USDA/APHIS Environmental Assessment

In response to permit applications (05-278-01r and 05-278-02r) received from Dr. Zengyu Wang of the Samuel Noble Foundation for a field-test to examine gene flow in genetically engineered tall fescue (*Festuca arundinacea*) and Italian ryegrass (*Lolium multiflorum*)

U.S. Department of Agriculture
Animal and Plant Health Inspection Service
Biotechnology Regulatory Services

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I. INTRODUCTION

A. Importance and Use of Tall Fescue and Italian Ryegrass

Two closely related grass genera, *Festuca* L. (fescues) and *Lolium* L. (ryegrasses) are of significant value in temperate grasslands. These plants are well-adapted, productive grasses widely distributed in temperate and cool climates where they are used for forage, lawns, and sports fields (Jauhar 1993).

Tall fescue (*Festuca arundinacea* Schreb.) is a perennial cool-season forage and turf grass. It was introduced to North America in the early to mid 1800's and has become the predominant cool-season pasture grass in the USA grown on over 14 million hectares (Buckner et al. 1979, Barnes 1990). Its wide-spread use in the US is due to its adaptation to a wide range of soil conditions, tolerance to continual grazing by animals such as cattle, high yields of forage and seed, long grazing season, compatibility with varied management practices, and low incidence of pest problems (Hanson 1979, Sleper and West 1996)

Italian ryegrass, also known as annual ryegrass (*Lolium multiflorum* Lam.), is a highly nutritious grass for grazers introduced to the US during colonial times (Schoth and Weihing 1962) and is now grown on more than one million hectares in the south-eastern US each year (Evers et al. 1995). This grass shows a rapid establishment from seed, good production in the seeding year, rapid recovery after grazing, and is one of the most important winter pasture species in mild climates (Buckner et al. 1967, Jauhar 1993, Moser and Hoveland 1996).

Both grasses are wind-pollinated and have a high potential to pass their genes to adjacent plants making breeding management possible but inefficient. Information regarding gene flow within the same species of plants and between tall fescue and Italian ryegrass has become extremely important for breeding and releasing transgenic and non-transgenic cultivars of both plants species.

B. Regulatory Authority

The authorities for regulation of genetically engineered tall fescue and Italian ryegrass are the Plant Protection Act of 2000, 7 U.S.C. 7701-7772, and USDA, APHIS regulations under 7 CFR § 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which are Plant Pests or Which There is Reason to Believe are Plant Pests." A genetically engineered organism is considered a regulated article if the donor organism, recipient organism, vector or vector agent used in engineering the organism belongs to one of the taxonomic groups listed in the regulation and is also a plant pest, or if there is a reason to believe it is a plant pest. In this submission, both plant species have been genetically engineered using the recombinant DNA technique of microprojectile bombardment. In tall fescue and Italian ryegrass, the introduced DNA sequence contains genes from *Escherichia coli* and the minor plant pest *Lolium perenne*, and regulatory sequences from the plant pests cauliflower mosaic virus, rice tungro virus, and

Agrobacterium tumefasciens. Tall fescue and Italian ryegrass are the recipient organisms and can also be considered minor plant pests in some environs.

This environmental assessment (EA) was conducted under the authority of the National Environmental Policy Act (NEPA), 42 U.S.C. 4321 and 7 CFR § 372, NEPA Implementing Procedures. Except for actions that are categorically excluded, approvals and issuance of permits for proposals involving genetically engineered or non-indigenous species normally require environmental assessments, but not necessarily environmental impact statements (7 CFR § 372.5(b)(4)). The actions described in the applications for permits 05-278-01r and 05-278-02r involve the release of two transgenic grass species. Analysis by APHIS of the conditions proposed in the permit applications suggests that these actions constitute a confined field release and thus are categorically excluded actions under 7 CFR 372. However, the recent scientific study in creeping bentgrass demonstrating pollen gene flow over large distances (Watrud et al. 2004) creates some uncertainty regarding the confinement of field trials of flowering grasses. APHIS is preparing an Environmental Assessment to address this new confinement issue.

II. NEED FOR THE PROPOSED ACTION

A. Proposed Action

The proposed action is for APHIS, Biotechnology Regulatory Services (BRS), to issue a permit for field-testing one line of tall fescue genetically modified to express hygromycin resistance and β-glucuronidase, and to issue a second permit for two lines of genetically modified Italian ryegrass: one ryegrass line engineered to express hygromycin resistance and one ryegrass line engineered to express hygromycin resistance, beta-glucuronidase and a gene for the down-regulation of a pollen allergen.

