainer, ii) near-isoline non-transgenic inbred control, and iii) three reference inbreds (Pioneer proprietary inbreds used to produce current commercial hybrid seed). All the tests were conducted in temperature-controlled growth chambers in the dark using a rolled towel test method. At the end of 10 days, the numbers of seeds germinated were counted and the percent germination was calculated.

Germination rates were reported as a percentage of germinating seed as follows: (number of seeds germinated /total number of seeds)*100.

Means were calculated for all germination data (Table 10). Statistical tests for differences were conducted at the significance level of $\alpha = 0.05$. For more details on the statistical analysis, refer to Appendix 11. No statistically significant differences were detected between the 32138 SPT maintainer, near-isoline inbred control, and the three reference inbreds for the warm and cold germination tests. No statistically significant differences were detected between the 32138 SPT maintainer, near-isoline inbred control, and two of the three reference inbreds for the diurnal test. A statistically significant difference was observed for the diurnal test between the 32138 SPT maintainer and one of the reference inbreds used (reference inbred B) as this reference inbred had a higher germination rate under the diurnal test.

Seeds that did not germinate in these tests were tested for viability using the Tetrazolium Chloride (TZ) test. The TZ test was developed as a color test for seed viability; viable but non-germinated seed would be an indicator of potential dormancy (http://www.seedlab.oscs.orst.edu/Page_Technical_Brochures/ValueTZTests.htm). All of the non-germinated seeds tested were non-viable (data not shown). Hence, there was no dormancy observed amongst any of the entries.

Germination and dormancy characteristics of the 32138 SPT maintainer were not altered when compared to other inbred maize. The 32138 SPT maintainer exhibited no dormancy tendencies and is unlikely to present an increased potential for weediness as compared to the conventional inbred maize.

Table 10. Summary of Germination Results for 32138 SPT Maintainer

a. Warm Germination Test

Parameter	32138 SPT Maintainer	Near-isoline Inbred	Reference Inbred A	Reference Inbred B	Reference Inbred C
	% Germination				
Mean	99	98.5	97.51	98.75	99.5
Range	98 - 100	98 - 99	95 - 99	97 - 100	98 - 100
P-value		0.549	0.153	0.752	0.441

b. Cold Germination Test

Parameter	32138 SPT Maintainer	Near-isoline Inbred	Reference Inbred A	Reference Inbred B	Reference Inbred C
	% Germination				
Mean	96.25	97	97.25	95.75	94.75
Range	94 - 98	96 - 99	96 - 99	95 - 96	93 - 96
P-value		0.565	0.437	0.723	0.322

c. Diurnal Test

Parameter	32138 SPT Maintainer	Near-isoline Inbred	Reference Inbred A	Reference Inbred B	Reference Inbred C
	% Germination				
Mean	96.83	93.66	97.79	99.75	97.34
Range	92 - 100	92 - 97	93 - 100	99 - 100	95 - 99
P-value		0.217	0.560	0.0344[1]	0.767

[1]Statistically significant difference, P-value <0.05.

VII-B. Field Trial Evaluations

The 32138 SPT maintainer has been field tested in the United States since 2005 as authorized by USDA permits and notifications listed in Appendix 12. The list compiles a number of test sites in diverse regions of the U.S. including the major crop-growing areas and off season nurseries in Hawaii and Puerto Rico. Agronomic data were collected to assess agronomic comparability as it relates to plant pest potential.

Also, throughout the crop growing season, 32138 SPT maintainer was observed for unexpected differences in response to abiotic stress (*e.g.*, drought, excess moisture, abnormal temperatures, *etc.*). Monthly observations for response to naturally occurring abiotic stressors indicated that 32138 SPT maintainer and near-isoline inbred controls were similar with respect to their response to abiotic stress.

Agronomic data were collected from 32138 SPT maintainer and control maize grown at six different locations in 2007 (Figure 47). The number of locations is sufficient because 32138 SPT maintainer will be grown on only a small number of acres (<5,000) that are under Pioneer control. The trial locations provide a range of environmental conditions representative of where 32138 SPT maintainer seed production is expected. Agronomic practices used to prepare and maintain each field site were determined based on the geographic location of each site.

The purpose of this experiment was to evaluate the agronomic characteristics of 32138 SPT maintainer (Table 11) and also to collect tissue samples for measurement of ZM-AA1 and DsRed2 protein concentrations (Section VIE, Tables 8 and 9) and nutrient composition analyses (Section VIII, Tables 13-19). Seed from the BC3S3 generation was used (Figure 12). The control plants were non-transgenic near-isoline inbred 705.

The following agronomic characteristics were measured: early population, final population, seedling vigor, time to silking, time to pollen shed, stalk lodging, root lodging, stay green, disease incidence, insect damage, plant height, ear height, and pollen viability (*i.e.*, shape and color) over time. Table 11 describes the quantitative field agronomic characteristics that were collected during the 2007 growing season.

Pollen collected was evaluated using a magnifying lens for shape and color at various time points in order to evaluate pollen viability of 32138 SPT maintainer and inbred control maize over time. The correlation between visual appearance and viability of pollen is well documented (Luna *et al*., 2001). Pollen can be viable, but not fertile as seen in the case of 50% of the pollen from 32138 SPT maintainer pollen (Figure 9). There were no morphological (shape, size, and color) differences between the pollen from inbred 705 control and fertile and infertile pollen from 32138 SPT maintainer.

Each site employed a randomized complete block design containing four blocks, of which three blocks were used for analysis of agronomic characteristics. Each block was separated by an alley distance of at least 36 inches (in.) (0.9 meters (m)) and contained one-row plots planted with maize 32138 SPT maintainer or the near-isoline inbred control. The 32138 SPT maintainer inbred and the near-isoline inbred control were planted at a rate of approximately 30 seeds per row. Each row was approximately 25 ft (7.6 m) in length, with seed spacing of approximately 10 in. (0.25 m). Spacing between rows was approximately 30 in. (0.76 m). Each one-row plot was bordered on either side by one row of maize inbreds of different genetic background. Normal agronomic practices were employed throughout the growing season.

A statistical analysis of agronomic data was conducted to test for differences in the mean values between the 32138 SPT maintainer and the near-isoline inbred control (refer to

Appendix 11 for statistical model used). The false discovery rate (FDR) adjustment of P-values being employed by Pioneer conservatively addresses the issues associated with multiple comparative testing while retaining the power for detecting real differences.

Results of the agronomic trials are summarized in Table 12. For all characteristics measured (early population, final population, seedling vigor, time to silking, time to pollen shed, stalk lodging, root lodging, stay green, disease incidence, insect damage, plant height, ear height, and pollen viability over time), no statistical differences in mean values were observed between 32138 SPT maintainer and near-isoline inbred control maize across locations (adjusted P-value > 0.05). These results indicate that 32138 SPT maintainer is agronomically comparable to near-isoline inbred control maize.

Agronomic performance of the reference inbreds was used to help determine the normal variation for the agronomic characteristics measured in the inbred. Using the data obtained from the reference inbreds, a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the inbred population. This statistical tolerance interval provided further context for the interpretation of the agronomic performance of 32138 SPT maintainer. The agronomic measurements in 32138 SPT maintainer that fell within the tolerance interval were considered to be within the range of normal variability of maize inbreds.

Figure 47. Identification of Agronomic Field Trials for 32138 SPT Maintainer

Red stars indicate approximate location of agronomic field trial sites

Table 11. Field Agronomic Characteristics Measured

General Characteristic	Characteristic Measured	Evaluation Timing[1]	Data Description	Scale
Germination / Emergence	Early Population	V2-V4	Number of plants emerged per plot	Actual count per plot
	Seedling Vigor	V2-V4	Visual estimate of average vigor of emerged plants per plot	From 1 to 9, where 1=short plants with small leaves, and 9=tall plants with large leaves
Vegetative Parameters	Plant Height	Approximately R6	Height from the soil surface to the tip of the tassel	Height in cm
	Ear Height	Approximately R6	Height from the soil surface to the base of the primary ear	Height in cm
	Stalk Lodging	Approximately R6	Visual estimate of percent of plants in the plot with stalks broken below the primary ear	0 to 100%
	Root Lodging	Approximately R6	Visual estimate of percent of plants in the plot leaning approximately 30˚ or more in the first 2 feet (0.6 m) above the soil surface.	0 to 100%
	Final Population	Approximately R6	The number of plants remaining per plot	Actual count per plot
	Stay Green	Approximately R6	Overall plant health	Ranging from 1-9 where 1 = No visible green tissue; 5 = approximately 50% green tissue remaining; 9 = very green, approximately 90% or greater green tissue remaining.

Table 11. Field Agronomic Characteristics Measured (continued)

General Characteristic	Characteristic Measured	Evaluation Timing[1]	Data Description	Scale
Reproductive Parameters	Time to Silking	Approximately 50% silking	From the time of planting until approximately 50% of the plants have emerged silks	Number of accumulated Growing Degree Units (GDU)
	Time to Pollen Shed	Approximately 50% pollen shed	From the time of planting until approximately 50% of the plants have tassels shedding pollen	Number of accumulated Growing Degree Units (GDU)
	Pollen Viability	Approximately 50% pollen shed	Pollen Shape	Percentage of pollen grains with collapsed walls at 0, 30, 60 & 120 minutes
			Pollen Color	Percentage of pollen grains with intense yellow color at 0, 30, 60 & 120 minutes
Ecological Interactions	Disease Incidence	Approximately R6	Visual estimate of foliar disease incidence	Ranging from 1-9 where 1 = poor disease resistance or high infection; 9 = best disease resistance or low infection
	Insect Damage	Approximately R6	Visual estimate of insect damage	Ranging from 1-9 where 1 = poor insect resistance or high damage; 9 = best insect resistance or low damage

Footnotes:
1= Refer to Ritchie *et al*., 1993 for a description of maize growth stages.

Table 12. Agronomic Performance of 32138 SPT Maintainer Across Six Locations

Agronomic Characteristic[1] (Unit)		Control	32138 SPT Maintainer	Tolerance Interval
Early Population (number of plants)	Mean[2]	28	28	8 - 26
	Range[3]	26 - 30	24 - 30	
	Adjusted P-value[4]		0.945	
	P-value[5]		0.728	
Final Population (number of plants)	Mean	26	27	11-29
	Range	17 - 29	23 - 29	
	Adjusted P-value		0.945	
	P-value		0.53	
Seedling Vigor (1-9 scale)	Mean	8	8	4 - 9
	Range	7 - 9	5 - 9	
	Adjusted P-value		0.799	
	P-value		0.138	
Time to Silking (growing degree units)	Mean	1420	1410	1120 - 1750
	Range	1330 - 1570	1340 - 1550	
	Adjusted P-value		0.975	
	P-value		0.874	
Time to Pollen Shed (growing degree units)	Mean	1450	1440	1060 - 1880
	Range	1350 - 1640	1340 - 1640	
	Adjusted P-value		0.956	
	P-value		0.791	
Stalk Lodging (% plants broken below primary ear)	Mean	2	1	0 - 29.3
	Range	0 - 15	0 - 5	
	Adjusted P-value		0.801	
	P-value		0.363	
Root Lodging (% plants leaning ~30˚ or more)	Mean	0	0.1	NA
	Range	0 - 0	0 - 2	
	Adjusted P-value		NA[6]	
	P-value		NA	
Stay Green (1-9 scale)	Mean	5	4	1 - 9
	Range	1 - 7	2 - 7	
	Adjusted P-value		0.799	
	P-value		0.276	
Disease Incidence (1-9 scale)	Mean	6	6	1 - 9
	Range	5 - 8	5 - 8	
	Adjusted P-value		0.945	
	P-value		0.711	
Insect Damage (1-9 scale)	Mean	6	6	1 - 9
	Range	5 - 8	5 - 7	
	Adjusted P-value		0.945	
	P-value		0.737	
Plant Height (cm)	Mean	212	211	128 - 299
	Range	181 - 230	189 - 224	
	Adjusted P-value		0.945	
	P-value		0.602	

Table 12. Agronomic Performance of 32138 SPT Maintainer across Six Locations (continued)

Agronomic Characteristic (Unit)		Control	32138 SPT Maintainer	Tolerance Interval
Ear Height (cm)	Mean	75	76	40.7 - 123
	Range	55 - 86	61 - 87	
	Adjusted P-value		0.799	
	P-value		0.187	
0 minutes (%)	**Pollen Viability (Shape) - % (0 to 100) pollen grains with collapsed walls**			
	Mean	10	12	0 - 94.9
	Range	0 - 90	0 - 90	
	Adjusted P-value		0.799	
	P-value		0.259	
30 minutes (%)	Mean	37	37	0 - 100
	Range	0 - 99	0 - 99	
	Adjusted P-value		1	
	P-value		1	
60 minutes (%)	Mean	71	68	0 - 100
	Range	30 - 100	10 - 100	
	Adjusted P-value		0.739	
	P-value		0.102	
120 minutes (%)	Mean	98	98	83.6 - 100
	Range	80 - 100	90 - 100	
	Adjusted P-value		0.801	
	P-value		0.363	
0 minutes (%)	**Pollen Viability (Color) - % (0 to 100) pollen grains with intense yellow color**			
	Mean	9	11	0 - 71.1
	Range	0 - 70	0 - 70	
	Adjusted P-value		0.799	
	P-value		0.275	
30 minutes (%)	Mean	35	38	0 - 100
	Range	0 - 90	0 - 90	
	Adjusted P-value		0.801	
	P-value		0.387	
60 minutes (%)	Mean	65	64	0 - 100
	Range	20 - 95	25 - 100	
	Adjusted P-value		0.945	
	P-value		0.741	
120 minutes (%)	Mean	94	93	54.3 - 100
	Range	75 - 100	60 - 100	
	Adjusted P-value		0.945	
	P-value		0.75	

Footnotes:
1. Refer to Table 11 for descriptions of each agronomic characteristic measured.
2. Least squares mean
3. Range denotes the lowest and highest individual value across sites.
4. False Discovery Rate (FDR) adjusted P-value
5. Non-adjusted P-value
6. Not applicable

VII-C. Ecological Observations

Ecological observations (plant interactions with insects and diseases) were recorded for all USDA-APHIS permitted field trials of 32138 SPT maintainer during the 2005-2008 growing seasons. Plant breeders and field staff that are experts in the fields of plant pathology and entomology observed 32138 SPT maintainer and control lines at least every four weeks for insect and disease pressure and recorded the severity of any stressor seen. Any unexpected differences in response between 32138 SPT maintainer and various control lines (null segregants, near-isoline and/or conventional maize inbred lines) were recorded.

The following scale was used when recording observations:

- mild – very little disease or insect injury (<10%) visible;
- moderate – noticeable plant tissue damage (10% to 30%);
- severe – significant plant tissue damage (>30%).

A summary of the insect and disease ecological observations is presented in Appendix 13. In every case, the severity of insect or disease stress on 32138 SPT maintainer was not qualitatively different from various control lines grown at the same location. These results support the conclusion that the ecological interactions for 32138 SPT maintainer were comparable to control maize lines with similar genetics or to conventional inbred maize lines.

VII-D. Conclusions on Agronomic Performance and Ecological Observations

32138 SPT maintainer was observed in experiments at six field locations to measure agronomic data. Data generated from these studies represent observations that are typically recorded by plant breeders and agronomists to evaluate the characteristics of maize over a range of environmental conditions that 32138 SPT maintainer would encounter. The measured characteristics provide crop biology data useful in establishing a basis to assess agronomic comparability and familiarity of 32138 SPT maintainer to conventional inbred maize in the context of ecological risk assessment.

The agronomic data demonstrated no biologically meaningful differences between 32138 SPT maintainer and inbred control maize (near-isoline of 32138 SPT maintainer and/or conventional maize inbred lines) with respect to germination/emergence, vegetative growth, reproductive parameters, yield, and ecological interactions. These data support the conclusion that 32138 SPT maintainer is comparable in agronomic characteristics to conventional inbred maize.

Monthly observations of all USDA-permitted field trials for responses of 32138 SPT maintainer to naturally occurring insect and disease stressors demonstrated no unexpected differences from inbred control maize. In addition, assessment of the ecological data detected no biologically significant differences between 32138 SPT maintainer and inbred control maize lines indicative of a selective advantage that would result in increased weed potential or plant pest risk for 32138 SPT maintainer.

VIII. Compositional Assessment

Compositional analysis of maize 32138 SPT maintainer was used to evaluate any changes in the levels of key nutrients, anti-nutrients, and secondary metabolites compared to the near-isoline inbred control and two reference inbreds. Along with agronomic data, compositional similarity is a general indicator that the 32138 SPT maintainer will not exhibit unexpected effects with respect to plant pest risk.

Comprehensive compositional analyses were performed on seed from 32138 SPT maintainer and a non-transgenic near-isoline inbred control (inbred 705), grown in 2007 at six field locations in maize growing areas of the Midwest (Figure 47, see Section VII-B for details on field trials).

Seed samples from were also collected from two non-transgenic, self-pollinated maize inbreds (reference inbreds) grown in 2007 at the same six field locations as the 32138 SPT maintainer (Figure 47). The reference inbreds were planted, harvested, processed, and analyzed using the same methods as those employed for the near-isoline inbred control and maize 32138 SPT maintainer. Compositional analysis of the reference inbreds was used to help determine the normal variation for the measured analytes in inbred seed. Using the data obtained from the reference inbreds, a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the inbred population. This statistical tolerance interval provided further context for interpretation of the composition results for 32138 SPT maintainer. The analyte ranges in 32138 SPT maintainer that fell within the tolerance interval were considered to be within the range of normal variability of maize inbreds.

The compositional assessment was conducted in accordance with the OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002). Compositional analyses included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, carbohydrates, fatty acids, amino acids, vitamins and minerals, key anti-nutrients (raffinose, phytic acid and trypsin inhibitor) and key secondary metabolites (furfural, ferulic acid, and *p*-coumaric acid).

Statistical analysis of nutrient composition data was conducted to test for differences in the analyte mean values between the 32138 SPT maintainer and the near-isoline inbred control (for details on the statistical methodology used, refer to Appendix 11). Pioneer applied False Discovery Rate (FDR) adjustment of p-values in order to conservatively address the issues associated with multiple comparative testing while retaining the power for detecting real differences.

VIII-A. Proximates

Proximates were analyzed in 32138 SPT maintainer and near-isoline inbred control maize seed. Results are shown in Table 13. No statistically significant differences were observed between 32138 SPT maintainer and inbred control maize mean values for fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash and carbohydrates (adjusted P-values > 0.05). A statistically significant difference was observed for crude protein (adjusted P-value < 0.05) however the individual protein values were within the tolerance interval.

In conclusion, analysis of proximates demonstrates that 32138 SPT maintainer is comparable to near-isoline and reference maize inbreds.

Table 13. Proximates in the Seed of Control and 32138 SPT Maintainer

Analyte (% Dry Weight)		Control	32138 SPT Maintainer	Tolerance Interval
Crude Protein[5]	Mean[1]	10.7	11.4	7.55-13.8
	Range[2]	9.59-11.3	9.00-12.4	
	Adjusted P-value[3]		0.0116[4]	
	P-value		0.0014	
Fat	Mean	3.14	3.36	1.91-4.38
	Range	2.03 - 3.61	2.47-4.25	
	Adjusted P-value		0.352	
	P-value		0.234	
ADF	Mean	3.76	3.81	0-8.12
	Range	2.82-4.59	2.92-4.66	
	Adjusted P-value		0.777	
	P-value		0.697	
NDF	Mean	9.88	10.0	4.35 – 27.4
	Range	7.79-12.4	6.31 – 14.8	
	Adjusted P-value		0.87	
	P-value		0.816	
Ash	Mean	1.36	1.49	0.559 - 2.80
	Range	1.02 – 1.54	1.23-1.79	
	Adjusted P-value		0.127	
	P-value		0.0559	
Carbohydrates	Mean	84.7	83.8	80.7-89.1
	Range	83.9-87.0	82.4-86.8	
	Adjusted P-value		0.0732	
	P-value		0.0247	

Footnotes:

1. Least squares mean
2. Range denotes the lowest and highest individual value across locations.
3. False Discovery Rate (FDR) adjusted P-value
4. Statistically significant difference, adjusted P-value < 0.05
5. The analyte values were transformed for statistical analysis. Means and confidence intervals have been back transformed to the data scale.

VIII-B. Fatty Acids

Concentrations of 29 fatty acids were measured in 32138 SPT maintainer and near-isoline inbred control maize seed. Levels of 16 fatty acids were below the limit of quantitation for the assay: caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), heptadecadienoic acid (C17:2), γ-linolenic acid (C18:3), nonadecanoic acid (C19:0), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), heneicosanoic acid (C21:0), erucic acid (C22:1) and tricosanoic acid (C23:0). Therefore, no statistical analyses were conducted on these fatty acids and data are not shown.

Results of the fatty acid analysis are presented in Table 14. No statistically significant differences (adjusted P-values > 0.05) were observed between the 32138 SPT maintainer and near-isoline inbred control maize for the fatty acid mean values for palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecenoic acid (C17:1), stearic acid (C18:0), linoleic acid (C18:2), and eicosenoic acid (C20:1). Statistically significant differences (adjusted P-values < 0.05), were observed for heptadecanoic acid (C17:0), oleic acic (C18:1), and linolenic acid (C18:3), arachidic acid (C20:0), behenic acid (C22:0) and lignoceric acid (C24:0), however the individual values for these fatty acids were within the respective tolerance interval.

In conclusion, fatty acid analysis demonstrates that 32138 SPT maintainer is comparable to near-isoline and reference maize inbreds.