The transgenic tall fescue line and the two transgenic lines of Italian ryegrass are part of a single field trial to investigate gene flow in transgenic grasses. All three transgenic grass lines will be evaluated for pollen dispersal, agronomic properties and out-crossing success between the two plant species. Thus, this environmental assessment (EA) will evaluate all regulated articles presented in the two permit applications (tall fescue in 05-278-01r and Italian ryegrass lines in 05-278-02r) within the context of a single field experiment. Granting approval to a single permit will not be considered as these two plant species are integral to the experimental design and the proposed research is contingent on both permit approvals.

B. Purpose of this Environmental Assessment

The purpose of this EA is to assess any potential adverse environmental effects of a field research study in Love County, Oklahoma. The permit applications were received by APHIS, BRS on October 5, 2005. They were submitted by the Dr. Zengyu Wang, Samuel Robert Noble Foundation, Ardmore, Carter County, Oklahoma. The application numbers are 05-278-01r and 05-278-02r.

C. Need for This Action

Under APHIS regulations, the receipt of a permit application to introduce a genetically engineered organism requires a response from the Administrator:

> *Administrative action on applications*. After receipt and review by APHIS of the application and the data submitted pursuant to paragraph (a) of this section, including any additional information requested by APHIS, a permit shall be granted or denied. 7 CFR 340.4(e)

III. ALTERNATIVES

A. No Action

Under APHIS/BRS regulations, the Administrator must either grant or deny permits properly submitted under 7 CFR 340. For the purposes of this Environmental Assessment, the No Action alternative would be the denial of permit applications 05-278-01r and 05-278-02r.

The transgenic tall fescue line was previously approved for planting in the fall of 2005 (permit number 05-291-01r). These plants were placed at outside a greenhouse for vernalization with the condition that they not be allowed to flower prior to approval of the two permits that are the focus of this environmental assessment (05-278-01r and 05-278-02r). An EA was not done for this permit (05-291-01r) because this permitted field trial meets the criteria of the categorical exclusion clause of the in 7 C.F.R. 372.5 (c) (3) (ii). Under the No Action Alternative, if these permits are denied, the transgenic tall fescue plants currently released will be removed and transferred back to the greenhouse facility at the Noble Foundation, as stated in the permit conditions for 05-291-01r.

B. Issue the Permits as Received

Issuing these permits would allow the following research to proceed at a grass field site in Love County, OK (see Appendix 1 for the detailed research plan) under the conditions provided by the applicant (see below, conditions a-l) and the standard permit conditions under 7 CFR §340.4 (see Appendix 5). Under this alternative, the field release of the genetically engineered tall fescue and Italian ryegrass plants would be authorized at the specified location with no additional conditions implemented by APHIS/BRS.

The following redundant mitigation measures are incorporated into the experimental procedures by the applicant to promote a confined field release and ensure the least amount of harm to the environment:

a. The test site is on private land owned by the Samuel Robert Noble Foundation and is expected to provide adequate physical security.
b. Sexually-incompatible grasses surround the 170 acre field site.
c. The field site has been managed to eliminate any potentially sexually-compatible grass species.

d. The 0.25 acre plot containing the transgenic tall fescue and Italian ryegrass plants, as well as the plots containing sexually compatible, non-transgenic tall fescue and Italian ryegrass plants are caged to prevent large animal grazing and discourage bird and mice grazing. The 0.25 acre transgenic plot also has a strong electrical fence to further prevent animals from entering the site.
e. Surrounding area (169.25 acres) will be mowed before surrounding grasses are allowed to flower, to further reduce the potential of pollen fertilizing neighboring plants. This will result in a minimum isolation distance of 900 ft.
f. Seed heads will be monitored daily during the seed ripening period.
g. Seeds in the transgenic plot and the non-transgenic recipient plots will be harvested immediately at maturity to prevent accidental seed dissemination by birds and/or rodents.
h. Seed heads from the transgenic and recipient plants will be bagged, cut from the plant, placed in labeled plastic containers, and transported back to the laboratory using a closed vehicle.
i. After termination of the experiment, all plants in the 170 acre field will be sprayed with an herbicide application and the field will be monitored monthly for a minimum of 2 years.
j. Warm season grasses (for example, bermudagrass) will be planted in the transgenic plot following termination of the experiment. Warm season grasses are easily distinguishable from any volunteer tall fescue or Italian ryegrass that may grow after termination of the experiment.
k. At harvest, the seed will be hand harvested, and moved by closed vehicle to a laboratory at the Samuel Robert Noble Foundation grounds.
l. Seed cleaning equipment will be used in the laboratory and will be cleaned before and after transgenic seed use. Any non-seed residues from the seed cleaning process or further analyses will be collected and autoclaved.