Table 14. Major Fatty Acids in the Seed of Control and 32138 SPT Maintainer

Analyte (% Total Fatty Acids)		Control	32138 SPT Maintainer	Tolerance Interval
Palmitic acid (C16:0)	Mean[1]	11.0	11.2	6.62 - 15.3
	Range[2]	10.4 - 11.5	10.5 - 12.1	
	Adjusted P-value[3]		0.111	
	P-value		0.0467	
Palmitoleic acid (C16:1)	Mean	0.195	0.183	0 - 0.319
	Range	0 - 0.395	0.124 - 0.343	
	Adjusted P-value		0.588	
	P-value		0.467	
Heptadecanoic acid (C17:0)	Mean	0.182	0.166	0 - 0.263
	Range	0.166 - 0.211	0.147 - 0.202	
	Adjusted P-value		0.011[4]	
	P-value		0.000835	
Heptadecenoic acid (C17:1)	Mean	0.125	0.104	0 – 0.120[6]
	Range	0 - 0.147	0 – 0.110	
	Adjusted P-value		NA	
	P-value		NA	
Stearic acid (C18:0)	Mean	2.17	2.06	1.04 - 3.31
	Range	1.97 - 2.37	1.89 - 2.34	
	Adjusted P-value		0.0558	
	P-value		0.0177	
Oleic acid (C18:1)	Mean	19.0	20.9	6.75 - 45.3
	Range	17.5 - 20.8	18.9 - 23.3	
	Adjusted P-value		0.0000571[4]	
	P-value		0.000000394	
Linoleic acid[5] (C18:2)	Mean	56.9	56.9	27.1 - 72.7
	Range	53.2 - 59.5	52.9 - 60.2	
	Adjusted P-value		0.985	
	P-value		0.985	
Linolenic acid (C18:3)	Mean	2.67	2.32	1.62 - 3.42
	Range	2.44 - 3.07	2.01 - 2.61	
	Adjusted P-value		0.000285[4]	
	P-value		0.00000399	
Arachidic acid (C20:0)	Mean	0.407	0.445	0.179 - 0.778
	Range	0.366 - 0.467	0.384 - 0.533	
	Adjusted P-value		0.0116[4]	
	P-value		0.00146	
Eicosenoic acid[5] (C20:1)	Mean	0.307	0.278	0.0899 - 0.656
	Range	0.281 - 0.350	0.258 - 0.312	
	Adjusted P-value		0.131	
	P-Value		0.0635	
Behenic acid (C22:0)	Mean	0.431	0.370	0.241 - 0.856
	Range	0.362 - 0.558	0.290 - 0.504	
	Adjusted P-value		0.0116[4]	
	P-value		0.00176	

Table 14. Major Fatty Acids in the Seed of Control and 32138 SPT Maintainer (continued)

Analyte (% Total Fatty Acids)		Control	32138 SPT Maintainer	Tolerance Interval
Lignoceric acid [5] (C24:0)	Mean	0.582	0.423	0.152 - 1.00
	Range	0.475 - 0.772	0.302 - 0.654	
	Adjusted P-value		0.00161[4]	
	P-value		0.0000668	

Footnotes:
1. Least squares mean
2. Range denotes the lowest and highest individual value across locations
3. False Discovery Rate (FDR) adjusted P-value
4. Statistically significant difference, adjusted P-value < 0.05
5. Analyte values were transformed for statistical analysis. Means and confidence intervals have been back transformed to the data scale.
6. No tolerance interval could be calculated; these values are minimum/maximum ranges

VIII-C. Total Amino Acids

Concentrations of 20 amino acids were measured in 32138 SPT maintainer and near-isoline inbred control maize seed. Results are presented in Table 15. No statistically significant differences (adjusted P-values > 0.05) were observed between 32138 SPT maintainer and near-isoline inbred control maize for the amino acid mean values for cystine, lysine, threonine, isoleucine, histidine, valine, leucine, arginine, phenylalanine, glycine, glutamic acid, proline, serine, and tyrosine. Statistically significant differences (adjusted P-values < 0.05), were observed for methionine, tryptophan, alanine, and aspartic acid, however the individual values for these amino acids were within the respective tolerance intervals.

In conclusion, total amino acid analysis demonstrates that 32138 SPT maintainer is comparable to near-isoline and reference maize inbreds

Table 15. Total Amino Acids in the Seed of Control and 32138 SPT Maintainer

Analyte (% Dry Weight)		Control	32138 SPT Maintainer	Tolerance Interval
Methionine	Mean[1]	0.238	0.208	0 - 0.459
	Range[2]	0.182 - 0.297	0.170 - 0.257	
	Adjusted P-value[3]		0.0133[4]	
	P-value[5]		0.00211	
Cystine	Mean	0.211	0.201	0.0199 - 0.376
	Range	0.128 - 0.269	0.132 - 0.277	
	Adjusted P-value		0.572	
	P-value		0.450	
Lysine[5]	Mean	0.317	0.314	0.131 - 0.509
	Range	0.215 - 0.417	0.274 - 0.405	
	Adjusted P-value		0.870	
	P-value		0.815	
Tryptophan	Mean	0.103	0.0880	0.0234 - 0.171
	Range	0.0860 - 0.126	0.0771 - 0.0991	
	Adjusted P-value		0.0163[4]	
	P-value		0.00281	
Threonine	Mean	0.366	0.381	0.294 - 0.475
	Range	0.282 - 0.399	0.302 - 0.419	
	Adjusted P-value		0.318	
	P-value		0.188	
Isoleucine[5]	Mean	0.396	0.411	0.287 - 0.498
	Range	0.330 - 0.422	0.326 - 0.455	
	Adjusted P-value		0.321	
	P-value		0.195	
Histidine[5]	Mean	0.304	0.313	0.211 - 0.401
	Range	0.235 - 0.344	0.243 - 0.338	
	Adjusted P-value		0.437	
	P-value		0.317	
Valine[5]	Mean	0.509	0.536	0.397 - 0.644
	Range	0.420 - 0.537	0.443 - 0.579	
	Adjusted P-value		0.0957	
	P-value		0.037	
Leucine	Mean	1.41	1.50	0.994 - 1.82
	Range	1.25 - 1.52	1.26 - 1.71	
	Adjusted P-value		0.0957	
	P-value		0.0357	
Arginine	Mean	0.437	0.451	0.175 - 0.694
	Range	0.330 - 0.494	0.383 - 0.515	
	Adjusted P-value		0.472	
	P-value		0.350	

Table 15. Total Amino Acids in the Seed of Control and 32138 SPT Maintainer (continued)

Analyte (% Dry Weight)		Control	32138 SPT Maintainer	Tolerance Interval
Phenylalanine	Mean	0.559	0.596	0.400 - 0.722
	Range	0.468 - 0.612	0.477 - 0.685	
	Adjusted P-value		0.119	
	P-value		0.0508	
Glycine[5]	Mean	0.396	0.403	0.256 - 0.537
	Range	0.295 - 0.448	0.330 - 0.438	
	Adjusted P-value		0.635	
	P-value		0.525	
Alanine	Mean	0.823	0.883	0.629 - 1.05
	Range	0.714 - 0.865	0.801 - 0.971	
	Adjusted P-value		0.0313[4]	
	P-value		0.00726	
Aspartic Acid	Mean	0.744	0.811	0.513 - 0.960
	Range	0.628 - 0.824	0.743 - 0.936	
	Adjusted P-value		0.0332[4]	
	P-value		0.00859	
Glutamic Acid[5]	Mean	2.23	2.39	1.70 - 2.85
	Range	1.80 - 2.40	1.77 - 2.63	
	Adjusted P-value		0.100	
	P-value		0.0395	
Proline	Mean	0.955	0.989	0.679 - 1.26
	Range	0.883 - 1.01	0.852 - 1.08	
	Adjusted P-value		0.209	
	P-value		0.114	
Serine	Mean	0.498	0.523	0.373 - 0.646
	Range	0.366 - 0.540	0.364 - 0.594	
	Adjusted P-value		0.326	
	P-value		0.207	
Tyrosine	Mean	0.252	0.268	0.127 - 0.377
	Range	0.188 - 0.303	0.199 - 0.349	
	Adjusted P-value		0.352	
	P-value		0.232	

Footnotes:
1. Least squares mean
2. Range denotes the lowest and highest individual value across locations
3. False Discovery Rate (FDR) adjusted P-value
4. Statistically significant difference, adjusted P-value < 0.05
5. The analyte values were transformed for statistical analysis. Means and confidence intervals have been back transformed to the data scale.

VIII-D. Vitamins and Minerals

Vitamins and minerals were measured in 32138 SPT maintainer and near-isoline inbred control maize seed. Based on OECD guidance, the following vitamins were analyzed: beta-carotene, vitamin B1 (thiamin), vitamin B2 (riboflavin), vitamin B6 (pyridoxine), vitamin B3 (niacin), vitamin B9 (folic acid) and α-tocopherol (OECD, 2002). The following minerals were also analyzed, based on OECD guidance: calcium, copper, iron, magnesium, phosphorus, potassium, sodium and zinc (OECD, 2002).

Vitamin results are shown in Table 16 and mineral results are presented in Table 17. The level of vitamin B2 (riboflavin) was below the limit of quantitation for the assay and therefore no statistical analysis was conducted on this analyte. No statistically significant differences (adjusted P-values > 0.05) were observed between the 32138 SPT maintainer and near-isoline inbred control maize mean values for beta-carotene, vitamin B1 (thiamin), vitamin B6 (pyridoxine), vitamin B9 (folic acid), vitamin B5 (pantothenic acid), α-tocopherol, copper, phosphorus, and sodium. Statistically significant differences (adjusted P-values < 0.05), were observed for vitamin B3, calcium, iron, magnesium, potassium, and zinc, however the individual values for these analytes were within the respective tolerance interval.

In conclusion, vitamin and mineral analyses demonstrate that 32138 SPT maintainer is comparable to near isoline and reference maize inbreds.

Table 16. Vitamins in the Seed of Control and 32138 SPT Maintainer

Analyte(% Dry Weight)		Control	32138 SPT Maintainer	Tolerance Interval
Beta-carotene[5]	Mean[1]	6.44	6.79	0 - 27.0
	Range[2]	<2.50[6]- 11.1	<2.50[6]- 13.8	
	Adjusted P-value[3]		0.709	
	P-value		0.599	
Vitamin B1 (Thiamin)	Mean	2.96	3.47	1.39 - 6.14
	Range	<1.80[6] - 4.43	2.84 - 3.93	
	Adjusted P-value		0.127	
	P-value		0.0571	
Vitamin B2 (Riboflavin)	Mean	<0.900[6]	<0.900[6]	NA[7]
	Range	<0.900[6]	<0.900[6]	
	Adjusted P-value		NA	
	P-value		NA	
Vitamin B6[5] (Pyridoxine)	Mean	6.52	7.03	0 - 14.7
	Range	4.52 - 10.1	5.39 - 8.84	
	Adjusted P-value		0.376	
	P-value		0.257	
Vitamin B3 (Niacin)	Mean	18.3	15.4	8.99 - 61.3
	Range	12.7 - 23.4	12.4 - 21.3	
	Adjusted P-value		0.0163[4]	
	P-value		0.00281	
Folic acid[5]	Mean	0.754	0.778	0 - 2.33
	Range	0.540 - 1.11	0.495 - 1.08	
	Adjusted P-value		0.820	
	P-value		0.746	
α-tocopherol[5]	Mean	8.54	9.81	0 - 16.5
	Range	3.81 - 17.7	3.05 - 22.3	
	Adjusted P-value		0.387	
	P-value		0.269	
Vitamin B5 Pantothenic acid (mg/kg)	Mean	5.25	5.44	0.600-10.1
	Range	3.57-7.41	4.89-6.16	
	Adjusted P-value		0.754	
	P-value		0.671	

Footnotes:
1. Least squares mean
2. Range denotes the lowest and highest individual value across locations
3. False Discovery Rate (FDR) adjusted P-value
4. Statistically significant difference, adjusted P-value < 0.05
5. The analyte values were transformed for statistical analysis. Means and confidence intervals have been back transformed to the data scale.
6. < Lower Limit of Quantitation (LLOQ); indicates that the values of the sample or samples were detected below the assay LLOQ
7. Not Applicable

Table 17. Minerals in the Seed of Control and 32138 SPT Maintainer

Analyte (% Dry Weight)		Control	32138 SPT Maintainer	Tolerance Interval
Calcium[5]	Mean[1]	0.00295	0.00340	0 - 0.0127
	Range[2]	0.00233 - 0.00382	0.00252 - 0.00505	
	Adjusted P-value[3]		0.0116[4]	
	P-value		0.00121	
Copper[5]	Mean	0.0000494	0.0000913	0 - 0.000373
	Range	<0.0000625[6] - 0.000205	<0.0000625[6] - 0.000161	
	Adjusted P-value		0.0539	
	P-value		0.0167	
Iron[5]	Mean	0.00167	0.00183	0.000721 - 0.00290
	Range	0.00139 - 0.00197	0.00154 - 0.00209	
	Adjusted P-value		0.0173[4]	
	P-value		0.00322	
Magnesium[5]	Mean	0.124	0.144	0.0303 - 0.178
	Range	0.0907 - 0.156	0.119 - 0.163	
	Adjusted P-value		0.0193[4]	
	P-value		0.00386	
Phosphorus	Mean	0.345	0.366	0.0807 - 0.506
	Range	0.245 - 0.409	0.319 - 0.412	
	Adjusted P-value		0.173	
	P-value		0.0905	
Potassium	Mean	0.321	0.362	0.191 - 0.446
	Range	0.255 - 0.380	0.322 - 0.416	
	Adjusted P-value		0.011[4]	
	P-value		0.00071	
Sodium[5]	Mean	0.0000104	0.0000510	0.0000625 - 0.000547[7]
	Range	<0.0000625[6] - 0.000139	<0.0000625[6] - 0.000281	
	Adjusted P-value		0.0957	
	P-value		0.0353	
Zinc[5]	Mean	0.00245	0.00282	0.000482 - 0.00426
	Range	0.00202 - 0.00330	0.00228 - 0.00322	
	Adjusted P-value		0.01164[4]	
	P-value		0.00157	

Footnotes:

1. Least squares mean
2. Range denotes the lowest and highest individual value across locations
3. False Discovery Rate (FDR) adjusted P-value
4. Statistically significant difference, adjusted P-value < 0.05
5. The analyte values were transformed for statistical analysis. Means and confidence intervals have been back transformed to the data scale.
6. < Lower Limit of Quantitation (LLOQ); indicates that the values of the sample or samples were detected below the assay LLOQ
7. No tolerance interval could be calculated; these values are minimum/maximum ranges.

VIII-E. Key Anti-nutrients

Maize seed contains several key anti-nutrients: raffinose, phytic acid and trypsin inhibitor (OECD, 2002). Raffinose is a low molecular weight carbohydrate that is non-digestible. Phytic acid binds most of the phosphorus in maize, which results in reduced bioavailability of phosphorus for non-ruminant animals. In addition, phytic acid chelates mineral nutrients including calcium, magnesium, potassium, iron and zinc, rendering them unavailable to monogastric animals. Trypsin inhibitor can interfere with the digestion of proteins, resulting in decreased animal growth.

Levels of key anti-nutrients were measured in 32138 SPT maintainer and near-isoline inbred control seed. Results are shown in Table 18. No statistically significant difference (adjusted P-value > 0.05) was observed between the mean values for 32138 SPT maintainer and near-isoline inbred control maize for trypsin inhibitor. Statistically significant differences (adjusted P-values < 0.05), were observed for raffinose and phytic acid, however the individual values were within the respective tolerance interval.

In conclusion, anti-nutrient analysis demonstrates that 32138 SPT maintainer is comparable to near isoline inbred control maize and reference maize inbreds.

Table 18. Key Anti-nutrients in the Seed of Control and 32138 SPT Maintainer

Analyte (% Dry Weight or as Indicated)		Control	32138 SPT Maintainer	Tolerance Interval
Raffinose	Mean[1]	0.227	0.141	0 - 0.339
	Range[2]	<0.0800[5]- 0.303	<0.0800[5] - 0.292	
	Adjusted P-value[3]		0.0116[4]	
	P-value		0.00175	
Phytic acid	Mean	1.04	0.841	0 - 2.19
	Range	0.395 - 1.60	0.470 - 1.08	
	Adjusted P-value		0.0499[4]	
	P-value		0.0144	
Trypsin Inhibitor (TIU[6]/mg)	Mean	3.45	2.78	1.16 - 5.17
	Range	2.17 - 4.21	2.02 - 3.76	
	Adjusted P-value		0.0539	
	P-value		0.0164	

Footnotes:
1. Least squares mean
2. Range denotes the lowest and highest individual value across locations
3. False Discovery Rate (FDR) adjusted P-value
4. Statistically significant difference, adjusted P-value < 0.05
5. <Lower Limit of Quantitation (LLOQ); indicates that the values of the sample or samples were detected below the assay LLOQ
6. Trypsin Inhibitor Units

VIII-F. Key Secondary Plant Metabolites in Control and 32138 SPT Maintainer

Secondary plant metabolites are neither nutrients nor anti-nutrients, but can be analyzed as indicators of the absence of unintended effects of the genetic modification on metabolism (OECD, 2002). Characteristic plant metabolites in maize are furfural and phenolic acids (ferulic acid and *p*-coumaric acid).

Furfural, *p*-coumaric acid and ferulic acid were measured in 32138 SPT maintainer and near-isoline inbred control maize seed. Results are shown in Table 19. The level of furfural was below the limit of quantitation for the assay and therefore no statistical analysis was conducted on this analyte. Statistically significant differences were observed between the mean values for 32138 SPT maintainer and near-isoline control maize for p-coumaric acid and ferulic acid (adjusted P-values < 0.05). However, the individual values for these analytes were within the respective tolerance interval.

In conclusion, metabolite analysis demonstrates that 32138 SPT maintainer is comparable to near-isoline control maize.

Table 19. Key Secondary Plant Metabolites in the Seed of Control and 32138 SPT Maintainer

Analyte (% Dry Weight)		Control	32138 SPT Maintainer	Tolerance Interval
Furfural	Mean[1]	<0.000100[7]	<0.000100[7]	NA[6]
	Range[2]	<0.000100[7]	<0.000100[7]	
	Adjusted P-value[3]		NA	
	P-value		NA	
p-Coumaric acid[5]	Mean	0.0277	0.0230	0.00421 - 0.0411
	Range	0.0210 - 0.0399	0.0149 - 0.0385	
	Adjusted P-value		0.0116[4]	
	P-value		0.00151	
Ferulic acid[5]	Mean	0.147	0.174	0.00769 - 0.354
	Range	0.120 - 0.194	0.110 - 0.245	
	Adjusted P-value		0.0307[4]	
	P-value		0.00635	

Footnotes:
1. Least squares mean
2. Range denotes the lowest and highest individual value across locations
3. False Discovery Rate (FDR) adjusted P-value
4. Statistically significant difference, adjusted P-value < 0.05
5. The analyte values were transformed for statistical analysis. Means and confidence intervals have been back transformed to the data scale.
6. Not applicable.
7. < Lower Limit of Quantitation (LLOQ); indicates that the values of the sample or samples were detected below the assay LLOQ

VIII-G. Conclusions on Compositional Assessment of Seed from 32138 SPT Maintainer

Extensive nutritional compositional analysis of 32138 SPT maintainer was conducted to evaluate the composition as compared to a non-transgenic near isoline inbred control and two conventional maize inbreds. In total, data from 59 different analytical components are presented here. Compositional analysis of 32138 SPT maintainer was used to evaluate any changes in the levels of key nutrients, anti-nutrients or secondary metabolites.

Compositional analyses included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, carbohydrates, fatty acids, total amino acids, minerals and vitamins, key anti-nutrients (raffinose, phytic acid and trypsin inhibitor) and key secondary metabolites (furfural, ferulic acid and *p*-coumaric acid). Based on the compositional evaluation, 32138 SPT maintainer is comparable to inbred maize with respect to nutrient composition. The detected statistical differences between 32138 SPT maintainer and near-isoline inbred control maize likely reflect the natural variability of the components in the inbreds since the levels of all the individual analytes for 32138 SPT maintainer are within the 99% tolerance intervals for inbred maize.

IX. Potential Environmental Impact Assessment of the Seed Production Technology Process

IX-A. Limited Use of and Exposure to the 32138 SPT Maintainer

The 32138 SPT maintainer will be grown on very small acreages (Sections I-A and I-D) and the Company's SPT process for hybrid seed production is extremely effective in virtually eliminating any transgenic seed of 32138 SPT maintainer from commercial seed (Section I-D and Appendices 2-4). The following are some of the unique use and exposure limiting attributes of 32138 SPT maintainer:

- **32138 SPT maintainer is not a commercial product.** Unlike other transgenic events that are deregulated and placed into commerce, 32138 maintainer will not be a commercial product, nor will the 32138 SPT insertion be present in commercial seed sold to growers. Cultivation of the 32138 SPT maintainer will be conducted entirely under Pioneer's control, by Company employees on Company-controlled land, as a part of Pioneer's SPT process for hybrid seed production. Specifically, the 32138 SPT maintainer will be increased in different genetic backgrounds (Figure 3, Step I) and then used in parent (inbred) seed production (Figure 3, Step II). As a consequence of the SPT process, the parent seed derived from Step II (Figure 3) of the process does not inherit any SPT genes and is not transgenic. Consequently, when those parents are used to produce F1 hybrid seed (Figure 3, Step III), no 32138 SPT insertion is present in the hybrid progeny seed that enters into commerce or the commercial maize grain produced by growers (Figure 3, Step IV). Since parent seed and hybrid seed that enter commerce does not contain the 32138 SPT insertion it need not be considered in any assessment of environmental impact.