C. Issue Permits with Supplemental Conditions

Issuing these permits would allow the following research to proceed at a grass field site in Love County, OK (see Appendix 1 for the detailed research plan) where supplemental permit conditions, based on APHIS scientific analysis of the permit applications, input from the State of Oklahoma, and public comment from this environmental assessment, would be required. If warranted, based on environmental risk of escape of the engineered organism, APHIS will require mitigating measures to prevent spread of the organism outside the field production area.

Currently APHIS proposes to include the following duplicative safety measures to promote a confined field release and to ensure no significant harm to the environment:

a. Seed heads from the transgenic and recipient plants will be bagged before removal from the plants, and placed into a second bag immediately after cutting seed head from plant.
b. The transgenic plot will be surrounded by a 10 ft fallow (bare ground) border to detect any potential vegetative reproduction by the transgenic tall fescue and Italian ryegrass plants.

c. Any plant material removed from the field site will be treated as a regulated article.

1. Purpose of the Research

Research on pollen movement is critical to addressing the issue of gene flow in transgenic releases, as well as for developing sound risk assessments for wind-pollinated transgenic grass species. The purpose of this proposed introduction is for research on transgenic tall fescue and Italian ryegrass plants, particularly to investigate:

1. the distance transgenic pollen can travel and still remain viable
2. the frequency of pollination at different distances from the pollen source
3. the probability/frequency of cross-hybridization between transgenic tall fescue, transgenic Italian ryegrass and related species under field conditions
4. the effects of down-regulation of a major pollen allergen on pollen dispersal in transgenic Italian ryegrass.

Additionally, the data gathered during this study will be used to assess the confined status of this field release and refine the confinement conditions necessary for future releases of these grass species.

2. Description of the Research

The three transgenic grass lines (a total of 1080 plants) will be released on no more than 0.25 acres, surrounded by 169.75 acres of sexually-incompatible grass species (see Figure 1). Within the 0.25 acre transgenic plot, different types of related, non-transgenic fescues and ryegrasses will be planted to assess the potential for hybridization. In addition, eight non-transgenic plants, four tall fescue and four Italian ryegrass plants, will be placed in each of 64 fenced enclosures and act as recipient plants to investigate transgenic gene flow, transgenic pollen movement, and out-crossing at different distances away from the transgenic plant species. There are 8 transects radiating from the center 0.25 acre transgenic plot and each transect will have an enclosure containing the non-transgenic recipient plants (recipient plots) every 25m, from 25m to 375m from the central plot (see Figure 2). For a detailed description of the experimental design for this research project, see Appendix 1.

IV. ENVIRONMENTAL CONSEQUENCES OF THE PROPOSED ACTION AND ALTERNATIVE

A. Deny the Permits

To deny the permit application would have no expected potential adverse environmental impact, would prevent the field research from proceeding, and prevent any benefits associated with the knowledge gained from this research study.

B. Issuance of the Permits as Received

The proposed action is not expected to have any adverse environmental impacts for the following biological and physical reasons:

1. No adverse consequences to non-target organisms or environmental quality are expected from the field release of these transgenic grass lines for the reasons stated below.
2. The proteins produced by genes introduced into these grass lines are not expected to have toxicological or allergenic properties.
3. None of the introduced genes provide the engineered tall fescue or Italian ryegrass plants with any selective advantage over non-engineered tall fescue or Italian ryegrass in the ability to be disseminated or to become established in the environment.

C. Issuance of the Permits with Additional Conditions

The proposed action is not expected to have any adverse environmental impacts for the following biological and physical reasons:

1. No adverse consequences to non-target organisms or environmental quality are expected from the field release of these transgenic grass lines.
2. The genes introduced into these grass lines are not expected to have toxicological or allergenic properties.
3. None of the introduced genes provide the engineered tall fescue or Italian ryegrass plants with any selective advantage over non-engineered tall fescue or Italian ryegrass in the ability to be disseminated or to become established in the environment.

Under this alternative, APHIS proposes to include the following duplicative safety measures to promote a confined field release and ensure no significant harm to the environment:

a. Seed heads from the transgenic and recipient plants will be bagged before removal from the plants, and placed into a second bag immediately after cutting seed head from plant. This additional measure will further reduce the risk of inadvertent loss of transgenic seeds during harvest.

b. The transgenic plot will be surrounded by a 10 ft fallow (bare ground) border to detect any potential vegetative reproduction by the transgenic tall fescue and Italian ryegrass plants. This additional measure will prevent vegetative propagules from establishing in the field site.

c. Any plant material removed from the field site will be treated as a regulated article. This additional measure will ensure contained movement of the transgenic seeds, plants, and plant parts between greenhouse facilities, laboratory facilities and the field site.