- **The amount of 32138 SPT maintainer cultivated is low**. Pioneer will implement the SPT process for the generation of female inbred parents incrementally. However, if all female inbred parents for Pioneer hybrid seed production were eventually produced using SPT (maximum internal use scenario), 32138 SPT maintainer would be grown on <5,000 acres each year in the U.S. A small portion of these acres will be used for crossing the 32138 SPT insertion into Pioneer elite inbred parents and increasing these SPT maintainers by self-pollination (Figure 3, Step I). The larger portion of these acres will be used for female inbred parent seed production (Figure 3, Step II). These two steps combined represent a total of <0.006% of the cultivated maize acres in the U.S. (Table 20).

  In the future, Pioneer might license the SPT system to third parties. If the SPT system were adopted across the entire U.S. seed industry (maximum industry use scenario), the total annual U.S. acreage planted to 32138 SPT maintainer would still not exceed 20,000 acres (Table 20).

Table 20. 32138 SPT maintainer Acres Relative to Total Maize Production in the U.S.

Current maize planting in the U.S.[1]	87,327,000 acres
Estimated 32138 SPT maintainer acres required by Pioneer (maximum internal use scenario)[2]	<5,000 acres (<0.006% of total U.S. maize acres)
Estimated 32138 SPT maintainer acres required by industry for (maximum industry use scenario)[3]	<20,000 acres (<0.023% of total U.S. maize acres)

Footnotes:
1. Source: USDA-NASS, 2008
2. Assumes that 100% of Pioneer maize hybrid production uses the SPT process
3. Assumes that 100% of maize hybrid seed production in the U.S. uses the SPT process

- **32138 SPT maintainer sheds virtually no fertile transgenic pollen.** The 32138 SPT maintainer produces virtually no fertile transgenic pollen and so does not pass any SPT genes when used as a pollinator. As discussed in Section I-D, 50% of the pollen produced by 32138 SPT maintainer lack the SPT insertion and is fertile, whereas, the other 50% is transgenic, but infertile. Since 32138 SPT maintainer does not shed fertile pollen that is transgenic for SPT, there is virtually no risk of gene flow to other maize crops or wild or weedy maize relatives (vertical gene flow).

- **32138 SPT maintainer seed is easily identified and removed.** As part of the SPT process, color sorting is used to identify seed containing 32138 SPT insertion (Appendix 3 describes the accuracy of this color sorting process.) All seeds of the 32138 SPT maintainer contain a color marker protein (DsRed2) that causes the seed to fluoresce a bright red color when illuminated by an appropriate excitation light source. When the SPT process is used to increase the 32138 SPT maintainer (Figure 3, Step I), 50% of the seed harvested after self-pollinating the 32138 SPT maintainer is transgenic. Color sorting is used to identify and separate the transgenic red fluorescent 32138 SPT maintainer seed from yellow seed that does not contain SPT derived DNA (Figure 3, Color Sort A).

  Parent inbred seed harvested in Step II (Figure 3) of the process does not contain the 32138 SPT insertion. However, a second color sorting step (Figure 3, Color Sort B) is performed for quality management to prevent any low-level presence of transgenic SPT seed in parent seed used for commercial seed production.

  Pioneer has considered unlikely scenarios whereby the genetic components of the SPT system might fail, for example, in the case of rare genetic recombination events where the *DsRed2(Alt1)* gene becomes de-coupled from the *Ms45* and *zm-aa1* male-sterility gene cassettes. As described in Appendix 7, the occurrence of genetic recombination mechanisms affecting the linkage of the cassettes of the 32138 SPT insertion are rare and would not be expected to occur based on the biological mechanisms or series of events that would need to occur for heritability. In the case that a linkage break did occur between the cassettes of the 32138 SPT insertion, Pioneer has considered the impact on resulting progeny and the overall SPT process. After considering each possible scenario and the phenotypes that result, Pioneer concludes that the progeny seed or plants would be easily identified, removed, or not propagated further.

- **32138 SPT maintainer is genetically, mechanically, and reproductively contained.** The SPT process employs a combination of genetic, mechanical, and reproductive measures that effectively contain the 32138 SPT maintainer within the Pioneer seed production process. The efficiency of the genetic containment is discussed in Appendix 2 and the accuracy of mechanical color sorting is discussed in Appendix 3. The combined efficiency of the genetic containment (*i.e*., the frequency with which the 32138 SPT insertion is not inherited through pollen) and mechanical color sorting accuracy (*i.e*., the accuracy of mechanical seed sorting to remove any transgenic 32138 SPT maintainer seed from non-transgenic male-sterile progeny maize parent seed) is calculated to be >99.9999995% (Appendix 5). In addition, inbred parent seed production is conducted under reproductive containment (spatial, temporal, *etc.*) to ensure genetic purity of the inbred parents (Appendix 6).

- **32138 SPT maintainer seed will be handled separately from other parent seed**. All 32138 SPT maintainer seed (Figure 3, Step I) will be processed and conditioned (cleaned, sized, and treated) in a facility dedicated to exclusively handling this seed, according to specially developed protocols that minimize any potential for cross contamination of non-transgenic seed with transgenic 32138 SPT maintainer seed (Appendix 6). After color sort A, 32138 SPT maintainer seed will be dyed a blue color to distinguish it from any other maize seed and facilitate separate handling. In addition, 32138 SPT maintainer seed will be bagged in bags clearly marked "Maintainer."

- ***De minimis* amounts of 32138 SPT maintainer seed and vegetative material may be used for feed**. Any unused 32138 SPT maintainer inbred seed or discarded seed from color sorting (Figure 3, Stage II) will be disposed of using established procedures for the destruction of proprietary inbred materials. Seed companies take precautions to insure that inbred parent lines are not misappropriated by third parties; special procedures have been established for the disposal of such material. Discarded parent seed and seed by-products (*e.g*., husks and cobs) will not enter into the commodity grain supply. Pioneer procedures for disposal of unwanted material include landfills, composting, and incineration. Discard maize seed and by-products can also be fed directly to animals, in which case, the animal feeder must sign a purchase agreement that the materials will be utilized for farm use only and cannot be delivered or sold commercially. Outdated 32138 SPT maintainer seed inventory (*i.e*., lines that are no longer used as parents in commercial hybrid seed products) will be disposed of using the discard procedures described above.

IX-B. Environmental Assessment of the Components of the 32138 SPT Maintainer: MS45, ZM-AA1, and DsRed2 Proteins

A very small percentage of the maize acreage will be planted to 32138 SPT maintainer (<5,000 acres), (Figure 3, Steps I and II), so there is minimal environmental exposure to the SPT gene expression products: MS45, α-amylase, and DsRed2 proteins. Two of the proteins, MS45 and α-amylase are derived from endogenous maize gene sequences that have no amino acid sequence similarities to protein toxins or allergens. The DsRed2 protein is from a non-food source, but does not have properties of protein toxins or allergens.

Seed products derived from the SPT process do not contain the 32138 SPT insertion (Appendix 2). Therefore, there is no exposure to the SPT gene expression products MS45, α-amylase, and DsRed2 proteins from the end of Step II of the SPT process, onwards (Figure 3).

Ms45 is an endogenous maize fertility gene expressing the MS45 protein in the developing anthers. Expression of MS45 protein in the developing anthers of 32138 SPT maintainer complements the naturally occurring male-sterile *ms45/ms45* mutation in the endogenous *Ms45* gene. MS45 protein is normally present in developing maize anthers and maize has an established history of exposure and safe use. Therefore, there is no novel environmental exposure to the MS45 protein, since the expression is targeted to the anther in the 32138 SPT maintainer.

The ZM-AA1 protein in 32138 SPT maintainer is comprised of maize endogenous protein sequences from the Brittle-1 transit peptide and the ZM-AA1 protein. The α-amylases are widely distributed in nature and are produced by plants, animals, and microorganisms. Multiple known forms of α-amylases exist in maize and are expressed in the endosperm and other tissues (Chao and Scandalios, 1971; James *et al.*, 2007). Amylases are reported to occur endogenously in the pollen of numerous plants including maize (Brewbaker, 1971; Agarwala *et al*., 1981; Wakhle *et al.* 1983). In the 32138 SPT maintainer, the *zm-aa1* gene is expressed in the developing pollen grains and expressed at very low-levels in seed, leaves, and whole plant due to incomplete tissue specificity of the promoter used. These tissues may represent novel routes of environmental exposure for ZM-AA1 to wildlife. Organisms directly ingesting fresh plant material would be regarded as pests (*e.g.,* European corn borer, corn rootworms, *etc.*), and would be purposely controlled using appropriate IPM (Integrated Pest Management) practices, as described in Section IX-F.3. Other non-pest organisms may also incidentally consume plant material (*i.e.* pollen or decaying crop residue). However, as shown in Table 8, expression of ZM-AA1 in whole plant and leaf tissue greatly diminishes to <0.28 ng/mg tissue dry weight by the R6 stage of maize development (Table 8), therefore exposure of decomposers to ZM-AA1 in crop residue is negligible. Pollinivorous insects inhabiting 32138 SPT maintainer-containing fields (<0.023% of maize acreage in the U.S.) may be exposed to elevated levels of ZM-AA1. However as previously described, α-amylases are ubiquitous enzymes present in many organisms including plants and insects (Campos *et al*., 1989; Raimbaud *et al*., 1989; Silva *et al*., 1999; Cristofoletti *et al*., 2001). Based on their endogenous nature, exposure to these enzymes and their byproducts is continuous for wildlife ingesting plant material (*e.g*., pollen from maize). Finally, the limited number of acres to be planted with 32138 SPT maintainer greatly reduces any exposure to populations of pollinivorous insects.

The DsRed2 protein is the only protein not derived from a maize gene sequence used in the 32138 SPT maintainer. DsRed2 belongs to a family of red fluorescent proteins that are used as *in vivo* color markers for a variety of applications in biological systems. The utility of the DsRed2 protein marker and other DsRed variants was demonstrated in both monocotyledonous and dicotyledonous plant species, (Jach *et al*., 2001; Dietrich and Maiss, 2002; Wenck *et al*., 2003; Mirabella *et al*., 2004; Stuitje *et al*. 2003). Plants stably

transformed with DsRed did not show any abnormalities, indicating that the protein did not interfere with plant growth, development, fertility, germination or morphogenesis (Jach *et al.*, 2001; Wenck *et al.* 2003).

As described earlier, the DsRed2 fluorescent protein is used as a color marker to facilitate identification and separation of 32138 SPT maintainer seeds. Seed expressing the DsRed2 protein will be contained within the Company-controlled parent inbred seed production process. The DsRed2 protein is not present in the parent seed product derived from the 32138 SPT maintainer (see Appendix 3) or in commercially sold hybrid seed products, therefore exposure to the DsRed2 protein in food and feed is unlikely. Exposure of wildlife such as birds, rodents, or beneficial arthropods in or near fields where the crop is grown to the DsRed2 protein will be very limited.

The DsRed2 protein has been extensively used as an *in vivo* marker of gene expression. An extensive search of published literature revealed no reports of adverse effects that would suggest a risk to human health and the environment. For example, there were no observed detrimental effects (Long *et al*. 2005) or signs of toxicity (Figueiredo *et al*., 2008), when DsRed variants were over-expressed in mice. Furthermore, transfected cell lines from an arachnid (*Ixodes scapularis*) expressing DsRed2 also exhibited no apparent adverse effects (Kurtti *et al*., 2008). Based on the data and information provided in the safety assessment for the DsRed2 protein (Section VI-I), we have determined that the DsRed2 protein is not likely to be allergenic or toxic to humans, and is also unlikely to cause adverse effects to other organisms which might possibly be exposed in the field. Therefore, with the combination of the safety assessment for DsRed2 and the very limited environmental exposure to DsRed2 protein in the seed and other tissues of 32138 SPT maintainer, we conclude that there will be no significant adverse environmental consequences.

We conclude that there is no potential risk to the environment due to the expression of the SPT genes in the 32138 SPT maintainer, or the deployment of the Pioneer SPT process. We anticipate no adverse effects on humans, domesticated animals or other organisms, including threatened and endangered species as a result of consuming seed or other tissues from 32138 SPT maintainer.

IX-C. Fate of Transgenic DNA in Humans and Animals

In general, SPT derived DNA from 32138 SPT maintainer will not be consumed by humans or animals because the 32138 SPT insertion will not be present in the commodity grain. *De minimis* amounts of discard grain of 32138 SPT maintainer and vegetative material may be fed to animals, but the material will not enter the food/feed commodity stream. In the very unlikely event that any SPT derived DNA is consumed by humans, the acid environment of the stomach rapidly degrades DNA. DNA is degraded within 30 seconds when it was mixed with human saliva and hydrochloric acid in a simulation of the conditions in the human stomach (Mercer *et al*. 1999). DNA from the 32138 SPT insertion is equivalent to other DNA consumed in the normal diets.

IX-D. Weediness Potential of 32138 SPT Maintainer

Various characteristics that might impart weediness to 32138 SPT Maintainer were evaluated (Section VII). Compared to the inbred control maize, no differences were seen in the characteristics such as seed germination, emergence, seedling vigor, disease and insect susceptibility. Assessment of these data detected no biologically significant differences between 32138 SPT maintainer and inbred control maize. Furthermore, post-harvest monitoring of field trial plots containing 32138 SPT maintainer showed no differences in survivability or persistence of 32138 SPT maintainer as compared to conventional maize.

These results support a conclusion of no increased weediness potential of 32138 SPT maintainer.

In general maize is not considered a weed. Maize seed cannot be disseminated without human intervention, nor can it readily survive from one growing season to the next. During the domestication of maize, traits associated with weediness such as seed dormancy, efficient dispersal mechanisms, or the ability to establish reproductive populations outside of cultivation, have not been selected. Maize is not listed on the noxious weed list distributed by the federal government (7CFR part 360). Commercial maize varieties in the U.S. are not effective in invading established ecosystems (CFIA, 1994). Based on the weediness potential evaluation, 32138 SPT maintainer has no additional weediness potential and is comparable to any other conventional maize.

IX-E. Gene Flow Assessment

IX-E.1. Vertical Transfer of the Introduced Genetic Material

Gene flow can occur to the same or a closely related species (vertical) through pollen dissemination. Maize is an open pollinated monoecious plant that produces abundant pollen, up to 50 million pollen grains per plant (Miller 1985). The 32138 SPT maintainer produces two types of pollen in 1:1 ratio: i) fertile, non-transgenic for SPT (do not contain the 32138 SPT insertion) and ii) infertile, transgenic for the 32138 SPT insertion. The occurrence of occasional red fluorescent seeds in batches of parent seed produced using SPT, before color sorting (Appendix 2) suggest that a minute fraction of the fertile pollen may carry SPT genes. Even in the extremely rare instance that another maize variety or another species was successfully pollinated by transgenic SPT pollen, the resulting plants would be unable to pass along the SPT genes, since any transgenic pollen produced would be infertile. Consequently, the risk of vertical gene flow of SPT genes from the 32138 SPT maintainer is essentially zero.

IX-E.2. Horizontal Transfer of the Introduced Genetic Material

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Nap *et al.*, 1992; Redenbaugh *et al.*, 1994). Because of the universality and the identical chemical make-up of DNA in all organisms, there is no reason to believe an organism is more likely to take-up a transgene than another gene. Many genomes have been sequenced from bacteria that are closely associated with plants (*e.g.*, *Agrobacterium* and *Rhizobium*), and there is no evidence that these organisms contain genes derived from plants (Kaneko *et al.*, 2002; Wood *et al*., 2001). Where sequence data indicate that horizontal gene transfer may have occurred, these events are estimated to occur on an evolutionary time scale on the order of millions of years (Koonin *et al*., 2001; Brown, 2003). In addition, transgene DNA promoters and coding sequences are optimized for plant expression and not bacterial expression, and it is therefore very unlikely that a protein corresponding to the transgene would be produced. The amount of 32138 SPT maintainer maize discard seed and by-products fed directly to animals is very minimal. Even if such a transfer were to take place and protein produced, the DNA and protein would not present a human health or plant pest risk. The gene encoding DsRed2 protein is already found in nature, and the *Ms45 and zm-aa1* gene sequences are endogenous to maize.

IX-F. Current Agronomic Practices for Cultivation of Maize Inbred Parent Seed

Because the 32138 SPT maintainer will only be grown on a small number of Company-controlled acres to increase maintainer seed (Figure 3, Step I) and as a pollinator for male-sterile female inbred parent seed (Figure 3, Step II), the scope of the agronomic practices assessment can be limited to maize parent inbred seed production.

In general, agronomic practices for maize parent inbred seed production are similar to practices used in commercial grain production (Wych, 1988). However, a parent inbred seed production field is monitored and managed more intensively than a typical grain production field in order to obtain the best possible yield of high quality, genetically pure seed. Seed production fields are carefully isolated from other fields and rogued (removal of off-type plants) to maintain genetic purity. A seed production field usually requires more agronomic inputs (fertilizers, herbicides, *etc.*) than a typical grain field. This is due to both the agronomic characteristics of inbred lines, which are typically less robust than hybrids, as well as the high value of quality parent inbred seed (Beck, 2002).

Maize for seed production is typically grown as a row crop, with an average row width of about 30 inches. In the Corn Belt, planting usually occurs in late-April to late-May, later than the typical hybrid grain production, to avoid early season stress. Harvesting of inbred seed generally occurs from late August to mid-October at higher seed moisture and usually happens earlier than the harvest of grain. The inbred ears are harvested at higher seed moistures (approximately 32% *vs*. 15 – 20% for grain) than with grain in order to avoid seed loss and to maintain optimum seed quality. A higher percentage of seed production acres are irrigated than grain production acres due to the value of seed, the reduced vigor of inbreds, their less well developed root systems, and to help ensure predictable nicking (synchronization of pollen production and silking).

IX-F.1. Fertilizer Use

Fertilizer use in seed production fields is calibrated for maximum yield and quality of seed. In the U.S., fertilizer use is generally quite similar to grain production fields except for nitrogen which is typically less than for grain production fields. The amount of nitrogen applied is proportional to the expected yield potential, which is lower for inbreds as compared to hybrids. Phosphate, potash, and to a lesser extent, sulfur, are added to soils in order to ensure the best seed yield and quality. Micronutrients (magnesium, boron, zinc, molybdenum) may also be supplemented if the soil tests indicate a need.

IX-F.2. Weed Control Practices

Control of weeds in seed production fields is critical because weeds compete with maize for water, nutrients, and sunlight and reduce crop yields. In particular, inbred lines used in seed production often have low vigor, and do not compete well with aggressive weeds. Weed species such as giant foxtail, barnyard grass and pigweed can reduce maize yields by up to 13, 35, and 50% respectively (Bosnic and Swanton, 1997; Fausey *et al*. 1997; Knake and Slife, 1965). Later in the process, weeds can interfere with harvesting, cleaning and conditioning of the seed crop. Weeds can also act as hosts to insects and diseases that may reduce seed yield and/or quality.

Pioneer must manage a wide array of broadleaf and grass weeds simultaneously and also consider the effect of the herbicide on the inbred line. A combination of weed management strategies is generally used: tillage, scouting, herbicide application (pre-emergence and post-emergence). The combination that Pioneer selects to control weeds depends on factors such as: weed spectrum, level of infestation, soil type, cropping system, weather, time, cost and labor availability.

IX-F.3. Insect Control Practices

Insect infestations can lead to a reduction in quality and quantity of seed in a seed production field. Insect control in seed production fields are usually achieved via insecticides (seed treatments, granular and/or foliar). Intensive Integrated Pest Management (IPM) programs

are practiced in order to scout for insects and determine if and when an insecticide may be needed.

IX-F.4. Disease Control Practices

In order to produce high yield and high quality seed, scouting and monitoring is needed to make sure plant pathogens do not spread. Inbreds are less vigorous and more susceptible to disease pressure than hybrids. Thus, foliar fungicides for diseases are more likely to be applied in seed production compared to hybrid grain production.

IX-F.5. Crop Rotation Practices

Approximately 60% of commercial hybrid maize is grown in rotation following soybean, with another 13% in rotation with other row crops and small grains (USDA-ERS ARMS, 2005). A greater percentage of maize seed production fields are typically rotated, especially in the Corn Belt, as crop rotation aids in the management of diseases, insects and weeds and increases organic matter and soil fertility. Importantly, for seed production fields, crop rotation also aids genetic purity, as there will be no volunteer corn from the previous year.

IX-G. Potential Impact of 32138 SPT Maintainer on Agronomic Practices for Cultivation of Inbred Maize Parent Inbred Seed

The introduction of the SPT process in hybrid seed production is expected to result in significant benefits. Using 32138 SPT maintainer to eliminate detasseling will increase worker safety by significantly reducing the number of seasonal workers, especially high school students, exposed to the hazards of working around heavy farm machinery. Use of 32138 SPT maintainer in female inbred parent seed production will also reduce fossil fuel use and soil compaction, as there will be no need to run heavy detasseling machinery across seed fields. Since detasseling damages plants and reduces plant health, seed yields will be increased by using the SPT process, resulting in a potential reduction in acres needed for parent inbred seed production. The high efficiency of the SPT process will improve seed quality resulting in a fewer batches of seed that fail to meet quality standards and better products for growers containing fewer off-types.

Because the SPT genes are not present in hybrid seed sold to farmers, there will be no potential impacts on agronomic practices for the cultivation of commercial (hybrid) maize. The 32138 SPT maintainer will only be grown on a small number of Pioneer-controlled acres during the increase and parent inbred seed production phases. Therefore, the scope of the agronomic practices assessment is limited to maize parent inbred seed production practices.

No changes in cultivation or management practices such as planting times, row spacing, irrigation amount and type, tillage intensity, or crop residue management are anticipated with Pioneer's use of the 32138 SPT maintainer in the production of female inbred parents. The 32138 SPT maintainer is comparable to conventional maize inbreds in phenotypic, agronomic, ecological and compositional characteristics. The only exceptions are the pinkish red aleurone color conferred by the DsRed protein and the reduced amount of fertile pollen that is shed. The pinkish red aleurone color does not confer any differences in the seed structure and function, so there is no potential agronomic impact. Although half the SPT maintainer pollen is infertile, maize produces such an abundance of pollen, about 14 to 50 million pollen grains per plant (Miller 1985), that there will be enough fertile pollen to insure complete fertilization of parent inbred seed plants. The 32138 SPT maintainer has the same level of resistance to insects and diseases as other inbreds and does not carry any novel tolerances to herbicides, so pest management practices are unaltered.