D. Analysis of Issues, Consequences, and Theoretical Risks of Field Research using Transgenic Tall Fescue and Transgenic Italian Ryegrass Grass Lines

1. Possibility of Gene Flow Outside of Field Test

Genes from transgenic grass may move outside of the test plot in two ways. The first pathway of escape is by pollen transfer. The second is by movement of propagative material, i.e., whole seeds.

a. Pollen Movement

Both tall fescue and Italian ryegrass require pollen movement by wind for successful reproduction. The pollen produced by these grasses is released, taken up by wind currents, and potentially lands on receptive, compatible plants. For transgenic tall fescue and transgenic Italian ryegrass, the pollen may successfully result in transgenic gene flow if the transgenic pollen fertilizes a receptive plant of the same species, or a receptive plant of a different but compatible species (i.e. interspecific hybrid). Tall fescue and Italian ryegrass are closely related and belong to a group of grasses called the *Festuca-Lolium* complex. Within this complex, there are 80 fescue species and 8 ryegrass species. Of these 88 grass species, those species that are wind-pollinated have the potential to form interspecific hybrid plants. For a more detailed description of the *Festuca-Lolium* complex, see Appendix 2.

The 170 acre (68.8 hectare) field site proposed for the experiment was sprayed with herbicide (glyphosate) in 2004 and 2005 to eliminate all non-transgenic fescue and ryegrass plants that may be compatible with the released transgenic plants. Since the herbicide application, the field has been surveyed by the applicant and no fescue or ryegrass plants have been found. Thus, outside of the experimental plants placed to detect gene flow, there should be no risk of transgenic gene flow and subsequent seed development within the 170 acre field site because only non-sexually compatible relatives are within field site. The applicant will take the additional steps of (1) mowing the 170 acre site to eliminate the production of flowers from any renegade fescue or ryegrass plant to decrease the likelihood that transgenic plants could reproduce with a non-transgenic relative, and (2) spraying the field site with additional herbicide applications at the end of the field experiment to kill any renegade fescue or ryegrass plant within the border of this field trial. Therefore, the only potential for gene flow by transgenic pollen movement is outside the boundary of the proposed field site.

Transgenic grass pollen from another grass species, creeping bentgrass, has been shown to hybridize to receptive bentgrass plants over distances as far as 21km from the source of the transgenic pollen (Watrud et al. 2004), indicating a theoretical risk exists for transgenic pollen movement beyond the field site boundary for any transgenic grass. However these experiments were based on data from 400 acres of transgenic grasses. Previous studies in tall fescue at a comparable scale to the proposed experiments have not detected gene flow over distances beyond

200 m where the potential for travel up to 2294 m was investigated (Wang et al. 2004b). Given that transgenic tall fescue pollen has not been found to travel beyond 200m, transgenic tall fescue pollen must travel 400m to traverse the field site boundary, and there are no sexually-compatible species within the 170 acre (68.8 hectare) field site, the transgenic tall fescue pollen should be effectively contained.

Unlike transgenic tall fescue, the properties of transgenic Italian ryegrass pollen have not been studied and are thus central to the research proposed in these permit applications. Pollen in a related but non-transgenic ryegrass (perennial ryegrass) has been shown to travel up to 144m (the farthest distance tested in the study) (Cunliffe et al. 2004) and studies in creeping bentgrass have reported pollen gene flow distances up to 13 miles where size of the field trial appears to have a marked influence on pollen gene flow distance (Wipff and Fricker 2000, Belanger et al. 2003, Watrud et al. 2004). The bentgrass studies raise some uncertainty with regard to the confinement of field releases of flowering transgenic grasses. Therefore, points **2, 3,** and **4** below will address the risks of the transgenes used in this field trial, in the unlikely event that transgenic grass pollen moves beyond the field trial boundaries.

b. Seed Movement

No transgenic seeds will be planted in this experiment, removing an important route for transgenic genes to move beyond the field site boundary. Entire transgenic tall fescue and Italian ryegrass plants will be transplanted into the proposed field site. Therefore, the only chance for gene flow migration by seed movement will be after seed maturation in the transgenic and recipient plots (see the field design Appendix for more information on these release plots). A strong electric fence and large cage surrounding the 0.25 acre transgenic plot, along with cages around the recipient plots, will eliminate potential seed movement by large animals that may forage on the seed heads. Seed-eating birds and mice will be dissuaded from entering the caged plots, but the potential exists for entry. The applicant is proposing to monitor the seed heads during the seed ripening period, and collect seeds immediately at maturation to further reduce the potential for mature seed movement by birds and/or rodents. The applicant is also proposing to immediately bag the seed heads after seed maturity, before removing the seed heads from the transgenic plants, to eliminate seed spillage during collection and transportation of transgenic seeds back to the laboratory for cleaning and analysis. To further eliminate the risk of inadvertent seed spillage, APHIS recommends that the applicant bag the seed heads before removal from the plant, and immediately place into a second bag during harvest (under Section III: Alternatives, Subsection: C. Issue Permit with Supplemental Conditions, Condition a). Therefore, because no transgenic seeds are planted during the experiment, large animals, birds and mice are discouraged from entering the transgenic and recipient plots, and seed heads will be monitored daily during the seed ripening period, collected immediately at maturity, and carefully transported from the field site, APHIS is confident that the current permit and supplementary permit conditions will confine the crop and make the risk of gene flow through seed movement negligible.

2. Risk of the Gene for Hygromycin Resistance to the Environment

The transgenic tall fescue and Italian ryegrass plants contain the gene for hygromycin resistance (see Appendix 3 for a more detailed discussion). In the unlikely event that the gene for hygromycin

resistance migrates to tall fescue or Italian ryegrass plants outside the field trial, the hygromycin phosphotransferase enzyme would not confer a selective advantage for a fescue or ryegrass plant outside the field trial because antibiotics are not used on pastures, hayfields or turf (Goldstein et al. 2005). In addition, resistance to antibiotics, including hygromycin, is already widely prevalent in enteric bacteria and soil-borne bacteria (Wang and Liu 2004; Sengelov et al., 2003; Jensen et al., 2001; Cole and Elkan, 1979; Bronstad et al., 1996) and resistance to hygromycin does not appear to confer resistance to other clinically relevant antibiotics (Wright and Thompson 1999).

The gene for hygromcin resistance has been evaluated in a line of transgenic corn that was approved for deregulation by USDA/APHIS (petition # 03-155-01p) in July, 2005 and was deemed not to pose a threat to agriculture or the environment. The Environmental Protection Agency (EPA) has also evaluated the safety of the enzyme produced by the gene for hygromycin resistance and has deemed it exempt from the requirement for tolerance of food residues under the Federal Food, Drug, and Cosmetic Act (FFDCA) (FR 69 18275-18278). Therefore, in the unlikely event that the gene for hygromycin resistance would migrate to fescue or ryegrass plants outside the field trial, APHIS concludes there would be no significant impact to the environment.

3. Risk of the Gene for β-glucuronidase to the Environment

The transgenic tall fescue plants and one line of the transgenic Italian ryegrass plants (see Appendix 3 for more detailed discussion) contain the gene (*gusA*) for expression of the β-glucuronidase enzyme as a screening tool for the applicant. In the unlikely event that the *gusA* gene migrates to tall fescue or Italian ryegrass plants outside the field trial, this gene would not confer a selective advantage because β-glucuronidase does not change any ecological or agronomic properties, apart from having β-glucuronidase enzymatic activity which confers no value to the plant (Gilissen et al. 1998).

The gene for β-glucuronidase expression has been evaluated in four transgenic crops that have been approved for deregulation by USDA/APHIS (petitions # 96-068-01p, # 97-008-01p, # 98-173-01p, and # 00-342-01p) , and deemed not to pose a threat to agriculture or the environment. In its review, the EPA concluded that there is a lack of similarity between the enzyme β-glucuronidase and known mammalian toxins or human allergens, thus the EPA deemed the enzyme β-glucuronidase exempt from tolerance requirement under the FFDCA (FR 66 42957-42962). Therefore, in the unlikely event that the gene for β-glucuronidase expression would migrate to fescue or ryegrass plants outside the field trial, APHIS concludes there would be no significant impact on the environment.

4. Risk of the Gene that Decreases Pollen Allergen Production to the Environment

Hay fever and seasonal allergenic asthma due to grass pollen are environmental diseases that afflict up to 25% of the population in cool temperate climates around the world. Ryegrass pollen is the most abundant pollen produced and is the major source of grass pollen allergens.

The major allergen of ryegrass pollen is a protein, toward which 95% of grass pollen allergic patients showed increased levels of IgE antibodies in their sera (Kahn and Marsh 1986). One line of transgenic Italian ryegrass carries a gene from perennial ryegrass (*Lol p1*) whose placement (antisense orientation, see Appendix 3) results in a decreased production of pollen allergen (Petrovska et al. 2004). A decrease in pollen allergen due to the presence of this transgene results in decreased pollen protein production normally produced in Italian ryegrass, and no additional or novel proteins are produced by the transgenic plants that bear this gene (Petrovska et al. 2004). Therefore, in the unlikely event that the *Lol p1* gene migrates to Italian ryegrass plants outside the field trial, this gene presents no additional toxicological or allergenic concerns, would not confer a selective advantage to other ryegrass plants, and would result in no significant impact on the environment.