We conclude that there will be no adverse impacts on the cultivation of maize parent inbred seed due to the deregulation of 32138 SPT maintainer. As described above, there will be a number of benefits arising from the elimination of detasseling, including improved worker safety, reduced fuel use and soil compaction as well as improved seed yields and improved hybrid seed quality.

IX.G.1. Potential Impact on Insect, Disease and Weed Control Practices

The use of the SPT process in hybrid seed production will not cause changes and/or shifts in the insect and weed populations in maize. The 32138 SPT maintainer is agronomically comparable to conventional parent inbred lines and does not carry herbicide tolerance or insect resistance traits. Pioneer will not have to make any adjustments to the Company's existing insect, disease and weed control practices.

IX.G.2. Potential Impact on Volunteer Management

We do not expect changes in volunteer management practices. Data from a warm and cold and diurnal germination study (Section VII-A) demonstrated that germination of 32138 SPT maintainer has not changed and no dormancy characteristics are displayed. In general, maize grows as a volunteer in the year following cultivation in the U.S. and only then under favorable environmental conditions. Volunteers, if they germinate, do not compete well with the succeeding crop and can easily be controlled by mechanical tillage or with herbicides.

IX.G.3. Potential Impact on Crop Rotation Practices

The 32138 SPT maintainer is agronomically no different than conventional maize parent inbred lines and hence no changes in the crop rotation are expected. Pioneer will continue to choose rotational crops based on internal practices in our breeding nurseries and parent inbred seed fields.

IX-H. Potential Impact on Organic or Conventional Farming

Because 32138 SPT maintainer is not a commercial product, and there will be no 32138 SPT insertion derived DNA in seed sold to growers, there will be no potential impacts on organic or conventional farming.

IX-I. Potential Impacts on Raw or Processed Agricultural Commodities

Because 32138 SPT maintainer is not a commercial product, and since there will be no detectable SPT genes or SPT proteins in seed sold to growers or grain harvested by growers, there should be no impacts on raw or processed agricultural commodities.

IX-J. Potential Impact on Biodiversity

The 32138 SPT maintainer does not have an increased weediness potential and cultivation should not lead to increased weediness of other sexually compatible relatives since 32138 SPT maintainer does not produce viable transgenic pollen. There would be no impact on any organisms including threatened or endangered species that came into contact with transgenic sterile SPT pollen because the SPT proteins are not known to be toxic. Importantly, any exposure would be limited to only those organisms in or near SPT maintainer fields. In addition, 32138 SPT maintainer will be grown on existing agricultural land, controlled and managed by Pioneer. No new land will be put into agricultural production because of the deregulation of 32138 SPT maintainer.

IX-K. Overall Environmental and Agronomic Practices Conclusions

It is appropriate to conduct a limited environmental and agronomic practices assessment for 32138 SPT maintainer because it will be used within Pioneer as a part of the Company's SPT process for hybrid seed production. Even if the SPT system is widely licensed and used for all female inbred seed increases across the entire seed industry, the total acreage will still not exceed 20,000 acres (<0.023% of the maize acres in the U.S.)

A thorough characterization of 32138 SPT maintainer was performed, including molecular analysis, protein detection analysis, phenotypic and ecological evaluation, and nutrient composition evaluation. Assessment of the data generated supports the conclusion of no increased plant pest potential, phenotypic comparability, and familiarity as they relate to ecological risk assessment.

No environmental effects due to the expression of *Ms45 and zm-aa1* α-amylase gene sequences introduced in 32138 SPT maintainer are expected due to the previous history of exposure and safe use. Based on the safety assessment the DsRed2 protein is not likely to be allergenic or toxic to humans, and is also unlikely to cause adverse effects to other organisms which might possibly be exposed in the field. Likewise, there is no impact on public health and safety expected due to the DNA introduced in 32138 SPT maintainer.

The 32138 SPT maintainer has been shown to be agronomically and ecologically similar to conventional maize inbred parent lines that have no weedy tendencies and are non-invasive in natural habitats. No differences were seen in characteristics such as seed germination and dormancy, emergence, seedling vigor, plant height, lodging, stay green, time to flowering, pollen viability, disease incidence and insect damage. Assessment of these data detected no biologically significant differences between 32138 SPT maintainer and inbred control maize indicative of a selective advantage that would result in increased weediness or outcrossing potential. On the basis of these data, it is concluded that there is no increased plant pest potential as a result of the use of 32138 SPT maintainer in seed production.

Because 32138 SPT maintainer is not a commercial product, there is no significant impact expected on raw or processed agricultural commodities or organic or conventional farming. We do not expect any changes in the current agronomic practices for inbred or hybrid seed production, beyond the advantages of eliminating the need to detassel female inbred parent seed plants. We anticipate no changes in the level of usage of insecticides or herbicides. The use of transgenic 32138 SPT maintainer in Pioneer's hybrid seed production process results in no adverse environmental impact. SPT technology advances agricultural sustainability by facilitating a more efficient hybrid production and the availability of a greater number of improved hybrids to growers to help achieve higher yields per acre.

IX-L. Non-Transgenic Status of SPT Derived Progeny

Pioneer is aware of the scrutiny of transgenic products in international commerce by the grain trade and importing countries as well as the various approvals and labeling requirements for transgenic foods in different jurisdictions.

Pioneer is requesting APHIS find that the SPT process, as described and operated by Pioneer, consistently and reliably gives rise to progeny seed that lack the 32138 SPT insertion and are not transgenic. This will help us to address questions regarding the movement of seed and grain in international commerce.

As discussed in Appendices 2-5, Pioneer has demonstrated that the overall SPT process is robust and highly efficient at generating progeny that lack the SPT genes. The genetic

efficiency of the SPT system was demonstrated to be >99.999% (Appendix 2) and the accuracy of the mechanical color sorting process was >99.95% (Appendix 3). The expression of the DsRed2 protein and corresponding red fluorescence of the seed are reliable and accurate markers to detect the presence of the 32138 SPT insertion (Appendix 3). The combined efficiency of the genetic containment (*i.e*., the frequency with which the 32138 SPT insertion is not inherited through pollen) and mechanical color sorting accuracy (*i.e*., the accuracy of mechanical seed sorting to remove any 32138 SPT maintainer seed from male-sterile progeny maize parent inbred parent seed) is calculated to be >99.9999995% (Appendix 5). In addition, enhanced quality management procedures will be used for parent inbred parent seed production to provide further checks and control on the system (Appendix 6). Combined, these procedures will effectively ensure the generation of progeny seed that do not contain the 32138 SPT insertion, even in the very rare event of recombination within the 32138 SPT insertion (Appendix 7). In view of this evidence, Pioneer asserts that progeny derived using the SPT process are non-transgenic and any environmental impacts from SPT progeny will be no different from the environmental impact of any other commercially grown maize.

X. Adverse Consequences of Introduction

Pioneer Hi-Bred International, Inc. is unaware of any information indicating that 32138 SPT maintainer may pose a greater plant pest risk than conventional maize. There are no adverse environmental consequences anticipated with its introduction and use in the Company's SPT process for hybrid seed production. Thus, we make the statement "unfavorable information: NONE," and on the basis of the substantial benefits that the SPT process brings to hybrid seed production (reduced labor and fuel costs, reduced soil compaction, and improved worker safety and yield/quality of inbred seed), Pioneer requests that 32138 SPT maintainer be granted non-regulated status under 7 CFR Part 340.
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XII. Appendices[†] 1-13

[†=] Data in the Appendices 2-7 were generated in response to the requests made by USDA-APHIS in their letter to Pioneer, dated 14 Sept, 2007.

Appendix 1

Other Male-Fertility Control Methods Used in Maize

1.1. Chemically Induced Male-Sterility

A number of chemicals (*e.g.*, auxins, antiauxins, halogenated aliphatic acids, gibberellins, arsenicals and ethepon) applied as a foliar spray prior to flowering have been investigated as sterilants in commercial production of hybrid maize seed (McRae, 1985) as well as coating tassels with resin to prevent pollen release (Newlin,1971). One product called Detasselor™ (Rohm & Haas; Zeneca) became commercially available in 1990. However, there has been little commercial use of chemical sterilants because of factors such as a failure to obtain complete pollen sterility, variation in response based on the genotype and environment as well as the challenges of accurate delivery of the chemicals and a narrow window of application. Furthermore, some chemicals reduced seed yields in excess of 20% because of damage to the female flower (Newhouse *et al*., 1996).

1.2. Biological Male-Sterility

Male-sterility describes a phenotype in which tassels are formed but no viable pollen is shed, or the pollen grains do not function normally during fertilization. Male-sterile maize plants can neither self-pollinate, nor cross-pollinate neighboring plants and therefore are very useful in the production of hybrid maize since they eliminate the need for detasseling. Cytoplasmic male-sterility (CMS) has been widely used, and nuclear male-sterility systems have also been investigated for use in hybrid maize production (Wych, 1988). Nuclear male-sterility is described in detail under section I-C.2.

CMS results from a specific interaction between a plant's nuclear and mitochondrial genomes. The CMS characteristic is inherited exclusively through the female maize plant, since only the female provides cytoplasm to the fertilized maize seed. CMS plants can be fertilized with pollen from a male inbred parent, but sometimes fertility may not be restored completely. CMS produced seed is usually blended with seed of the same hybrid produced by detasseling to insure that an adequate amount of pollen is available for fertilization in the grower's field and to contribute to cytoplasmic diversity. Cytoplasmic male-sterility does not work in all types of maize germplasm, depending upon the presence or absence of nuclear restoration genes, thereby restricting genetic combinations available for use in hybrid seed production. Furthermore, the CMS based male-sterility can break down and fail, particularly under stressful environmental conditions, resulting in some level of pollen production by the male-sterile lines and reduced hybrid seed quality.

Major investments of time and resources are required to backcross a male-sterility inducing cytoplasm into those elite female inbred parent lines where CMS will work so these females may be used for production of commercial hybrids. A major challenge is increasing seed of the male-sterile female inbred parent since self-pollination is not possible. Thus, CMS lines must be multiplied by repeated crossing to a specific maintainer that has the same genetics as the CMS line, but carries a normal fertile cytoplasm.

Several CMS types (*e.g.,* CMS-T, CMS-S, and CMS-C) have been used for commercial hybrid maize seed production. The CMS-T type was widely used for more than a decade and 70 to 90% of the hybrid maize grown in the United States carried T-cytoplasm. When the Race T of the Southern maize leaf blight epiphytotic swept the United States in 1970, plants that carried T-type cytoplasm were more susceptible to the blight than plants carrying normal cytoplasm (Havey, 2004). This prompted a re-evaluation of the use of CMS-T and limited its use in hybrid seed production (Wych 1988 and Kaul 1988). CMS-C and CMS-S are in somewhat common use today.

1.3. Male-Sterility Systems Developed using Biotechnology

Research has been underway to develop alternatives to the conventional methods of inducing male-sterility in maize. For example, transgenic dominant sterility systems have been described and developed that rely on the introduction of cytocidal genes regulated by anther-specific promoters to confer male-sterility (Albertsen *et al*., 1993; Greenland *et al*., 1997; Unger *et al*., 2001, 2002; and Havey 2004).

Pioneer previously developed a male-sterility system (Tissue-Specific Sterility, or TSS) based on the DNA adenine methylase (*dam*) gene from *E.coli* that disrupts normal pollen development when placed under control of an anther-specific promoter. The *dam* gene is linked to a selectable marker PAT (Phosphinothricin Acetyltransferase) gene conferring tolerance to the herbicidal active ingredient glufosinate-ammonium. When a TSS transgenic female inbred parent is crossed to a normal parent of the same genotype during female inbred parent increase, 50% of the progeny plants are male-sterile and herbicide tolerant and 50% are male-fertile and herbicide susceptible. During hybrid production, these male-fertile plants can be eliminated by glufosinate treatment as they lack herbicide tolerance gene. The surviving plants are male-sterile and serve as the female inbred parent in hybrid production with normal male inbred parents. Resulting hybrid plants are 50% transgenic, male-sterile and herbicide tolerant and 50% male-fertile and herbicide sensitive. This 1:1 blend of male-fertile plants and male-sterile plants results in enough pollen to insure all plants are fully pollinated in the grower's field.

Another approach uses barnase, an extracellular ribonuclease (RNAse) from *Bacillus amyloliquefaciens,* to induce sterility. Placed under the control of an anther-specific promoter, the *barnase* gene blocks pollen development by interfering with RNA production resulting in a male-sterile plant. Barnase is also linked to the PAT selectable marker and male-sterile female inbred parents are selected by herbicide treatment. A variation of the barnase technology uses a transgenic fertility restorer line homozygous for barstar, the *B. amyloliquefaciens* intracellular inhibitor of barnase for use on the male inbred parent. When the transgenic barnase male-sterile line and the barstar fertility restorer line are crossed, barstar binds to and inhibits barnase, resulting in restoration of fertility. Hybrids derived from the barnase/barstar system are fully fertile (50% because they lack the barnase gene and 50% because of the presence of the barstar gene that inhibits barnase).

Both the TSS and barnase approaches to male-sterility involve added cost and complexity, such as the need to remove non-transgenic plants by herbicide application. Moreover the product is a mixture of transgenic and non-transgenic hybrid seed. Neither the TSS or barnase systems have been adopted in commercial hybrid maize seed production.

USDA-APHIS has deregulated several transgenic male-sterility systems in various crops (Table 1).

Table 1. Transgenic Male-Sterility Systems Deregulated by USDA-APHIS.

Crop	Event(s)	Developer	Petition No.	Mechanism
Maize	MS3	Plant Genetic System	95-228-01p	barnase
Chicory	RM3-3, RM3-4, RM3-6	Bejo	97-148-01p	barnase
Maize	676, 678, 680	Pioneer Hi-Bred	97-342-01p	dam (TSS)
Rapeseed	MS8 x RF3	AgrEvo	98-278-01p	barnase/barstar
Maize	MS6	AgrEvo	98-349-01p (95-228-01p)	barnase
Rapeseed	MS1, RF1/RF2	Aventis	01-206-01p	barnase/barstar

Appendix 2

Genetic Efficiency and Stability of the SPT System

To confirm the genetic efficiency of the SPT system, Pioneer conducted a study to measure the efficiency and stability of the SPT system across several generations and in different genetic backgrounds, under hand-pollination conditions (Study 1) and under open-pollinated field conditions (Study 2). This is important since Pioneer is asking USDA-APHIS to concur that the SPT process reliably gives rise to progeny that are non-transgenic or lack the 32138 SPT insertion.

The SPT system is highly genetically efficient, so virtually all of the fertile pollen from the 32138 SPT maintainer should lack the 32138 SPT insertion, so therefore virtually all of the harvested female inbred seed should also be non-transgenic for SPT.

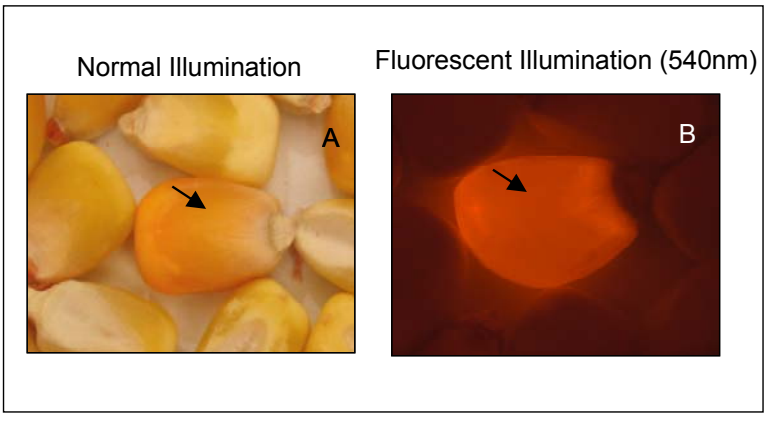
The transgenic SPT seed contains the *DsRed2* gene cassette tightly linked to the *Ms45* and *zm-aa1* male-sterility cassettes. Expression of the DsRed2 protein imparts a pinkish red color to seed containing the 32138 SPT insertion and causes the seed to emit a strong red fluorescence when appropriately illuminated. Therefore, expression of the DsRed2 protein serves as a highly sensitive visible marker for seed containing the 32138 SPT insertion and it enables SPT seed to be identified and separated from non-SPT seed.

2.1. Study 1

Hand-pollinated crosses were made between 32138 SPT maintainer (used as a pollen donor, or pollinator) and female maize lines that were non-transgenic for SPT. The seed from the female lines was hand harvested and examined for the presence of any transgenic SPT seed.

The harvested seed was manually sorted using a light box, Leica KL2500 light systems (Schott Glas, Mainz, Germany) equipped with a fluorescent excitation filter to look for red fluorescing and hence transgenic SPT seed. As shown in Figure 1A (Appendix 2), pinkish red transgenic SPT seed could be distinguished from yellow seed (non-transgenic for SPT) under normal light (Appendix 2-Figure 1A) and fluoresced a bright red when viewed under fluorescent illumination (Appendix 2-Figure 1B).

Figure 1. Appearance of Seed Mixture under Normal and Fluorescent Illumination



Arrow shows the 32138 SPT maintainer seed amongst the yellow seed.

In order to assess the efficiency and stability of the SPT system, this study was conducted within a single genotypic background over six generations (Appendix 2-Table 1), and across five different elite inbred backgrounds designated A-E (Appendix 2-Table 2).

Table 1. Genetic Efficiency of the SPT System across Five Generations

Generation	Total no. of Seed	No. of Red Fluorescing Seed	No. of Yellow Seed	Genetic Efficiency† (%)
T0	362	0	362	100
T1	19,012	0	19,012	100
T2	86,126	2	86,124	99.998
T3	213,689	4	213,685	99.998
T4	437,298	3	437,295	99.999
T5	348,909	1	348,908	99.999
Total	**1,105,396**	**10**	**1,105,386**	**>99.999**

† = {100 – (# of red fluorescing seed/ total # of seed) x100}

Table 2. Genetic Efficiency of the SPT System across Five Genetic Backgrounds

Generation	Genetic Background	Total no. of Seed	No. of Red Fluorescing Seed	No. of Yellow Seed	Genetic Efficiency† (%)
T3	A	84,178	0	84,178	100
	B	129,511	4	129,507	99.997
T4	C	130,606	0	130,606	100
	D	38,421	0	38,421	100
	B	320,520	3	320,517	99.999
T5	E	78,354	1	78,353	99.999
	B	218,303	0	218,303	100
Total		**999,893**	**8**	**999,885**	**>99.999**

† = {100 – (# of red fluorescing seed/ total # of seed) x100}

2.2. Study 2

Genetically male-sterile (*ms45/ms45*) plants were open pollinated in the field with transgenic 32138 SPT maintainer pollen (Step II-Figure 3). More than 380,000 seeds were harvested and subjected to machine color sorting using optimal conditions and procedures (Appendix 2-Table 3). The harvested seed was expected to be all non-transgenic for SPT (do not contain the 32138 SPT insertion) and hence are yellow in color. In the first pass, five red fluorescing seeds were identified and removed. In the second pass of the remaining seed, no additional red fluorescing seeds were found, confirming that the first pass identified all of the SPT seed.

Table 3. Genetic Efficiency of the SPT Process under Open-Pollinated Field Conditions

Pass #	Total no. of seed[1]	No. of Red Fluorescing Seed	No. of Yellow seed	Genetic Efficiency
1	380,005	5	380,000	99.999%
2	380,000	0	380,000	N/A[2]

Footnotes:
1. Total number of seeds was estimated based on the known test weight value for inbred used and the amount of seed (in kg) that passed thru the sorter.
2. N/A= not applicable

2.3. Conclusions

These studies demonstrate that the SPT process generates progeny seed that are non-transgenic for SPT in a highly stable and efficient manner. In the first experiment (using hand-pollinated seed), the SPT system was efficient and stable across six generations and across five different genetic backgrounds. In the second experiment (open-pollinated field conditions), only five out of 380,005 seeds were fluorescing red, indicating that the genetic efficiency of the SPT system was 99.999% in the field. Taking into account the results from both the studies (1 & 2), genetic efficiency of the SPT system to deliver the progeny that are non-transgenic for SPT was never less than 99.997% and overall was >99.999%.

Appendix 3

Mechanical Color Sorting of the 32138 SPT Maintainer: Process and Facilities

Mechanical color sorting is an integral component of the SPT process, and takes place in Pioneer seed conditioning facilities.

As described (Appendix 2), the *DsRed2* gene is tightly genetically linked to the *Ms45* and *zm-aa1* genes in the 32138 SPT maintainer and serves as a visible marker for seed containing the 32138 SPT insertion. The DsRed2 protein emits a strong red fluorescence when appropriately illuminated (Appendix 2-Figure 1B). Therefore, seeds containing the 32138 SPT insertion can be readily detected and separated from seeds that lack the 32138 SPT insertion in a highly efficient manner using mechanical color sorters equipped with appropriate optical filter sets.

At two important steps in the SPT process (Figure 3, Steps I & II) color sorters are used to separate yellow seed (non-transgenic for SPT) from red flourescing transgenic SPT seed (Figure 3, Color Sort A & B).

Color sorting is accomplished using Satake ScanMaster II series, 40-channel sorters (Satake Corporation, Houston, TX, USA) (Appendix 3-Figure 1). Each sorter is equipped with an excitation light source (Satake P/N 0912360660, green), optical band pass filters [Satake P/N 4676200110] and a Satake background filter (P/N 4661840160) to enable color sorting of maize seeds based on DsRed2 fluorescence. The sorter is equipped with multiple high resolution charge coupled device imaging systems that view each seed from the front and back. Seed can pass through the sorter at rates of up to 5,000 kg per hour (Appendix 3-Figure 2).

Color Sort A, which takes place in Pioneer's dedicated SPT maintainer conditioning facility (Appendix 3-Figure 3), is designed to separate and collect the transgenic 32138 SPT maintainer seed for use in female inbred parent seed increase (Figure 3, Step I). In Color Sort A, seed is expected to be segregating 1:1 for the SPT genes, and therefore 50% of the seed will be yellow and 50% will be red fluorescent. The harvested seed is sorted twice (two passes). After the first pass, the red fluorescent transgenic SPT seed is collected and re-run through the sorter, in order to make sure all yellow (non-SPT) seed is removed.

Color Sort B, which takes place in Pioneer's inbred seed conditioning facilities, is a quality control check to identify and remove any low-levels of unwanted fluorescent red 32138 SPT maintainer seed from female inbred parent seed (Figure 3, Step II). In Color Sort B, seed is expected to be virtually free of fluorescent red (non-transgenic for SPT) seed. The harvested seed is sorted twice (two passes) to identify and remove any adventitious fluorescent red transgenic SPT seed.

Figure 1. Satake ScanMaster II Color Sorter

Figure 2. Seed Passing Through Multiple Channels of a Seed Sorter

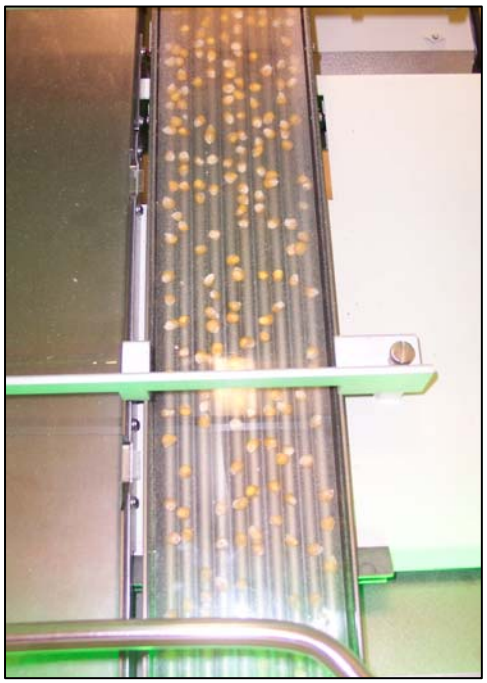


Figure 3. Pioneer SPT Maintainer Conditioning Facility (Color Sort A)

3.1. Sorter Calibration and Optimization

Prior to using the mechanical sorters, it is important to optimize their settings and confirm their accuracy. In other words, determine the accuracy with which the mechanical sorters correctly identify and sort a known (*i.e.* a representative and very low) amount of transgenic SPT (fluorescent red) seed from large batches of yellow seed that is non-transgenic for SPT.

First, the optimal settings for sorting SPT seed were determined experimentally. More than 3 tons (>3000 kg or >6720 lbs) of maize seed was passed through the sorter in multiple batches. Each batch contained an average of 51,000 seeds that were non-transgenic for SPT physically mixed with a pre-determined amount (0.05%) of transgenic 32138 SPT maintainer seed. Sorter parameters (*e.g.,* feed rate) were adjusted so that all transgenic 32138 SPT maintainer seeds were consistently separated from each batch of seed.

The accuracy of the sorter at the optimal settings identified was then evaluated by passing through another 50 batches of seed non-transgenic for SPT mixed with 0.05% transgenic 32138 SPT maintainer (fluorescent red) seeds and determining the machine's ability to detect and separate transgenic 32138 SPT maintainer (red fluorescing seed) from the non-SPT (yellow) seed. The number of red fluorescing 32138 SPT maintainer seed detected in each run (comprising of two passes) were counted and recorded. Sorter accuracy was calculated as the percentage of transgenic 32138 SPT maintainer seed detected by the sorter relative to a known number of transgenic 32138 SPT maintainer seed added. Sorter accuracy was recorded after two passes and confirmed as >99.95% (data not shown).

3.2. Conclusions

Mechanical color sorters provide a fast, efficient and accurate way of identifying and sorting transgenic SPT seed from the seed that is non-transgenic for SPT. Under the optimal settings identified, the accuracy of the color sorters to detect and separate fluorescing transgenic SPT seed was determined to be at >99.95%.

Appendix 4

Molecular Analysis to Confirm the Absence of the 32138 SPT Insertion in SPT Progeny

In addition to the phenotypic studies described in Appendix 2, Pioneer also conducted a molecular experiment to confirm the absence of the 32138 SPT insertion in the male-sterile progeny derived from the SPT system. Pioneer is requesting that USDA-APHIS find that progeny from the SPT process lack the 32138 SPT insertion and are therefore non-trasngenic for SPT.

As previously discussed (Appendix 2), the SPT system is highly genetically efficient. Nearly all of the functional pollen from 32138 SPT maintainer should not contain the SPT insertion, and therefore, as described in Appendix 2, >99.999% of the seed made using the pollen from the SPT maintainer do not contain the SPT insertion. For the seeds used in the molecular experiment described below, a transgenic 32138 SPT maintainer (used as a pollen donor or pollinator) was crossed to a male-sterile *ms45/ms45* maize line non-transgenic for SPT. The seed from the male-sterile line was hand harvested and passed twice through an optical sorter to identify and separate any red fluorescent SPT transgenic seed from the non-fluorescent, non-transgenic SPT progeny seed (Appendix 2).

Non-fluorescent (yellow) seeds (15,000 seed) were randomly selected from a sorted population and planted in the greenhouse. Two leaf samples (punches) were collected from each seedling and genomic DNA was extracted from each leaf sample. Each genomic DNA sample was analyzed using Real-Time PCR assays, for the three introduced SPT genes from the T-DNA region of plasmid PHP24597 (Figure 13): *Ms45*, *zm-aa1*, and *DsRed2(Alt1)*. The three gene assays on the samples were run in independent PCR reactions. Nearly every plant was analyzed in duplicate, with ten samples giving low DNA results in the first round of analysis and 25 samples in the second round of analysis (Appendix 4-Table 1). In total, all 15,000 plants were analyzed at least once in the Real-Time PCR analysis.

In summary for both rounds of analysis, 14,998 plants tested negative for all three gene PCR assays (Appendix 4-Table 1). Positive and negative control samples gave the expected results during each run (data not shown). Two of the tested plants had unexpected results. One progeny maize plant was negative for the *Ms45* and *DsRed2(Alt1)* PCR assays, but tested positive in both rounds of the analysis for the *zm-aa1* PCR assay. A second plant that had been negative for all assays in the first round of analysis was positive for the *DsRed2(Alt1)* assay and negative for the *zm-aa1* and *Ms45* assays in the second PCR round.

Table 1. Results of Qualitative Real-time PCR Analysis

Analysis Round[1]	Total Samples Analyzed	Negative for All PCR Assays	Low DNA Result (No Sample)[2]	Low DNA Result (Other)[3]	Positive for *zm-aa1* PCR[4]	Positive for *DsRed2(Alt1)* PCR[5]
1	15,000	14,989	4	6	1	0
2	15,000	14,973	21	4	1	1

Footnotes:
1. Two individual leaf samples were collected from each plant and DNA was extracted from each sample and analyzed by Real-Time PCR.
2. Missing leaf punch or cracked extraction tube caused absence of DNA sample.
3. No reason determined for low DNA result.
4. Negative for *Ms45* and *DsRed2(Alt1)*.
5. Negative for *Ms45* and *zm-aa1*.

The single plant that was positive for the *zm-aa1* but negative for the *Ms45* and *DsRed2(Alt1)* in the both the first and second round of PCR assays was investigated further to determine the origin of the *zm-aa1* gene. Sequencing of the DNA insertion in this plant demonstrated that the positive *zm-aa1* PCR assay was the result of the presence of a different T-DNA source plasmid that contained a similar *zm-aa1* cassette and was not from plasmid PHP24597. Therefore, the *zm-aa1* positive result was attributed to the presence of a single seed containing a different T-DNA and was not due to the transmission of the 32138 SPT insertion to the male-sterile progeny.

The plant that was positive for only the *DsRed2(Alt1)* PCR assay in the second round of analysis was attributed to sample contamination by DNA positive for *DsRed2(Alt1)*, as the same plant was negative for all three assays in the first round of analysis and negative for the *zm-aa1* and *Ms45* PCR assays in the second round. Duplicate leaf punches were prepared independently and it was possible that contamination could have occurred during extraction or during the individual set-up of the *DsRed2(Alt1)* assay. This sample was positive for the maize endogenous reference gene in all PCR assays in the first round of analysis, indicating that the DNA sample was adequate and it was not a false negative result. The negative results for the *Ms45* and *zm-aa1* assays in both rounds of analysis of this plant also indicate the absence of the PHP24597 T-DNA insertion.

Conclusions

Based on the two independent rounds of PCR screening, none of the 15,000 male-sterile progeny plants originating from 32138 SPT maintainer event DP-32138-1 contained cloned genes derived from plasmid PHP24597. This demonstrates the extremely high efficiency of the SPT system in preventing the transfer of the 32138 SPT insertion to the male-sterile maize progeny.

Appendix 5

Overall Efficiency of the SPT Process

In order to calculate the overall efficiency of the SPT Process, it is necessary to take into account both the genetic efficiency and the mechanical sorting accuracy.

Genetic efficiency is defined as the ability of the 32138 SPT maintainer to prevent transmission of the 32138 SPT insertion to progeny seed when the 32138 SPT maintainer is used as a pollen source (pollinator). As shown in Appendix 2, under both hand-pollinated and open-pollinated field conditions, the genetic efficiency of the SPT system was never less than 99.997% and overall was >99.999%.

Mechanical color sorting accuracy is defined as the ability of the mechanical color sorters to accurately identify and separate red fluorescing seed from yellow seed. As demonstrated in Appendix 3, the mechanical color sorting accuracy was determined to be >99.95%. Embedded in this accuracy is the fact that the DsRed2 protein is an accurate and reliable color marker for determining the presence of 32138 SPT insertion, which was demonstrated in Appendix 4.

The overall efficiency of the SPT process can be calculated by combining the genetic efficiency and mechanical sorter accuracy:

> The genetic efficiency of the SPT system is >99.999%.
>
> The mechanical sorter accuracy is >99.95%.
>
> This means that of the possible <0.001% of the SPT progeny that might contain the 32138 SPT insertion (*i.e.* red fluorescing seed), the color sorter will accurately identify and sort these with >99.95% accuracy, or will only fail <0.05% of inaccuracy to separate red fluorescing seed from yellow seed.

Therefore, the overall efficiency of the SPT process is 100% - (0.001% × 0.05%) or >99.9999995%.

Appendix 6

Inbred Parent Seed Production Practices Using the SPT

As part of its normal inbred parent inbred seed production process, Pioneer adheres to many practices designed to produce high-quality, genetically pure inbred seed. With the use of the SPT process, additional procedures will be put in place to ensure that seeds containing the 32138 SPT insertion are not inadvertently carried forward into hybrid seed production or commercial seed.

6.1. Planting and Isolation

- All 32138 SPT maintainer inbred seed will be uniquely dyed with a blue color.
- All seed production will meet minimum isolation distances required by government regulations for certification of seed products.
- For male-sterile female inbred parent increase, 32138 SPT maintainer will be planted in either a 2:2 or 4:4 or 4:2 planting pattern (male-sterile female: 32138 SPT maintainer rows). The first row and the last row must be 32138 SPT maintainer (pollinator) rows.

6.2. Roguing (Removal of Off-Types in the Field)

- Roguing needs are determined and documented according to inbred parent corn seed production location and upon visual inspection for off types by experienced and qualified field personnel.
- Any off-type plants (rogues) identified will be destroyed.

    i. Male-sterile rows: Any fertile plants detected in the male-sterile rows will be destroyed.
    ii. 32138 SPT maintainer rows: Any sterile plants detected in the maintainer rows will be destroyed.

6.3. Harvesting

- 32138 SPT maintainer and male-sterile inbreds will be harvested and kept separately.

6.4. Sorting, Drying, and Shelling of Maize Ears

- All off-type ears will be removed at sorting for both 32138 SPT maintainer and male-sterile lines.
- For quality control purposes, sorting, drying and shelling of 32138 SPT maintainer inbreds will be done in a small lot handling system.

6.5. Optical Seed Sorter Calibration

- The calibration of the seed sorter (Sataki ScanMaster II, Satake Corporation, Houston, TX, USA) will be verified daily before use according to the manufacture's instructions for maintaining the equipment and to specifications described in a Pioneer standard operating procedure (SOP).
- Calibration records will be maintained locally and archived.

6.6. Seed Conditioning, Treatment, and Packaging

32138 SPT maintainer (Color Sort A):

- 32138 SPT maintainer seed will be conditioned in a building dedicated exclusively to conditioning SPT maintainers.
- All 32138 SPT maintainer seed will be color sorted using two sequential sorting steps. Records from each production run will be archived.
- The reject portion of the maintainer shall be discarded using Regulatory Compliance ISO 9000 discard procedures and shall not be used for any other purpose.
- Following the second color sorting process of SPT materials, samples will be collected for a purity check.
- All 32138 SPT maintainer inbreds will be dyed with a blue color.
- Parent corn seed designated as MAINTAINER will be packaged in bags clearly marked MAINTAINER and labeled per instructions.

Male-Sterile Progeny (Color Sort B):

- All SPT male-sterile progeny will be color sorted twice to remove 32138 SPT maintainer (red fluorescent) seed.
- Following color sorting, a sample will be collected for purity check.
- If an 32138 SPT maintainer seed is detected, the seed lot will be color sorted again.
- Results will be recorded and archived.
- To monitor the efficacy of the SPT process, discarded seed will be collected and analyzed further for the presence of any maintainer seed. Results will be recorded and archived.
- Any discard seed shall be destroyed using Regulatory Compliance ISO 9000 procedures and shall not be used for any other purpose.

6.7. Record Maintenance

- All records will be retained for a minimum of 5 years.
- Records will be available for audit upon written request.

Appendix 7

Potential for Linkage Break between the Cassettes of the 32138 SPT Insertion and Consequences for the SPT Process

Pioneer has demonstrated that the SPT process is extremely stable and efficient in ensuring that the 32138 SPT insertion is not transmitted during the seed production process. However, there are potential scenarios, described below, under which the genetic components of the SPT system might become decoupled (*i.e.* linkage break between the cassettes *via* genetic recombination). These occurrences are rare and would not be expected to occur based on the biological mechanisms or series of events that would need to occur for heritability. If a linkage break between the cassettes does occur in the 32138 SPT insertion the effects of such occurrences could be detected and these seed eliminated from the seed supply, thus not compromising the efficiency of the SPT process.

7.1. Genetic Recombination Mechanisms

The possibility of a linkage break between the gene cassettes of the 32138 SPT insertion in 32138 SPT maintainer would be rare. Research on meiotic recombination, crossover rates based on genomic location, and somatic recombination are summarized below and indicate that such occurrences resulting in linkage break are rare.

The insertion in 32138 SPT maintainer is maintained in the hemizygous state because of α-amylase expression. As discussed previously, pollen containing the insertion will be non-functional because of the α-amylase enzyme activity and, therefore, only pollen without the insertion will be successful in pollination. Because 32138 SPT maintainer plants can only be hemizygous (SPT/-) for the insertion, the likelihood of a linkage break by meiotic recombination mechanisms within the T-DNA insertion is rare. In plants, meiotic recombination occurs between regions of identical homology on the chromosomes that pair and form chiasmata (Hamant *et al.*, 2006). Furthermore, strict pairing of chromosomes and chiasmata formation occurs because of the protein complexes and machinery involved. This strict pairing of chromosomes has been observed in allopolyploid species and is evidence of the tightness of these conserved meiotic mechanisms; only identical chromosomes will pair and form chiasmata and genetically-related non-identical chromosomes (homeologous chromosomes) will not pair with each other (Hamant *et al.*, 2006). The lack of a homologous region for pairing in the 32138 SPT insertion would make crossovers within the insertion rare; any recombination that might occur would have to take place via illegitimate recombination mechanisms.

Furthermore, the 32138 SPT insertion is located near a centromeric region of the maize genome. Centromeric regions have a low level of recombination that has been confirmed by molecular mapping as well as by microscopic examination of maize chromosomes (Anderson *et al.*, 2003). Based on the chromosomal location of the 32138 SPT insertion and positions of nearby mapping markers, in general, the average frequency of meiotic recombination in this region was calculated to be approximately 0.09 cM/Mb, 10-fold lower than the average maize meiotic recombination frequency of 1 cM/Mb (Fengler *et al.*, 2007; personal communication, K. Fengler). However, based on the above discussion, hemizygosity of the insertion would eliminate the possibility of meiotic crossover via legitimate recombination mechanisms, thus these numbers are applicable only to surrounding genomic regions and not representative of recombination within the 32138 SPT insertion.

Recombination in somatic cells may have an impact on linkage within the 32138 SPT insertion, but would also be expected to be extremely rare. The primary concern for such recombination would be a double-strand break repair in genomic DNA that could potentially

become heritable (Puchta, 2005). Somatic recombination mechanisms occur primarily as a result of DNA damage and double-strand break repair (Puchta, 2005; Schuermann *et al.*, 2005). In general, these repair mechanisms rely on template information from homologous regions located at an allele on a sister chromatid (allelic recombination), elsewhere on the chromosome (interchromasomal recombination), or elsewhere in the genome (ectopic recombination) (Puchta, 2005). Based on scientific research, rates of allelic and ectopic recombination are similar and have been estimated to be about 10^{-3} to 10^{-5} in tobacco transformants with double-strand breaks induced experimentally via an active maize *Ac* transposon element or a rare cutting enzyme (Shalev and Levy, 1997; Puchta, 1999; Puchta, 2005). In these same experiments, the rarity of somatic recombination was also shown in the absence of induced double-stranded breaks, since no changes to the transgenic insertion were detected (Shalev and Levy, 1997; Puchta, 1999). Repair from non-homologous regions can also occur, creating repair junctions that have been altered from the original region (Salomon and Puchta, 1998; Puchta, 2005). Non-homologous recombination mechanisms have also been studied via the induction of double-strand breaks (Salomon and Puchta, 1998), so the frequency of these types of repairs is not well known in the endogenous genome. Further, the probability of a somatic recombination event occurring in the 32138 SPT maintainer and being inherited is rare, since it requires the chance of a double-stranded break repair within a small pool of cells of the flower meristem

In addition, transposons (*Ac* and *Ds* elements) have been implicated in maize genome breakage, recombination and rearrangements (Ralston *et al.*, 1989; Dooner and Belachew, 1991; Weil and Wessler, 1993; Zhang and Peterson, 1999). In general, chromosomal changes are associated with linked elements and the presence of active *Ac* elements (Ralston *et al.*, 1989; Dooner and Belachew, 1991; Weil and Wessler, 1993; Zhang and Peterson, 1999). While there is evidence from these studies that these elements are points of chromosomal breaks, recombination, and rearrangement if all the necessary elements are present, any rearrangements that may occur in the 32138 insertion in maize 32138 SPT maintainer would not likely result in a linkage break within the insertion. Transposon-like elements are not present within the 32138 SPT insertion but may be present in the neighboring genome and any rearrangements that may occur would move the insertion as a unit, thus not affecting the linkage of the gene cassettes.

7.2. Description of Few Possible Scenarios

Given this body of research and knowledge about meiotic recombination, somatic recombination, and transposon rearrangement, the rare possibility exists for a linkage break within the 32138 SPT insertion. The impact of several possible scenarios has been assessed to determine the ability to detect recombination of the SPT insertion (Appendix 7-Table 1). In most cases, deletion of one or two of the three SPT genes (*Ms45*, *zm-aa1* and *DsRed2*) from a single break (Appendix 7-Figure 1Scenarios 1 and 2) gives rise to seed of the wrong color that will be removed during sorting, or to plants with phenotypes opposite to those expected (*i.e.,* male-fertile or male-sterile) that will be removed after routine inspection as part of quality control practices (Appendix 7-Table 1). In the extraordinarily rare occurrence of two breaks between each of the cassettes deleting *zm-aa1* (Appendix 7-Figure 1 Scenario 3), the proportion of seed observed on each ear in the sorting process would indicate an aberrant plant genotype and would be removed from use (Appendix 7-Table 1). In the case of the deletion of the *Ms45* and *DsRed2* genes, the *zm-aa1* gene from the SPT insertion would likely be passed on to the next generation, however the progeny would be sterile and not propagated further (Appendix 7-Table 1).

7.3. Conclusions

The occurrence of genetic recombination mechanisms affecting the linkage of the cassettes of the 32138 SPT insertion are rare and would not be expected to occur based on the

biological mechanisms or series of events that would need to occur for heritability. In the case that a linkage break did occur between the cassettes of the 32138 SPT insertion, Pioneer has considered the impact on resulting progeny and the overall SPT process. After considering a few representative possible scenarios and the resultant phenotypes, Pioneer concludes that in these scenarios the progeny seed or plants would most likely be identified, removed, or not propagated further.