5. Persistence of Transgenic Grasses

It is highly unlikely that transgenic tall fescue and Italian ryegrass will persist within the field site. During the experiment, the applicant will mow the 170 acre site to eliminate the production of flowers from any renegade fescue or ryegrass plant. Seeds will be carefully harvested from transgenic and recipient plants, and transported from the field site, reducing the risk of potential germination of a transgenic fescue or ryegrass during the next growing season. In addition, after completion of the field release, the entire 170 acre field plot will be sprayed with an effective herbicide to remove all cool-season grasses involved in the experiment. If the applicant removes transgenic or non-transgenic plants from the field site, APHIS proposes to require the applicant to treat all plants removed from the field site as regulated articles (under Section III: Alternatives, Subsection: C. Issue Permit with Supplemental Conditions, Condition c). Thus the removed plants will be transported under contained conditions, eliminating the risk of persistence of a transgenic plant by way of escape during transportation. After completion of the experiment, the field site will be monitored monthly for volunteer plants for two years. Cool season grasses look distinctly different from warm season grasses, thus any volunteer fescue or ryegrass will be easily found and killed with herbicide.

It is also highly unlikely that transgenic pollen will travel outside the field boundary (see section D. 1. a. for a discussion on Pollen Movement). Recent research suggesting long distance pollen movement used a relatively large, 400 acre transgenic pollen source (Watrud et al. 2004). The proposed field experiment will release a relatively small amount of pollen from 1080 transgenic plants within 0.25 acres. A previous study using a similar field design to the one proposed in these permit applications found that transgenic pollen did not travel beyond 200m (Wang et al. 2004b), well within the 400 m field site boundary.

Finally, the availability of receptive, compatible plant species outside the proposed field site must also be examined. The area surrounding the proposed field site is inhospitable to cool season forage grasses such as fescues and ryegrasses and contains sexually incompatible warm season grasses. Thus, the risk of transgenic pollen reaching a sexually compatible plant is low.

Furthermore, because of the dry climate, any offspring of tall fescue and ryegrass plants outside the field boundary accidentally fertilized with inadvertently released transgenic pollen would also

require irrigation for establishment. Thus, the risk of establishment and persistence of any transgenic tall fescue and Italian ryegrass plants is extremely low.

Thus, for successful establishment of fescue and ryegrass plants outside the field site boundary, transgenic pollen must (1) travel a distance of greater than 400m, which is unlikely, and (2) find and pollinate remote, isolated, sexually compatible plants within large expanses of non-compatible plants and (3) survive a climate inherently unfriendly to tall fescue and Italian ryegrass. Given the low probability of each of these requirements in regards to transgenic pollen movement, APHIS finds that there is minimal risk of the persistence of transgenic plants outside the proposed field site boundary.

6. Weediness of Tall Fescue and Italian Ryegrass

Weedy characteristics of grasses include properties such as aggressive growth or the production of stolons (plant shoots that produce roots and thus establish faster than a plant that does not produce stolons), or increased ability for seed shattering (resulting in a increased ability for seed dispersal for a plant compared to a plant with lesser ability for seed shattering) (Baker 1965, 1974). Transgenic tall fescue plants do not exhibit any greater weediness potential than non-transgenic, commercially available tall fescue plants (Wang et al. 2003).

Previous studies have found transgenic tall fescue has similar agronomic performance as non-transgenic tall fescue (Wang et al. 2003). Although flowering time is slightly earlier and transgenic tall fescue is marginally taller compared to non-transgenic tall fescue (Wang et al. 2003), modeling studies have shown that these two traits do not significantly affect gene flow in *Festuca* (Nurminiemi et al. 1998), and should not increase the weediness potential of transgenic tall fescue.

As pasture and forage grasses, both tall fescue and Italian ryegrass are not as heavily domesticated as crop plants and thus retain some characteristics that allow the non-transgenic versions of these two grasses to become weeds in particular crops (Stubbendieck et al. 1994, Whitson et al. 1996, Uva et al. 1997, SWSS 1998, CEPPC 1999). Tall fescue is considered a weed when grown in Kentucky bluegrass lawns or sod farms (Dernoeden 1990) but there are measures available to reduce tall fescue invasion of Kentucky bluegrass (Larocque and Christians 1985, Dernoeden 1986, Maloy and Christians 1986, Dernoeden 1990). Any of the measures used to control conventional tall fescue would still be effective to control the transgenic version.

Italian ryegrass is known to be a minor weed in soybean, corn, and tall fescue turf (Brede and Brede 1988, Carruthers et al. 1998, Beam et al. 2005) and can be a troublesome weed in winter wheat (Elmore et al. 1995, Stone et al. 1998, Hoskins et al. 2005). Control of Italian ryegrass in winter wheat grown in Arkansas and Oregon can be hindered by herbicide (diclopfop) – resistant populations the ryegrass (Ghersa et al. 1994, Hoskins et al. 2005). Resistance of Italian ryegrass to another herbicide, glyphosate, has also been discovered in Brazil and Chile (Perez and Kogan 2003, Christoffoleti et al. 2005). However, none of the genes inserted into Italian ryegrass for this experiment are expected to increase the weediness potential or herbicidal tolerance that is already present in commercially available Italian ryegrass. Any of the measures used to control conventional Italian ryegrass would still be effective to control the transgenic version.