Figure 1. Aberrant Gene Recombination in the SPT Insert—A Few Possible Scenarios

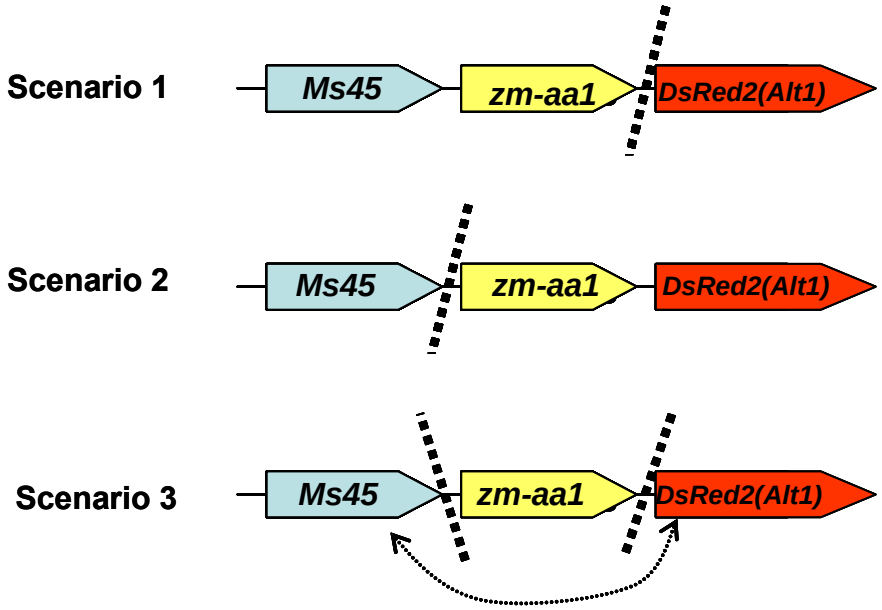


Table 1. Representative Scenarios of Recombination within the 32138 SPT Insertion and Traceability in the SPT Process

		Seed Increase of SPT maintainer by Self-Pollination		**Seed Increase of the Male-Sterile Female Inbred Parent using SPT Maintainer as a Pollinator (in the Case of a Recombination Event in the SPT Pollen).**		
Scenario #	**Gene(s) Deleted**	**Will Color Sort A be Able to Detect and Discard the Seed?**	**Impact on 32138 SPT maintainer Seed Increase (Step I, Figure 3)**	**Will Color Sort B be Able to Detect and Discard the Seed?**	**Impact on Male-Sterile Female Inbred Parent Seed Increase (Step II, Figure 3) and Commercial Hybrid Seed Production (Step III, Figure 3)**	**Impact on Commercial Maize Seed and Grain Production (Step III & IV, Figure 3)**
1	*DsRed2(Alt1)*	**Yes**	**None** Will be discarded as yellow seed during Color Sort A	**No**	Yellow seed planted in female inbred parent rows (Step III, Figure 3), but is an obvious off-type plant (fertile) that will be removed (rougued) before pollen shed	**None**
	Ms45 + zm-aa1	**No**	**None** Pinkish red color seed may be planted in maintainer rows but is an obvious off-type (male-sterile) that will be removed (rougued).	**Yes**	**None** The red fluorescing seed will be removed during Color Sort B and will not enter Hybrid Seed Production Fields (Step III, Figure 3).	**None**
2	*Ms45*	**No**	**None** Pinkish red color seed may be planted in maintainer rows but is an obvious off-type (male-sterile) that will be removed (rougued).	**Yes**	**None** The red fluorescing seed will be removed during Color Sort B and will not enter Hybrid Seed Production Fields (Step III, Figure 3).	**None**
	zm-aa1+ DsRed2(Alt1)	**Yes**	**None** Will be discarded as yellow seed during Color Sort A	**No**	Yellow seed planted in female inbred parent rows (Step III, Figure 3), but is an obvious off-type plant (fertile) that will be removed (rougued) before pollen shed	**None**

Table 1. Representative Scenarios of Recombination within the SPT Insertion and Traceability in the SPT Process (continued)

		Seed Increase of SPT maintainer by Self-Pollination		Seed Increase of the Male-Sterile Female Inbred Parent using SPT Maintainer as a Pollinator (in the Case of a Recombination Event in the SPT Pollen).		
Scenario #	**Gene(s) Deleted**	**Will Color Sort A be Able to Detect and Discard the Seed?**	**Impact on 32138 SPT maintainer Seed Increase (Step I, Figure 3)**	**Will Color Sort B be Able to Detect and Discard the Seed?**	**Impact on Male-Sterile Female Inbred Parent Seed Increase (Step II, Figure 3) and Commercial Hybrid Seed Production (Step III, Figure 3)**	**Impact on Commercial Grain Production (Step IV, Figure 3)**
3	*zm-aa1*[4]	**No**	Pinkish Red seed may be planted in maintainer rows. Upon self-pollination, instead of 50:50 (red:yellow) segregation, it will result in 75:25 (red : yellow) seed. Incorrect ratio can be detected during visual inspection of 32138 SPT maintainer ears prior to Color Sort A (Appendix 6.4) and ears will be discarded.	**Yes**	**None** The red fluorescing seed will be removed during Color Sort B	**None**
	Ms45 + *DsRed2(Alt1)*[4]	**Yes**	**None** Will be discarded as yellow seed	**No**	**None** Pollen containing the gene deletions will be infertile due to α-amylase and therefore virtually no transmission of transgene to male- sterile female inbred seed and hybrid seed (Steps II and III Figure 3).	**None**

[4]Two recombination events would be required to delete *Ms45* and *DsRed2(Alt1)* while retaining α-amylase, which would be extraordinarily rare

Appendix 8

Materials and Methods for Molecular Characterization of 32138 SPT Maintainer

To characterize the DNA insertion in 32138 SPT maintainer, Southern blot analysis was conducted. Individual plants of the T1S1 generation were analyzed to determine the copy number of each of the genetic elements inserted and to verify the integrity of the PHP24597 T-DNA. The insertion in 32138 SPT maintainer was characterized using *Bmt* I, *Bam*H I, *Bam*H I/*Hin*d III, and *Eco*R I restriction enzyme digestion. Southern blot analysis was conducted on individual plants of two generations, T1S1 and BC3, to confirm insert stability across generations and to verify the absence of backbone sequences from plasmid PHP24597. An additional generation, BC4, was analyzed to verify insertion stability and to confirm Mendelian segregation of the insertion (discussed in Section V-E). All probes used for the analysis are indicated on the schematic maps of plasmid PHP24597 and its T-DNA (Section V, Figures 15 and 16, respectively) and outlined in Section V, Table 3.

8.1. 32138 SPT Maintainer Material

Seeds from the T1S1, BC3, and BC4 generations (Figure 12—breeding diagram) of 32138 SPT maintainer were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction. For the T1S1, red (*i.e.* seeds containing the 32138 SPT insertion) and yellow seeds were planted and only the plants from the pinkish red seed were used for analysis. For the BC4 generation, both red and yellow seeds (a segregating population) were planted and analyzed (Shown in Figures 33, 34, and 35).

8.2. Control Material

Seeds from the unmodified lines of Hi-II, Hi-II (*ms45*), and inbred 705 were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction. These control DNA samples were used as negative controls to help interpret hybridization results since the *5126* promoter, *Ms45*, *Pg47* promoter, *zm-bt1*, *zm-aa1*, and *In2-1* terminator probes cross-hybridize with endogenous maize sequences.

8.3. Reference Material

Plasmid DNA from PHP24597 was amplified in *E. coli* and was used as a positive control for Southern analysis to verify probe hybridization and to verify sizes of internal fragments. The plasmid stock was a copy of the plasmid used for *Agrobacterium*-transformation experiments to produce 32138 SPT maintainer and was digested with restriction enzymes to confirm the plasmid map. The probes used in this study (Table 3) were derived from plasmid PHP24597 or from a plasmid containing equivalent genetic elements.

DNA molecular weight markers for gel electrophoresis and Southern blot analysis were used to determine approximate molecular weights. For Southern analysis, DNA Molecular Weight Marker VII, digoxigenin (DIG) labeled (Roche, Indianapolis, IN), was used as a size standard for hybridizing fragments. ΦX174 RF DNA/Hae III Fragments (Invitrogen, Carlsbad, CA) was used as a molecular weight standard to determine sufficient migration and separation of the fragments on the gel.

8.4. Genomic DNA Extraction

Genomic DNA was extracted from leaf tissue harvested from individual plants as described above. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep, Inc., Metuchen, NJ) instrument and the genomic DNA isolated using a

urea-based procedure (modification from Chen and Dellporta, 1994). Approximately 1 gram of ground tissue was extracted with 5 mL Urea Extraction Buffer (7 M Urea, 0.34 M NaCl, 0.05 M Tris-HCl, pH 8.0, 0.02 M EDTA, 1% N-Lauroylsarcosine) for 12-30 minutes at 37°C, followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and one extraction with water saturated chloroform. The DNA was precipitated from the aqueous phase by the addition of 1/10 volume of 3 M NaOAc (pH 5.2) and 1 volume of isopropyl alcohol, followed by centrifugation to pellet the DNA. After washing the pellet twice with 70% ethanol, the DNA was dissolved in 0.5 mL TE buffer (10mM Tris, 1 mM EDTA, pH 7.5) and treated with 10 μg Ribonuclease A for 15 minutes at 37°C. The sample was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with water saturated chloroform, followed by precipitation with isopropyl alcohol and washing with 70% ethanol. After drying, the DNA was re-dissolved with 0.5 mL TE buffer and stored at 4°C.

8.5. Quantitation of Genomic DNA

Following extraction, the DNA was quantified on a spectrofluorometer using PicoGreen® reagent (Molecular Probes, Inc., Eugene, OR) following a standard procedure. The DNA was also visualized on an agarose gel to confirm quantitation values from the PicoGreen® analysis and to determine DNA quality.

8.6. Phenotypic Identification of 32138 SPT Maintainer Seed

Phenotypic analysis of 32138 SPT maintainer was carried out by visual observation of the seed prior to planting. The color of the seed was determined and documents as either red (containing the 32138 SPT insertion) or yellow (not containing the 32138 SPT insertion) as an indication of the seed and plant phenotype.

A preliminary Southern blot analysis of DNA isolated from all 32138 SPT maintainer plants was used to confirm the presence of the 32138 SPT insertion and the *Ms45, ZM-AA1,* and *DsRed2(Alt1)* genes. Methods for this preliminary characterization are described below. For the T1S1 and BC3 generations, the final Southern blot analysis was carried out on a subset of 32138 SPT maintainer plants.

8.7. Digestion of DNA for Southern Blot Analyses

Genomic DNA samples extracted from selected 32138 SPT maintainer and inbred control maize plants were digested with restriction enzymes following a standard procedure. Approximately 2 μg of genomic DNA was digested in a volume of 100 μL using 50 units of enzyme according to manufacturer's recommendations. The digestions were carried out at 37°C for three hours, followed by ethanol precipitation with 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of 100% ethanol. After incubation at 4°C and centrifugation, the DNA was allowed to dry and re-dissolved in TE buffer. The reference plasmid, PHP24597, was spiked into a control plant DNA sample in an amount equivalent to approximately one or three gene copies per maize genome and digested with the same enzyme to serve as a positive control for probe hybridization and to verify sizes of internal fragments on the Southern blot.

8.8. Electrophoretic Separation and Southern Transfer

Following restriction enzyme digestion, the DNA fragments produced were electrophoretically separated by size through an agarose gel and a molecular weight standard [ΦX174 RF DNA/Hae III Fragments (Invitrogen)] was used to determine sufficient migration and separation of the fragments on the gel. DIG labeled DNA Molecular Weight Marker VII (Roche), visible after DIG detection as described below, was used to determine hybridizing fragment size on the Southern blots.

Agarose gels containing the separated DNA fragments were depurinated, denatured, and neutralized in situ, and transferred to a nylon membrane in 20x SSC buffer (3M NaCl, 0.3 M Sodium Citrate) using the method as described for the TURBOBLOTTER™ Rapid Downward Transfer System (Schleicher & Schuell, Keene, NH). Following transfer to the membrane, the DNA was bound to the membrane by UV crosslinking (Stratalinker, Stratagene, La Jolla, CA).

8.9. DNA Probe Labeling for Southern Blot Hybridization

Probes homologous to the *5126* promoter, *Ms45*, *Pg47* promoter, *zm-bt1*, *zm-aa1*, *In2-1* terminator, 35S enhancer, *Ltp2* promoter, *DsRed2(Alt1)*, and *pin*II terminator were used to detect genes and elements within the insertion (Table 3). Backbone probes (*spc*, *tet*, *vir*G, LB, and RB) for the PHP24597 plasmid were used to verify absence of plasmid backbone DNA in 32138 SPT maintainer (Table 3). DNA fragments of the probe elements were generated by PCR from plasmid PHP24597 (Figure 15) or a plasmid with equivalent elements using specific primers. PCR fragments were electrophoretically separated on an agarose gel, excised and purified using a gel purification kit (Qiagen, Valencia, CA). DNA probes were generated from these fragments by PCR that incorporated a DIG labeled nucleotide, [DIG-11]-dUTP, into the fragment. PCR labeling of isolated fragments was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

8.10. Probe Hybridization and Visualization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized to a labeled probe. Labeled probes were hybridized to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG Easy Hyb solution (Roche). After stringent washes, the hybridized DIG-labeled probes and DIG-labeled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Blots were exposed to X-ray film for one or more time points to detect hybridizing fragments and to visualize molecular weight standards. Images were then digitally captured by detection with the Luminescent Image Analyzer LAS-3000 (Fujifilm Medical Systems, Stamford, CT). Digital images were compared to original X-ray film exposures as verification for use in this report. The sizes of detected bands were documented for each digest and each probe.

8.11. Stripping of Probes and Subsequent Hybridizations

Following hybridization and detection, membranes were stripped of DIG-labeled probe to prepare the blot for subsequent re-hybridization to additional probes. Membranes were rinsed briefly in distilled, de-ionized water and then stripped in a solution of 0.2 N NaOH and 1.0% SDS at 40°C with constant shaking. The membranes were then rinsed in 2x SSC and either used directly for subsequent hybridizations or stored at 4°C or -20°C for later use. The alkali-based stripping procedure effectively removes probes labeled with the alkali-labile DIG.

Appendix 9

Materials and Methods for the Determination of MS45, ZM-AA1 and DsRed2 Protein Concentrations in 32138 SPT Maintainer

The MS45 protein levels in 32138 SPT maintainer was visualized using Western blot analysis and the concentration of ZM-AA1 and DsRed2 proteins were determined using an enzyme linked immunosorbent assays (ELISA) and a fluorometric assay, respectively.

9.1 Storage and Processing of Maize Tissue Samples

Upon receipt, all plant tissue samples were stored in temperature-monitored freezers at <-10°C. Forage samples were coarsely homogenized on dry ice using a Stephan VCM 12 (Stephan Machinery Singapore Pte Ltd, Singapore) blender for approximately 2 minutes and sub-sampled. All samples were lyophilized at <-12°C under vacuum until dry. The lyophilization time varied between 18 to 72 hours depending on the sample size and tissue type. Forage and seed tissues were finely ground for approximately 60 seconds using a GenoGrinder (BT&C/OPS Diagnostics, Metuchen, NJ, USA).

Two samples of four anthers each were collected for each developmental stage from plants derived from the test, control, and reference lines and used for protein extraction. Between lyophilization and grinding, samples were stored frozen in temperature-monitored freezers at <-10°C.

Tissue samples, with the exception of developing anther samples for Western blot analysis, were weighed into 1.2 ml tubes at the following target weights (+/- 5%); 5 mg for pollen, 10 mg for leaf, 20 mg for grain and 30 mg for whole plant and stalk tissue. Developing anther samples were transferred to a 1.2 ml tube (4 anthers per stage per tube) without weighing and were kept frozen <-10°C until analysis.

Inbred seed samples derived from BC1S2 and BC3S2 generations of 32138 SPT maintainer consisted of red and yellow seed in ~1:1 ratio. Each seed sample was color sorted by hand into red and yellow seeds and the purity of red kernel sample was confirmed by a fluorescence assay using a light box. All kernel samples remained frozen during the color sorting process. Yellow kernel samples were used as negative controls for MS45 Western blot analysis. Red kernel samples (32138 SPTmaintainer) were lyophilized under vacuum until dry, and were finely ground and stored frozen until analysis.

9.2 Protein Extraction from Processed Maize Tissues for MS45 Western Blot Analysis

For MS45 western blot analysis, all samples were extracted in 600 µl of 1X LDS sample buffer (prepared using the following ratio of 65% PBST, 25% 4X LDS sample buffer and 10% 10X reducing buffer containing DTT) with the addition of two 5/32” steel balls per tube in a genogrinder for a single 30 sec cycle at a setting of 1500 strokes per minute. Extracts were centrifuged until separated in a refrigerated centrifuge and supernatants were prepared for electrophoresis.

9.3. Preparation of MS45 Standard Solution for Western Blot Analysis

MS45 protein standard was diluted to the following concentrations; 200, 100, and 50 ng/ml in 1XLDS sample buffer prepared using the following ratio of 65% PBST, 25% 4XLDS sample buffer and 10% 10X reducing buffer containing DTT.

9.4. SDS-PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 4-12% Bis-Tris Gels (Invitrogen, #NP0322). Samples that were heated for 5-15 minutes at 70°C then stored on either ice, or kept frozen were briefly warmed (less than 2 minutes) at 70°C prior to loading. Samples were loaded at 20 μl /well and SeeBlue® Pre-stained Standard (Invitrogen, #LC5625) was loaded at 10 μl/well. Electrophoresis was conducted using a XCell *SureLock*™ Mini-Cell electrophoresis unit (Invitrogen, #EI0001) with NuPAGE® MES SDS Running Buffer (Invitrogen, #NP0002) and a constant voltage setting of 200V for approximately 30 min or until the dye front neared the bottom of the gel.

9.5. Western Blot Analysis

Following SDS-PAGE, gels were removed from the gel cassette and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Invitrogen) for approximately 7 minutes using an iBlot Module (Invitrogen Catalog # IB1001) according to manufacturer's instructions. Following the transfer, the PVDF membrane blots were blocked with a 5% low fat milk/PBST solution followed by at least 60 minute at room temperature or overnight at 4ºC incubation with a primary antibody diluted in 1% low fat milk/PBST solution (mouse monoclonal antibody 6B3.F7 lot# 090104 diluted at 1:2,500). The blots were washed with PBST at least three times, with each wash for at least 10 minutes and then incubated for at least 60 minutes with the secondary antibody conjugated to horseradish peroxidase (HRP) from Promega, (Anti-mouse IgG-HRP conjugate diluted 1:25,000. The unbound secondary antibody-HRP conjugate was removed with additional 3 washes of at least 10 minutes each in PBST.

The blots were developed using a chemiluminescent detection kit (Thermo Scientific, IL, USA) followed by detection using a Kodak 4000R Pro Imaging system utilizing the Kodak Molecular Imaging Software. Developed blots were evaluated for the presence or absence of bands with the expected migration.

9.6. Protein Extraction from Processed Maize Tissues for Determination of ZM-AA1 Protein Concentration

Each sample was extracted with 600 microliters (µl) of chilled (2-8°C) buffer solution which is comprised of 50 millimolar (mM) HEPES, 150 mM NaCl, 0.15% Tween-20, 0.5 mM $CaCl_2$. 0.5% polyethylene glycol and 5 mM Sodium metabisulfite. Two 5/32" steel balls were added to each tube, and the samples were homogenized with a single 30 second cycle at a setting of 1500 strokes per minute using a SPEX Certiprep GenoGrinder (BT&C/OPS Diagnostics). Following centrifugation in Jouan GR-422 model centrifuge at a setting of 4,000 rpm and 4°C for 10 min supernatants were removed, diluted and analyzed for ZM-AA1 protein concentrations using specific enzyme linked immunosorbent assay (ELISA).

9.7. Determination of ZM-AA1 Protein Concentration

The ZM-AA1 ELISA utilized a "sandwich" Enzyme-Linked ImmunoSorbent Assay (ELISA) for the determination of the concentration of ZM-AA1. In this assay, standards and samples were incubated in stabilized plates that have been pre-coated with a ZM-AA1-specific antibody. Unbound substances were washed from the plate and the bound ZM-AA1 was

incubated with a different ZM-AA1-specific antibody that had been conjugated to the enzyme horseradish peroxidase (HRP). After about one hour of incubation, unbound substances were washed off from the plate. The detection of the ZM-AA1-antibody complex was accomplished by the addition of a substrate that generated a colored product in the presence of HRP. The reaction was stopped after 30 minutes with 1 N Hydrochloric Acid and the optical density of each well was determined using a Molecular Devices plate reader (Molecular Devices Corporation, Sunnyvale, CA) with a wavelength setting of 450 nm minus 650 nm. SoftMax Pro software[5] was used to perform the calculations that generated the quadratic fit of the standard curve and converted the sample optical density (OD) values to ZM-AA1 protein concentration values. The mean duplicate well values in ng/ml were used in the calculation of the reported ZM-AA1 concentration of each sample (ng/mg dry weight). A characterized sample extract (QCE) was included on each plate as a control for data acceptance.

A quantitative range for the assay was 0.23 ng/ml to 5.5 ng/ml. The lower limit of quantitation (LLOQ) in ng/mg dry weight for each tissue was based on extraction volume (µl) to weight ratios, the limit of quantitation for the ELISA in ng/ml, and the dilutions used for analysis. The sample LLOQs on a ng/mg dry weight basis for ZM-AA1 were 0.28ng/mg dry weight for leaf; 0.092 ng/mg dry weight for whole plant and forage; 0.55 ng/mg dry weight for pollen; and 0.14ng/mg dry weight for seed.

9.8. Protein Extraction from Processed Maize Tissues for DsRed2 Fluorometric Analysis

Lyophilized plant tissue samples were weighed into 1.2 ml microtiter tubes at the following target weights: pollen 5 mg, leaf 10 mg, grain 10 mg, stalk 20 mg, and whole plant 20 mg. Each sample was extracted in 0.8 ml Assay Buffer (1X PBST pH 7.45, 25% StabilZyme Select[6] Buffer) and centrifuged until separated. Following centrifugation, the supernatants were removed to new tubes and diluted as applicable.

9.9. DsRed2 Fluorometric Assay – Experimental Design

Each standard sample (analyzed in triplicate wells on each assay plate), tissue sample (analyzed in duplicate wells), and quality control (QC) sample (see description below for QC samples) was dispensed into a separate assay plate well, with each well containing a final volume of 300 µl. Assay Buffer was added to unoccupied wells and the plate was analyzed within 30 minutes of plating using a fluorometer at 563 nm excitation, 600 nm emission with a 590 nm cutoff filter. Each well was read six times, and the fluorometer output was presented as a mean Relative Fluorescence Unit (RFU) value per well. SoftMax[7] Pro software was used to perform the calculations required to convert the RFU values obtained by the fluorometer to protein concentration values.

The DsRed2 fluorometric assay was validated for quantification of DsRed2 protein in lyophilized maize tissues by an assessment of the following: specificity (cross-reactivity and matrix effects), accuracy and precision (fortification recovery and assay ruggedness), extraction efficiency, and sample dilution agreement. Minimum dilutions were established based on the outcome of the dilution agreement, matrix effects, and fortification recovery results. In addition, a database was established for standard curve quality control purposes. Control of experimental bias was achieved through use of replicate testing. Quality Control (QC) samples were included in the analysis to monitor assay performance during the validation testing, and consisted of diluted standard (Assay Buffer fortified with DsRed2 at 4.0

[5] Registered trademark of Molecular Devices Corporation. Sunnyvale CA
[6] StabilZyme Select is a registered trademark of SurModics, Inc.
[7] Registered trademark of Molecular Devices Corporation.

and 0.5 µg/ml, hereafter referred to as QC1 and QC2, respectively) and diluted extracts of a DsRed2-positive grain sample (referred to as QCE). Information gathered on the QCE samples was used to help establish a baseline value and range for potential use as part of the acceptance criteria for the DsRed2 assay. The DsRed2 fluorometric assay demonstrated an acceptable level of specificity, accuracy, precision, and extraction efficiency for the quantification of DsRed2 protein from lyophilized maize tissues.

9.10. Calculations for Determining Tissue Concentration of DsRed2 Protein

a. Standard Curve

A standard curve was included on each assay plate. The equation for the standard curve was generated by the software, which used a quadratic fit to relate the mean RFUs obtained from the standards to the respective standard concentrations in nanograms per milliliter (ng/ml).

The quadratic regression equation was applied as follows: $y = Cx^2 + Bx + A$

Where x = known standard concentration and y = respective mean Relative Fluorescence Unit (RFU).

b. Sample Concentration

Interpolation of the sample concentration (µg/ml) was solved using the values for A, B, and C that were determined for the standard curve.

$$\text{Sample concentration (µg/ml)} = \frac{-B + \sqrt{B^2 - 4C(A - \text{sample RFU})}}{2C}$$

e.g.: given curve parameters of A = 0.143, B = 0.00625, C= -0.00000399 and a sample RFU = 0.249,

$$\text{Sample concentration} = \frac{-0.00625 + \sqrt{0.00625^2 - 4\left(-0.00000399\right)\left(0.143 - 0.249\right)}}{2\left(-0.00000399\right)} = 17 \text{ µg/ml}$$

c. Adjustment for Dilution Factor

Concentration values were adjusted for a dilution factor expressed as 1:N by multiplying the interpolated concentration by N.

e.g.: given a dilution factor of 1:8 and an interpolated concentration of 0.63 µg/ml,

Adjusted concentration = 8 * 0.63 µg/ml = 5.0 µg/ml

Appendix 10

Equivalency of Plant Derived and Microbially Expressed DsRed2 Protein

10.1. Method for Purification of the DsRed2 Protein from 32138 SPT Maintainer

Approximately 50 g of ground seed derived from 32138 SPT maintainer was homogenized in 250 ml Buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM sodium chloride and EDTA-free Complete Protease Inhibitor Cocktail [Roche Applied Science, Indianapolis, IN]). Particulate material in the resulting slurry was removed by filtering through four layers of cheese cloth. The extract was further clarified by centrifugation for 20 minutes at 30,500 *g* and filtration through 0.45 μm membrane.

Proteins in the extract were subjected to ammonium sulfate fractionation (45-65% saturation). The precipitated protein was resuspended in Buffer B (50 mM Tris, pH 8.0, 50 mM NaCl, 0.8 M ammonium sulfate and 1 mM EDTA). The sample was then applied to a Phenyl Sepharose[c] column (Cat. # 17-1355-01, GE Healthcare Bio-Sciences, Piscataway, NJ). The bound protein was eluted from the column with a linear ammonium sulfate gradient mixed with Buffer B and Buffer C (50 mM Tris, pH 8.0, 50 mM NaCl and 1 mM EDTA). The peak fractions containing DsRed2 protein were pooled and the buffer of the sample was exchanged to Buffer D (50 mM Tris, pH 8.5, 50 mM NaCl and 1 mM EDTA). The resulting sample was loaded on to a Mono Q[8] column (Cat. # 17-5166-01, GE Healthcare Bio-Sciences). The bound protein was eluted from the column with a linear NaCl gradient obtained by mixing Buffer D with Buffer E (50 mM Tris, pH 8.5, 1 M NaCl and 1 mM EDTA). DsRed2 protein fractions were pooled and diluted with Buffer F (25 mM MES, pH 5.9, 50 mM NaCl and 1 mM EDTA). Proteins in the resulting sample were separated by a Mono S[8] column (Cat. # 17-5168-01, GE Healthcare Bio-Sciences). After buffer exchange into Buffer D, the DsRed2 protein sample was loaded back to the Mono Q[8] column for further purification as described above. The purified protein was stored at -80°C with or without concentration by Nanosep[9] 3K concentrators (Pall Corporation, NY).

10.2. Method for Purification of the DsRed2 Protein Expressed in *E. coli*

The microbial DsRed2 protein was expressed in *E. coli* strain BL21 (DE3) as a fusion protein containing a His-thioredoxin tag and was purified using an immobilized metal affinity column (TALON[10] Metal Affinity resin, Clontech, Cat. # 635503,1290 Terra Bella Avenue, Mountain View, CA 94043, USA). After the fusion protein was eluted from the TALON column, the tag was cleaved in solution with thrombin (Calbiochem, Cat. # 605195, EMD Biosciences, Inc.). Thrombin cleavage resulted in two additional N-terminal amino acid residues (glycine and serine) that are not part of the DsRed2 protein. Following thrombin cleavage, the protein was concentrated and buffer exchanged by tangential flow filtration. Thrombin was removed by chromatography on HiTrap[b] SP Sepharose[11] HP columns (Cat. # 17-1151-01, GE Healthcare Bio-Sciences, Piscataway, NJ). Diafiltration was used to remove the cleaved fusion tag and exchange the buffer to 50 mM ammonium bicarbonate, pH 7.5. The purified DsRed2 protein solution was lyophilized and the resulting powder was stored at -80˚C.

[8] Registered trademark of GE Healthcare Bio-Sciences AB
[9] Registered trademark of Pall Corporation
[10] Registered trademark of Clontech
[11] Registered trademark of GE Healthcare Bio-Sciences AB

10.3. SDS-PAGE Analysis Method

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by first mixing DsRed2 protein samples with Laemmli sample buffer (Gradipore Limited, 22 Rodborough Road, Frenchs Forest NSW 2086, Australia) containing 100 mM dithiothreitol and heating the solution to 100°C for approximately five minutes. The prepared protein samples were loaded into a 10-20% gradient Tris-HCl Ready Gel[12] (Bio-Rad Laboratories, Inc., CA). PageRuler[13] Prestained Protein Ladder (#SM0671; Fermentas, Inc., Maryland, USA) molecular weight markers were loaded into the gel to provide a visual estimate of molecular weight. Electrophoresis was conducted using the Ready Gel Cell system (Bio-Rad Laboratories, Inc) with Tris-glycine running buffer (Gradipore Limited) at a constant 150 volts (V) for ~60 minutes or until the dye front neared the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassette and washed three times with deionized water for approximately five minutes each. The gels were then stained for approximately 60 minutes with GelCode[14] Coomassie Blue stain reagent (Pierce Biotechnology, Inc., IL), and rinsed with deionized water at least four times for approximately ten minutes each, or until the background of the gel was clear.

10.4. Western Blot Analysis Method

Following SDS-PAGE, the resulting gel was soaked in Transfer buffer (48 mM Tris-HCl, pH 8.6, 39 mM glycine, 0.0375% sodium dodecyl sulfate, and 20% methanol) for approximately 20 minutes. A polyvinylidene difluoride (PVDF, Bio-Rad Laboratories, Inc.) membrane was briefly placed in 100% methanol, followed by immersion in Transfer buffer for 10-15 minutes. A Trans-Blot[15] SD Semi-Dry Electrophoretic Transfer Cell system (Bio-Rad Laboratories, Inc.) was used to transfer proteins from the gel to the membrane at 10 V for ~45 minutes. Following protein transfer, the membrane was then washed three times for five minutes each in Classic buffer (50 mM Tris-HCl, pH 7.0, 500 mM NaCl, 0.5% Tween[16]-20), and then blocked by incubating in phosphate-buffered saline solution with Tween-20 (PBST: 8.1 mM phosphate buffer, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20) containing 5% (w/v) non-fat dry milk for 60 minutes. The blocked membrane was washed three times for five minutes each in Classic buffer, and then incubated for 60 minutes with a DsRed rabbit polyclonal primary antibody (Clontech, Cat. #632496) diluted 1: 5,000 in PBST containing 5% non-fat dry milk. The unbound antibody was rinsed from the membrane with three washes of Classic buffer for five minutes each. The membrane was then incubated for 60 minutes with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate) diluted 1:10,000 in Classic buffer. The membrane was then washed three times with Classic buffer for five minutes each and developed with Pierce Super Signal[14] West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., 3747 N. Meridian Road, Rockford, IL 61105, USA) according to the manufacturer's instructions. The molecular weight of the recognized protein band was estimated using PageRuler Prestained Protein Ladder (#SM0671; Fermentas).

10.5. Method for N-Terminal Amino Acid Sequence Analysis

The 32138 SPT maintainer derived DsRed2 protein sample was separated by SDS-PAGE and then electrophoretically transferred to a PVDF membrane as previously described. The resulting blot was stained with Ponceau S solution (Sigma-Aldrich, St. Louis, MO) to visualize the protein band. The DsRed2 band was excised and sent to Crop Genetics (DuPont

[12] Registered trademark of Bio-Rad Laboratories, Inc.
[13] Registered trademark of Fermentas, Inc.
[14] Registered trademark of Pierce Biotechnology, Inc.
[15] Registered trademark of Bio-Rad Laboratories, Inc.
[16] Registered trademark of ICI Americas, Inc.

Experimental Station) for Edman N-terminal amino acid sequencing with the Procise[17] 494 cLC analyzer (Applied Biosystems, Inc., Foster City, CA) equipped with an online high performance liquid chromatography system.

The microbially expressed DsRed2 protein sample was sent to Crop Genetics (E. I. du Pont de Nemours and Company, DuPont Experimental Station, Wilmington, DE 19880, USA) as lyophilized powder. The protein was resuspended in water and transferred to PVDF membrane using ProSorb[18] Inserts (Applied Biosystems, Foster City CA 94404, USA) and washed with 200 µl 0.1% TFA in water. The membrane was removed and used for Edman N-terminal amino acid sequencing with the Procise[17] 494 LC analyzer (Applied Biosystems) equipped with an online high performance liquid chromatography system.

10.6. Method for LC/MS Identification of Tryptic Peptides

Following SDS-PAGE, the 32138 SPT maintainer derived DsRed2 protein was visualized by staining with Coomassie Blue. The bands (~27 kDa and ~18 kDa) were then excised from the gel. The gel slices were digested with trypsin for approximately 14 hours at 37°C. Tryptic peptides were analyzed using a Thermo Fisher Scientific LCQ[19] ion trap mass spectrometer (Thermo Fisher Scientific, Inc., USA) equipped with an Agilent 1100 HPLC (Agilent, 5301 Stevens Creek Blvd, Santa Clara, CA) and a New Objective PicoView[20] ESI source (New Objective, 2 Constitution Way, Woburn, MA 01801, USA). The sample was injected into an Agilent 1100 HPLC nanoflow system, separated on a C18 capillary column, and eluted at a flow rate of 200 nl/min directly into the mass spectrometer using a reverse phase gradient. Peptides were identified by analyzing the tandem MS spectra of the peptides using Turbo SEQUEST software. Peptide matches were made by comparing identified peptides with the expected DsRed2 peptides as determined by *in silico* trypsin cleavage of the protein sequence. In addition, the identified peptides were screened to detect a blocked N-terminal sequence.

In the case of microbially expressed DsRed2 protein, following SDS-PAGE and Coomassie Blue staining, the gel slice containing the sample was excised, placed in an Eppendorf tube, and digested with trypsin for approximately 14 hours at 37°C. Tryptic peptides were analyzed using a ThermoFisher Scientific LCQ[21] Classic ion trap mass spectrometer (Thermo Fisher Scientific, Inc.) equipped with an Agilent 1100 HPLC (Agilent, Santa Clara, CA) and a New Objective PicoView[22] ESI source (New Objective, Woburn, MA). The sample was injected into an Agilent 1100 HPLC nanoflow system, separated on a C18 capillary column and eluted at a flow rate of 200 nl/min directly into the mass spectrometer using a reverse phase gradient. Peptides were identified by analyzing the tandem MS spectra of the peptides using Turbo SEQUEST software. The peptide matches were made by comparison to the DsRed2 peptides as determined by *in silico* trypsin cleavage of the protein sequence. Allowances were made for the modifications to the peptides caused by oxidation of methionine (observed value is 15.999 Da greater than the theoretical value).

10.7. Method for Protein Glycosylation Analysis

A GelCode glycoprotein staining kit (Pierce Biotechnology, Inc.) was used according to the manufacturer's instructions to determine whether the DsRed2 proteins was glycosylated. The microbially expressed and the maize derived DsRed2 proteins, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor) were run

[17] Registered trademark of Applied Biosystems
[18] Registered trademark of Applied Biosystems
[19] Registered trademark of Thermo Fisher Scientific, Inc.
[20] Registered trademark of New Objective
[21] Registered trademark of ThermoFisher Scientific Inc.
[22] Registered trademark of New Objective

by SDS-PAGE as described in the above methodology. Following electrophoresis, the gel was fixed with 50% methanol for 30 minutes and washed with 3% acetic acid. The gel was then incubated with oxidizing solution for 15 minutes and washed three times with 3% acetic acid. The gel was incubated with GelCode glycoprotein staining reagent (Pierce Biotechnology, Inc.) for 15 minutes and then incubated in reducing reagent. The gel was then extensively washed with 3% acetic acid and deionized water. Glycoproteins were detected as magenta colored bands on the gel. Following glycoprotein detection, the gel was scanned and the image was captured electronically. The same gel was then stained with Coomassie Blue, as described previously, to visualize all protein bands.

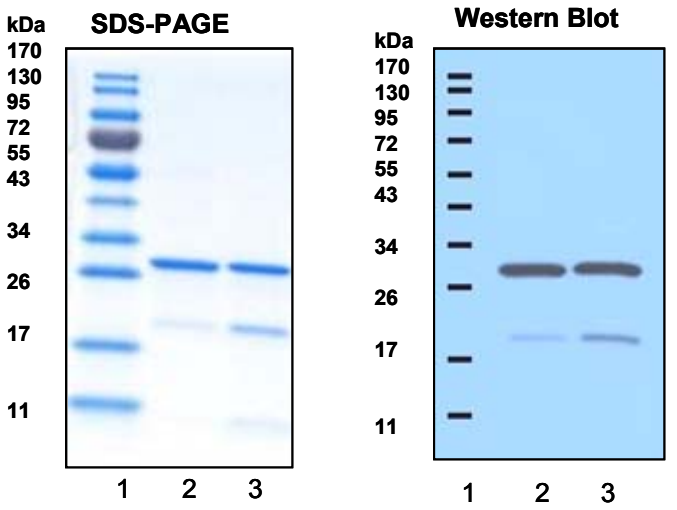
10.8. Results of SDS-PAGE and Western Analyses of DsRed2 Protein

SDS-PAGE analysis indicated that the partially purified 32138 SPT maintainer derived DsRed2 protein (Appendix 10-Figure 1, lane 2) and the microbially expressed DsRed2 protein (Appendix 10-Figure 1, lane 3) samples had predominant bands migrating at approximately 27 kDa. For both the 32138 SPT maintainer derived DsRed2 protein and the microbially expressed DsRed2 protein, a minor band migrating at approximately 18 kDa was detected. A faint band migrating at approximately 10 kDa was also detected in the microbially expressed and 32138 SPT maintainer derived DsRed2 proteins. The 18 kDa and 10 kDa minor bands were likely truncated DsRed2 fragments resulted from partial fragmentation of the protein during denaturation.

Western blot analysis of the 32138 SPT maintainer derived and microbially expressed DsRed2 proteins resulted in detection of a major band migrating at approximately 27 kDa. For both the 32138 SPT maintainer derived DsRed2 protein and the microbially expressed DsRed2 protein, a minor band migrating at approximately 18 kDa was also recognized by the antibody, suggesting that the band was a truncated DsRed2 fragment.

In conclusion, DsRed2 proteins from the two sources demonstrated equivalency in SDS-PAGE and Western analyses.

Figure 1. SDS PAGE and Western Blot Analysis of Microbially Expressed and 32138 SPT Maintainer Derived DsRed2 protein



Lane	Sample ID
1	Fermentas PageRuler Prestained Protein Ladder
2	DsRed2 Derived from 32138 SPT Maintainer (~1 µg for SDS-PAGE and ~100ng for the Western Blotting
3	DsRed2 Derived from Microbial Expression System, (~1 µg for SDS-PAGE and ~100ng for the Western Blotting)

10.9. Results of N-terminal Amino Acid Determination

Analysis by LC/MS following trypsin digestion of the 32138 SPT maintainer derived DsRed2 protein indicated that the first methionine residue (M) was cleaved off and the second residue, alanine (A), was exposed. LC/MS analysis also demonstrated that this alanine residue was acetylated, confirming a blocked N-terminus of the protein. Therefore, the actual N-terminal sequence of the 32138 SPT maintainer derived DsRed2 protein was found to be ASSENVITEFMR (Appendix 10-Table 1). As expected, N-terminal sequencing analysis indicated that the microbially expressed DsRed2 had two additional N-terminal amino acid residues (glycine and serine) resulting from thrombin cleavage of the His-thioredoxin fusion tag used for protein expression and purification. Therefore, after accounting for the missing N-terminal methionine residue of the 32138 SPT maintainer derived DsRed2 and the two additional N-terminal amino acid residues of the microbially expressed DsRed2, the N-terminal sequences of the microbially expressed and 32138 SPT maintainer derived DsRed2 proteins matched the expected sequence of the DsRed2 protein.
.

Table 1. N-terminal Amino Acid Sequence of the Microbially Expressed and 32138 SPT Maintainer derived DsRed2 protein.

Actual	M-A-S-S-E-N-V-I-T-E-F-M-R
32138 SPT Maintainer Derived	A-S-S-E-N-V-I-T-E-F-M-R
Microbially Expressed	G-S-M-A-S-S-E-N-V-I-T-E-F-M-R

10.10. Results of the LC/MS Identification of Tryptic Peptides of DsRed2

LC/MS analysis of the trypsin digest of the 32138 SPT maintainer derived and microbially expressed DsRed2 proteins identified 11 and 9 peptides, respectively, that matched the expected tryptic peptides of the DsRed2 protein, resulting in coverage of 67% and 51% of the DsRed2 amino acid sequence (Appendix 10-Table 2). The 32138 SPT maintainer derived DsRed2 protein and the microbially expressed DsRed2 protein each possessed two of the identified peptides with an oxidized methionine residue. The 18 kDa minor band from the 32138 SPT maintainer derived DsRed2 protein sample was also analyzed by LC/MS following trypsin digestion. Seven peptides (87 amino acid residues) were identified matching the DsRed2 protein sequence (data not presented), indicating that this band was a truncated fragment derived from the DsRed2 protein

Table 2. Identified Peptides of the Microbially Expressed and 32138 SPT Maintainer Derived DsRed2 Proteins Using LC/MS Analysis

Amino Acid Residue #	Microbially Expressed DsRed2	32138 SPT Maintainer Derived DsRed2
GS[1]+1-13	GSMASSENVITEFMR	ND [2]
2-13	ND	ASSENVITEFMR [3]
18-45	ND	MEGTVNGHEFEIEGEGEGRPYEGHNTVK
18-36	MEGTVNGHEFEIEGEGEGR [4]	ND
37-45	PYEGHNTVK	ND
71-83	ND	VYVKHPADIPDYK
75-83	HPADIPDYK	ND
84-95	KLSFPEGFKWER	KLSFPEGFKWER
122-138	ND	VKFIGVNFPSDGPVMQK[4]
124-138	FIGVNFPSDGPVMQK	ND
139-149	ND	KTMGWEASTER[4]
140-149	TMGWEASTER [4]	ND
150-158	ND	LYPRDGVLK
154-163	ND	DGVLKGETHK
167-178	LKDGGHYLVEFK	LKDGGHYLVEFK
185-198	KPVQLPGYYYVDAK	KPVQLPGYYYVDAK
199-216	ND	LDITSHNEDYTIVEQYER

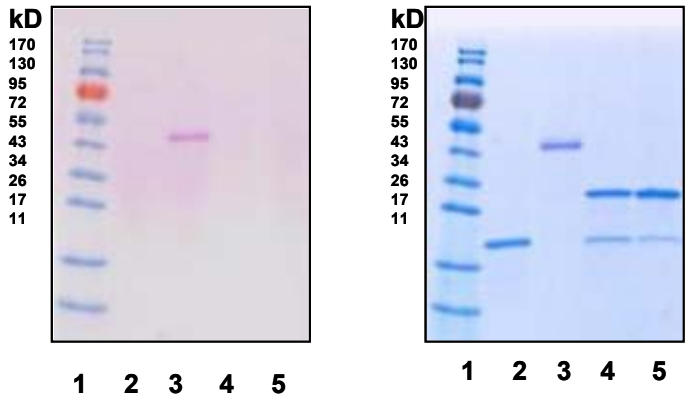
Footnotes:

1. The N-terminal glycine and serine residues are not a part of the DsRed2 sequence but remain following thrombin cleavage of the His-thioredoxin fusion tag used for protein expression and purification of the microbially expressed DsRed2.
2. ND is an abbreviation for not detected
3. Peptide identified following correction for alanine acetylation (addition of 42.0370 Da to theoretical mass)
4. Peptide identified following correction for methionine oxidation (addition of 15.995 Da to theoretical mass)

10.11. Results of the Protein Glycosylation Analysis of DsRed2 Protein

Glycosylation was not detected for either the 32138 SPT maintainer derived or microbially expressed DsRed2 proteins (Appendix 10-Figure 2). The glycoprotein positive control (horseradish peroxidase) was clearly visible as a magenta colored band and the negative control (soybean trypsin inhibitor) was not visible after staining.