As an additional permit condition, APHIS would require the applicant to include a 10 ft wide fallow, bare ground border to determine the extent of vegetative spread of the transgenic grasses (under Section III: Alternatives, Subsection: C. Issue Permit with Supplemental Conditions, Condition b). Neither tall fescue nor Italian ryegrass is known to quickly spread vegetatively, and this additional measure will allow the researcher to confirm the absence of this weedy trait from the transgenic grasses used in the experimental design.

7. Alteration in Susceptibility to Disease or Insects

There has been no intentional genetic change in these plants to affect their susceptibility to disease or insect damage. Wang et al. (2003) found no observable changes concerning the incidence of pests, beneficial insects or pathogens in the transgenic tall fescue. Neither the gene for hygromycin resistance nor β-glucuronidase activity, nor the insertion of the *Lol p1* gene resulting in the decrease of a pollen allergen in Italian ryegrass is expected to alter the susceptibility of the transgenic grass plants to disease or insect damage.

Execution of the prescribed periodic monitoring of the field plots will allow the detection of any unexpected infestation by plant disease organisms or animal pests. The applicant is required to report any such unanticipated effects to APHIS under the terms of the permit. See 7 CFR § 340.4(f)(10)(ii).

8. Horizontal Gene Transfer to Other Organisms

Horizontal gene transfer and expression of DNA from the two plant species to bacteria is unlikely to occur. First, many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Kaneko et al. 2000, Wood et al. 2001, Kaneko et al. 2002). There is no evidence that these organisms contain genes derived from plants. Second, in cases where review of sequence data implied that horizontal gene transfer occurred, these events are inferred to occur on an evolutionary time scale on the order of millions of years (Koonin et al. 2001, Brown 2003). Third, transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. Thus even if horizontal gene transfer occurred, proteins corresponding to the transgenes are not likely to be produced. Fourth, the FDA has evaluated horizontal gene transfer from the use of antibiotic resistance marker genes, and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment, is remote (http://vm.cfsan.fda.gov/~dms/opa-armg.html). Furthermore, hygromycin was withdrawn from the market in 2002 for sales and marketing reasons (Dawe and Hofacre 2002) and has no clinical utility at this time or in the foreseeable future (Goldstein et al. 2005). Therefore APHIS concludes that horizontal gene transfer poses no significant environmental risk.

9. Fate of Transgenic DNA

As the applicant has taken steps to reduce animal access to the transgenic and recipient plots, and that these transgenic and recipient fescue and ryegrass plants will not be used for food or feed, the information presented in this section is for the unlikely event of accidental consumption.

Transgenic DNA is no different from other DNA consumed as part of the normal diet. Genetically engineered organisms have been used in drug production and microbial fermentation (cheese and yogurt) since the late 1970's. More than 500 million cumulative acres of engineered food and feed crops have been grown and consumed world wide in the past seven years (International Service for the Acquisition of Agri-biotech Applications, (ISAAA) at: http://www.isaaa.org/kc/CBTNews/press_release/briefs30/es_b30.pdf. The FDA has not reported any significant concerns with bioengineered food and feed currently on the market. The EPA has exempted from a tolerance DNA that encodes currently registered plant incorporated protectants because of a lack of toxicity (FR 66 37817-37830).

There have been several studies in humans and animals following the fate of DNA once consumed (Mercer et al. 1999, Beever and Kemp 2000, Duggan et al. 2000, Einspanier et al. 2001, Chambers et al. 2002, Netherwood et al. 2002, Duggan et al. 2003). The majority of DNA consumed is degraded in the gastro-intestinal tract although the degradation is not 100% efficient. There is evidence that DNA from consumed food can move from the GI tract lumen to other areas of the body and that this is a normal occurrence. No risks have been identified as a result of this movement.

10. Effects on Chemical (pesticide, herbicide, fungicide) Load on the Environment

The only additional chemical load on the environment due to proposed research is a single herbicide application on the 170 acre field site following termination of the experiment. The applicant proposes to use glyphosate products, as herbicides containing this ingredient are registered for use on tall fescue and Italian ryegrass by the Environmental Protection Agency (EPA) (http://www.epa.gov/oppsrrd1/REDs/factsheets/0178fact.pdf). Glyphosate, compared to other herbicides, has favorable environmental safety characteristics, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence) (Franz et al. 1997), as well as extremely low toxicity to mammals, birds and fish (Malik et al. 1989). Glyphosate has been applied to this same field site in 2004 and 2005, thus the proposed 2006 application of glyphosate does not constitute a change in field management practices for this site. Additionally, the field site is small (170 acres) compared to the agricultural production acreage in Oklahoma (350,000 acres of herbicide-tolerant crops (http://usda.mannlib.cornell.edu/reports/nassr/field/pcp-bba/acrg0605.pdf)) that may use glyphosate as the primary herbicide. Therefore, APHIS concludes that there is no significant impact on the environment due to the glyphosate application proposed in the permit applications.