Figure 2. Glycosylation Analysis of the Microbially Expressed and 32138 SPT Maintainer Derived DsRed2 protein



[a] This gel was stained with the glycoprotein staining kit
[b] This gel was stained with the glycoprotein staining kit followed by staining with Coomassie Blue

Lane	Sample ID
1	Fermentas PageRuler Prestained Protein Ladder
2	Negative Control, Soybean Trypsin Inhibitor (1.5 µg)
3	Positive Control, Horseradish Peroxidase (1.5 µg)
4	DsRed2 Derived from Microbial Expression System, (~1.5 µg)
5	DsRed2 Derived from 32138 SPT Maintainer (1.5 µg)

Appendix 11

Description of Statistical Analyses

11.1. Trait Inheritance Data (Section V-E, Table 7)

Based on Mendel's segregation law, the expected segregation ratios in Table 7 were tested by the statistic:

$$\chi^2 = [\Sigma(o - e)^2 / e]$$

where o = observed frequency of the genotype,
e = expected frequency of the genotype, and

For the binomial case, chi-squared values are calculated by

$$\chi^2 = \Sigma [(o - e)^2 / e]$$
$$= (n_{obs(pos)} - n_{exp(pos)})^2 / n_{exp(pos)} + (n_{obs(neg)} - n_{exp(neg)})^2 / n_{exp(neg)}$$

where n is the total of the positive ($n_{obs(pos)}$) and negative ($n_{obs(neg)}$) frequencies.

χ^2 follows a chi-squared distribution with one degree of freedom (df) for binomial cases (Agresti, 2002).

χ^2 values > 3.84 ($\chi^2(df=1)$) have probability $p< 0.05$, indicate a significant difference between observed and expected frequencies.

11.2. Agronomic Data (Section VII-B)

Agronomic data (Table 12) were analyzed using a linear mixed model designed to account for the design effects of location and blocks within location. The linear mixed model assumed the entries were a fixed effect while the locations, blocks within locations and the entry by location interaction were random effects.

Early population, seedling vigor, time to silking, time to pollen shed, pollen viability, stalk lodging, root lodging, final population, stay green, disease incidence and insect damage data were analyzed using the following linear mixed model:

$$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$$

$\ell_j \sim iid\ N(0, \sigma^2_{Loc})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent})$, and $\varepsilon_{ijk} \sim iid\ N(0,\sigma^2_{plot})$

Where μ_i denotes the mean of the i^{th} entry (fixed effect), ℓj denotes the effect of the j^{th} site (random effect), $r_{k(j)}$ denotes the effect of the k^{th} block within the j^{th} site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect) and ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} site (random effect or residual). Notation ~iid $N(0,\sigma^2_a)$ indicates random variables that are identically independently distributed (iid) as normal with zero mean and variance σ^2_a.

Plant height and ear height data were analyzed using the following linear mixed model:

$$y_{ijkl} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk} + \delta_{ijkl}$$

$$\ddot{y}_j \sim iid\ N(0,\sigma^2_{Loc}),\ r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep}),\ (\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent}),\ \varepsilon_{ijk} \sim iid$$

$$N(0,\sigma^2_{plot}),\ \text{and}\ \delta_{ijkl} \sim iid\ N(0,\sigma^2_{observations})$$

Where μ_i denotes the mean of the ith entry (fixed effect), ℓj denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the ith entries and jth sites (random effect), ε_{ijk} denotes the effect of the plot assigned the ith entry in the kth block of the jth site (random plot), and δ_{ijkl} denotes the effect of the plant assigned the lth plant in the ith entry in the kth block of the jth site (observational error).

A significant difference between the mean of 32138 SPT maintainer and the means of near-isoline control was established with a false discovery rate (FDR)-adjusted P-value <0.05. When numerous comparisons are being made, it is important to control the rate of false positive results. Since the introduction of the FDR approach in the mid-1990's, it has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science and signal/image processing (*e.g.*, Pawitan *et al*., 2005; Spelman and Bovenhuis, 1998). A false positive result occurs when two means are deemed significantly different when, in fact, they are not. If one uses a 5% type I error rate for each agronomic characteristic measured, then the number of false positives increases as the number of characteristics increase. In order to help manage the false positive rate, the FDR method of Benjamini and Hochberg was applied to account for making multiple comparisons (Benjamini and Hochberg, 1995; Westfall *et al*., 2006). P-values were adjusted accordingly. This resulted in the false positive rate being held to 5%. Both adjusted and unadjusted P-values are provided for the agronomic data. Pioneer's adjustment of p-values using the FDR method is appropriate for the statistical analysis of agronomic and nutrient composition data because the adjustment controls the chance of declaring "false significant differences" when numerous comparisons are conducted in a single study. In particular, the FDR method offers a potential gain in power compared to other multiplicity adjustment procedures (Bonferroni, Holm's, Hochberg's step-up, *etc.*) (Westfall, 2006), and the potential for an increase in power is larger when more of the hypotheses are non-true.

11.3. Nutrient Composition Data (Section VIII-A through VIII-F)

Data were analyzed using a linear mixed model designed to account for the design effects of location and blocks within location. The linear mixed model assumes the entries are a fixed effect while the locations, blocks within locations and the entry by location interaction are random effects. A significant difference between the mean of 32138 SPT maintainer and the means of the near-isoline control was established with an FDR-adjusted P-value <0.05 for each analyte.

Composition data presented in Tables 13-19 were analyzed using the following linear mixed model:

$$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$$
$$\ell_j \sim iid\ N(0,\sigma^2_{Loc}),\ r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep}),\ (\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent}),\ \text{and}\ \varepsilon_{ijk} \sim iid\ N(0,\sigma^2_{plot})$$

Where μ_i denotes the mean of the ith entry (fixed effect), ℓj denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect) and ε_{ijk} denotes the

effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} site (random effect or residual). Notation ~iid $N(0,\sigma^2_a)$ indicates random variables that are identically independently distributed (iid) as normal with zero mean and variance σ^2_a.

Appendix 12

USDA Field Trials of 32138 SPT Maintainer [1, 2]

Year of Planting	Permit Name	Permit Valid Date	State	# of Counties Where 32138 SPT Maintainer was Planted	Acreage[3]
2005	05-024-04r	5/25/2005	HI	1	0.02
2006	05-024-04r	5/25/2005	HI	1	0.083
2006	06-019-03R	5/2/2006	IA	1	0.025
			PR	1	0.022
2006	06-019-04R	5/3/2006	HI	1	0.35
2007	06-019-03R	5/2/2006	PR	1	0.003
2007	06-019-04R	5/3/2006	HI	1	0.032
2007	07-040-101rm	5/3/2007	HI	2	0.364
			IL	3	0.058
			IA	4	0.742
			MN	1	0.023
			NE	1	0.035
			PR	1	0.101
2008	07-040-101rm	5/3/2007	HI	2	5.448
			PR	1	0.6
2008	08-011-101n[4]	4/16/2008	IL	2	0.0459
			IA	1	0.002
			KS	1	0.046
			NE	1	2.819
2008	08-011-102n[4]	4/2/2008	OK	1	0.046
2008	08-014-105n[4]	4/16/2008	IL	1	0.046
			IA	1	0.022
			PR	1	0.138
2008	08-014-124n[4]	3/17/2008	HI	1	1.003
2008	08-023-101n[4]	3/1/2008	HI	2	11.926
			IA	1	0.011
			WA	1	0.69
2009	08-014-105n[4]	4/16/2008	PR	1	0.06
2009	08-014-124n[4]	3/17/2008	HI	1	0.068
2009	09-012-101n[4]	3/1/2009	HI	1	0.443
2009	09-013-107n[4]	4/1/2009	IA	2	0.01
2009	09-035-107n[4]	3/19/2009	HI	1	0.023
			WI	1	0.006

Footnotes:
1. Plantings through June 5, 2009 are listed.
2. In USDA final reports, 32138 SPT Maintainer is called E6611.32.1.38.
3. Total acreage for all 32138 SPT Maintainer (acreage includes non-transgenic 32138 progeny plants)
4. Final field test report not yet due to USDA.

Appendix 13

Ecological Observations of 32138 SPT Maintainer

Key to "Range of severity in 32138 SPT Maintainer" in Appendix 13-Tables 1 and 2:

mild – very little disease or insect injury (<10%) visible;
moderate – noticeable plant tissue damage (10% to 30%);
severe – significant plant tissue damage (>30%).

Table 1. Insect Stressor Comparison between 32138 SPT Maintainer and Control

Year of Planting	Permit number	State	County	Stressor	Range of Severity in 32138 SPT maintainer	Difference with Control?
2005	05-024-04r	HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Beet armyworm (*Spodoptera exigua*)	mild to moderate	no
2006	05-024-04r	HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
2006	06-019-03R	IA	Polk	Corn flea beetle (*Chaetocnema pulicaria*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
		PR	Salinas	Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Corn earworm (*Helicoverpa zea*)	mild	no
2006	06-019-04R	HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
2007	06-019-03R	PR	Salinas	Fall armyworm (*Spodoptera frugiperda*)	moderate	no
				Corn earworm (*Helicoverpa zea*)	mild	no

Table 1. Insect Stressor Comparison between 32138 SPT Maintainer and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT maintainer	Difference with Control?
2007	06-019-04R	HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Spidermite (Tetranychidae)	mild	no
				Leafhopper (Cicadellidae)	mild	no
2007	07-040-101rm	HI	Honolulu	Leafhopper (Cicadellidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Spidermite (Tetranychidae)	mild	no
				Green lacewing *(Chrysoperla carnea)*	mild	no
				Syrphid Flies (Syrphidae)	mild	no
				Spidermite (Tetranychidae)	mild	no
			Kauai	Green lacewing *(Chrysoperla carnea)*	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Syrphid Flies (Syrphidae)	mild	no
				Spidermite (Tetranychidae)	mild	no
				Aphids (Aphididae)	mild	no
		IL	Bureau	Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
			Clinton	Leafhopper (Cicadellidae)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Stark	Western bean cutworm (*Richia albicosta*)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no

Table 1. Insect Stressor Comparison between 32138 SPT Maintainer and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT Maintainer	Difference with Control?
2007	07-040-101rm	IA	Green	Cutworms (coleopteran)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Aphids (Aphididae)	mild	no
			Jefferson	Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Aphids (Aphididae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
			Linn	Japanese beetle (*Popillia japonica*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
			Polk	Beetles (Coleoptera)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no

Table 1. Insect Stressor Comparison between 32138 SPT Maintainer and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT Maintainer	Difference with Control?
2007	07-040-101rm	MN	Freeborn	European corn borer *(Ostrinia nubilalis)*	mild	no
				Aphids (Aphididae)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
		NE	York	European corn borer *(Ostrinia nubilalis)*	mild	no
				Western bean cutworm (*Richia albicosta*)	mild to moderate	no
				Leafhopper (Cicadellidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Picnic beetle *(Glischrochilus quadrisignatus)*	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Beetles (Coleoptera)	mild	no
				Butterflies (Lepidoptera)	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no
		PR	Guayama	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
2008	07-040-101rm	HI	Honolulu	Corn earworm (*Helicoverpa zea*)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Common thrips (Thripidae)	mild	no
			Kauai	Leafhopper (Cicadellidae)	mild	no
				Spidermite (Tetranychidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
		PR	Salinas	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no

Table 1. Insect Stressor Comparison between 32138 SPT Maintainer and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT Maintainer	Difference with Control?
2008	08-011-101n	IL	Clinton	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Stark	Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
		IA	Polk	Japanese beetle (*Popillia japonica*)	mild	no
		KS	Pawnee	European corn borer *(Ostrinia nubilalis)*	mild	no
		NE	York	Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
2008	08-011-102n	OK	Caddo	Fall armyworm (*Spodoptera frugiperda*)	mild	no
2008	08-014-105n	IL	Ogle	Japanese beetle (*Popillia japonica*)	mild	no
				Corn rootworm (*Diabrotica* spp.)		
		IA	Polk	European corn borer *(Ostrinia nubilalis)*	mild	no
		PR	Juana Diaz	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
2008	08-014-124n	HI	Kauai	Spidermite (Tetranychidae)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no

Table 1. Insect Stressor Comparison between 32138 SPT Maintainer and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT Maintainer	Difference with Control?
2008	08-023-101n	HI	Kauai	Spidermite (Tetranychidae)	mild	no
				Leafhopper (Cicadellidae)	mild	no
			Honolulu	Leafhopper (Cicadellidae)	mild	no
				Common thrips (Thripidae)	mild	no
		IA	Polk	Japanese beetle (*Popillia japonica*)	mild	no
		WA	Franklin	No Disease	N/A	no

N/A= not applicable

Table 2. Disease Stressor Comparison between 32138 SPT Maintainer and Control

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT Maintainer	Difference with Control?
2005	05-024-04r	HI	Kauai	Aspergillus ear and kernel rot (*Aspergillus* sp.)	mild	no
				Fusarium (*Fusarium* sp.)	mild	no
				Gibberella ear rot (*Gibberella zeae*)	mild	no
2006	05-024-04r	HI	Kauai	No disease	N/A	no
2006	06-019-03R	IA	Polk	Stewart's seedling wilt (*Pantoea stewartii*)	mild to severe	no
				Common smut (*Ustilago zeae*)	mild to severe	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Southern corn rust (*Puccinia polysora*)	mild	no
		PR	Salinas	No disease	N/A	no
2006	06-019-04R	HI	Kauai	No disease	N/A	no
2007	06-019-03R	PR	Salinas	No disease	N/A	no
2007	06-019-04R	HI	Kauai	Common smut (*Ustilago zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no

Table 2. Disease Stressor Comparison between 32138 SPT Maintainer and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT Maintainer	Difference with Control?
2007	07-040-101rm	HI	Honolulu	Common corn rust (*Puccinia sorghi*)	mild	no
			Kauai	Common smut (*Ustilago zeae*)	mild	no
		IL	Bureau	Common corn rust (*Puccinia sorghi*)	mild	no
			Clinton	Root rot (*Phytopthora spp.*)	mild	no
				Seedling Blight (*Pythium Spp*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			Stark	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Anthracnose (*Colletotrichum graminicola*)	mild	no
		IA	Green	Common smut (*Ustilago zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Jefferson	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			Linn	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
		IA	Polk	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Stewart's seedling wilt (*Pantoea stewartii*)	mild to moderate	no
				Common smut (*Ustilago zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Anthracnose (*Colletotrichum graminicola*)	mild	no
				Southern corn rust (*Puccinia polysora*)	mild	no

Table 2. Disease Stressor Comparison between 32138 SPT Maintainer and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT Maintainer	Difference with Control?
2007	2007-040-101rm	IA	Polk	Northern corn leaf blight (*Exserohilum turcicum*)	moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	moderate	no
		MN	Freeborn	Common smut (*Ustilago zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		NE	York	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
		PR	Guayama	No Disease	N/A	no
2008	07-040-101rm	HI	Honolulu	Common corn rust (*Puccinia sorghi*)	mild	no
			Kauai	Leafhopper (Cicadellidae)	mild	no
				Spidermite (Tetranychidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
		PR	Salinas	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
2008	08-011-101n	IL	Clinton	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Stark	Common corn rust (*Puccinia sorghi*)	mild	no
		IA	Polk	No Disease	N/A	no
		KS	Pawnee	Common corn rust (*Puccinia sorghi*)	mild	no
		NE	York	Eyespot (*Aureobasidium zeae*)	mild	no
2008	08-011-102n	OK	Caddo	No Disease	N/A	no
2008	08-014-105n	IL	Ogle	No Disease	N/A	no
		IA	Polk	No Disease	N/A	no
		PR	Juana Diaz	No Disease	N/A	no

Table 2. Disease Stressor Comparison between 32138 SPT Maintainer and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Range of Severity in 32138 SPT Maintainer	Difference with Control?
2008	08-014-124n	HI	Kauai	No Disease	N/A	no
2008	08-023-101n	HI	Kauai	No Disease	N/A	no
			Honolulu	No Disease	N/A	no
		IA	Polk	No Disease	N/A	no
		WA	Franklin	No Disease	N/A	no

N/A=not applicable

PIONEER.
A DUPONT BUSINESS

October 11, 2010

Dr. Margaret Jones
Biotechnology Regulatory Services
USDA-APHIS
4700 River Road
Riverdale, MD 20737

RE: Pioneer Hi-Bred International, Inc., Petition Number 08-338-01p for the Determination of Non-regulated Status for Maize 32138 SPT Maintainer Used in the Pioneer Seed Production Technology (SPT) Process

Dear Dr. Jones,

As a follow-up to our telephone conversation of October 6, 2010, I am writing to make two minor updates to Pioneer's revised petition 08-338-01p that was submitted on June 18, 2009.

These updates affect the base pair positions and sizes of two of the genetic elements found in Table 2 on pages 41 and 42 of the revised petition. The updates to the positions and sizes are bolded and underlined in red text in the following attachment. These updates in no way affect the safety of the 32138 SPT maintainer or the data interpretation and conclusions of the petition.

This letter and attachment do not contain any confidential business information.

Please feel free to contact me should you have any questions.

Sincerely,

Natalie Weber

Natalie Weber
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cc: Cynthia Eck (e-mail, hard copy)
Craig Roseland (e-mail)

Update to p. 41:

Table 2. Description of Genetic Elements in the T-DNA Region of Plasmid PHP24597

Location on T-DNA (base pair position)	Genetic Element	Size (base pairs)	Description
1 to 25	Right Border	25	T-DNA Right Border region from Ti plasmid of *Agrobacterium tumefaciens*
26 to 177	Ti Plasmid Region	152	Non-functional sequence from Ti plasmid of *A. tumefaciens*
178 to 203	Polylinker Region	26	Region required for cloning genetic elements
204 to 706	*5126* Promoter	503	Maize anther-preferred *5126* promoter (Cigan and Albertsen, 1997)
707 to 2658	*Ms45* Genomic Region	1952	Maize genomic DNA including the *Ms45* coding sequence and associated 3' untranslated region as indicated below: Exon 1 bp 708 to 1086 Intron 1 bp 1087 to 1215 Exon 2 bp 1216 to 1499 Intron 2 bp 1500 to 1596 Exon 3 bp 1597 to 1764 Intron 3 bp 1765 to 1850 Exon 4 bp 1851 to 2258 3' UTR bp 2384 to 2658 (Albertsen *et al.*, 1993; Albertsen *et al.*, 1995)
2659 to 2730	Polylinker Region	72	Region required for cloning genetic elements
2731 to 5466	*Pg47* Promoter	2736	Promoter from the maize, pollen polygalacturonase (*Pg47*) gene (Allen and Lonsdale, 1993)
5467 to 5468	Polylinker Region	2	Region required for cloning genetic elements
5469 to 5693	*zm-bt1* Transit Peptide	225	Amyloplast-targeting transit peptide from the maize *brittle-1* gene (Sullivan *et al.*, 1991)
5694 to 6956	*zm-aa1* Gene	1263	Maize α-amylase gene
6957 to 7033	Polylinker Region	77	Region required for cloning genetic elements
7034 to 7377	*In2-1* Terminator	344	Terminator sequence from the maize *In2-1* gene (Hershey and Stoner, 1991)
7378 to 7411	Polylinker Region	34	Region required for cloning genetic elements
7412 to 7886	CaMV 35S Enhancer	475	Enhancer region from the Cauliflower Mosaic Virus genome (Franck *et al.*, 1980; Odell *et al.*, 1985; Odell *et al.*, 1988).

Update to p. 42:

Table 2. Description of Genetic Elements in the T-DNA Region of Plasmid PHP24597 (continued)

Location on T-DNA (base pair position)	Genetic Element	Size (base pairs)	Description
7887 to 7905	Polylinker Region	19	Region required for cloning genetic elements
7906 to 8761	*Ltp2* Promoter	856	Promoter from barley lipid transfer protein (*Ltp2*) gene (Kalla *et al.*, 1994)
8762 to 8809	Polylinker Region	48	Region required for cloning genetic elements
8810 to 9487	*DsRed2(Alt1)* Gene	678	Modified DsRed2 gene (Wasson-Blader T, 2001) with internal *Bst*E II restriction site removed
9488 to 9528	Polylinker Region	41	Region required for cloning genetic elements
9529 to 9839	*pin*II Terminator	311	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (Keil *et al.*, 1986; An *et al.*, 1989).
9840 to 9872	Polylinker Region	33	Region required for cloning genetic elements
9873 to 9925	Ti Plasmid Region	53	Non-functional sequence from Ti plasmid of *A. tumefaciens*
9926 to 9950	Left Border	25	T-DNA Left Border region from Ti plasmid of *Agrobacterium tumefaciens*