11. Potential Impacts on Humans, Including Minorities, Low Income Populations, and Children

Because the field test is on an isolated property owned by the Samuel Robert Noble Foundation, the public will not be exposed to these transgenic plants. The fescue and ryegrass seeds collected at the termination of the experiment are unlikely to be mixed with any seeds intended for human or animal consumption because of numerous measures described in above text and APHIS inspections during harvesting. All the harvested seeds will be stored in dedicated storage bags on site and seeds transferred to a laboratory setting for seed cleaning and analysis.

Consideration of these potential impacts are specified in Executive Orders 13045 an 12898 and address the identification of health or safety risks that might disproportionately affect children or have adverse impacts on minorities and low-income populations. The proposed actions are not expected to adversely affect any of these groups.

12. Risks to Threatened and Endangered Species

BRS has reviewed the data in accordance with a process mutually agreed upon with the U.S. Fish and Wildlife Service to determine when a consultation, as required under Section 7 of the Endangered Species Act, is needed. APHIS has reached a determination that the release under the permits 05-278-01r and 05-278-02r would have no effects on listed threatened or endangered species and consequently, a written concurrence or formal consultation with the Fish and Wildlife Service is not required for this EA. Appendix 4 includes the BRS analysis of threatened and endangered species in the area of the field release.

13. Effects on Native Floral and Faunal Communities

a. Native Floral Communities

The field site proposed in the permit applications and the surrounding fields have a history of growing warm season grasses as the climate is too dry for continual successful and competitive growth of cool season grasses, such as tall fescue and Italian ryegrass. As climate, in combination with the confinement conditions imposed by the applicant and APHIS, will successfully limit the invasiveness and competitiveness of tall fescue and Italian ryegrass, APHIS concludes there would be no significant effect on any native floral species.

b. Terrestrial Vertebrate Animals

The most likely vertebrates to encounter the transgenic tall fescue and Italian ryegrass plants in this field experiment are grazers (e.g. pasture animals) and seed-eaters (e.g. birds and rodents). Analysis conducted by APHIS suggests these vertebrates will not be significantly affected by this transgenic release for the following reasons:

1. In order to minimize exposure by plant consumption, the applicant will be required under the permit conditions to surround the 0.25 acre transgenic plot with a strong electrical fence as well as caging the transgenic plots and the recipient plots to eliminate grazers and to discourage seed-eaters from entering these plots.

2. All seed heads will be bagged immediately at seed maturation to reduce the consumption of mature seeds. As a duplicative measure, APHIS would require the applicant to place the bag containing the cut seed head immediately into another bag before removal from the field site.

3. In the unlikely event of accidental consumption of plant material or seeds, the enzyme β-glucuronidase and the enzyme for hygromycin resistance do not have any similarity to known allergens or toxins, as stated by EPA review (FR 66 42957-42962, FR 69 18275-18278). The gene for the down regulation of pollen does not produce any protein and thus does not confer any toxicological or allergenic properties.

4. APHIS will inspect the site during planting and harvesting to ensure all permit conditions are met.

c. Terrestrial Invertebrate Animals

The most likely invertebrate animals exposed to the transgenes in the grass seed would be seed-eating invertebrates. In the unlikely event of accidental consumption of seeds by terrestrial invertebrates, the enzyme β-glucuronidase and the enzyme for hygromycin resistance do not have any similarity to known toxins, as stated by EPA review (FR 66 42957-42962, FR 69 18275-18278). The gene for the down regulation of pollen does not produce any protein and thus does not confer any toxicological properties. APHIS therefore concludes there would be no significant effect on any invertebrate species.

d. Aquatic Organisms

Within the proposed 170 acre field site, there is a small pond that will be used as an irrigation source for the experimental tall fescue and Italian ryegrass plants. As stated above, there is no expectation of toxicological effects on any organism due to the ingestion of the transgenic plant material in this study. APHIS therefore concludes there would be no significant effect on any aquatic species.

14. Impact on Existing Agricultural Practices

In 2002, 73 farms in Oklahoma produced 587,557 kg of fescue seed on 6595 acres, with no production located in Love County OK or surrounding counties. This small field test will not have any significant impact on existing agricultural practices because this test is solely for research purposes.

15. Consistency of proposal with other environmental requirements

The proposal is believed to be consistent with other environmental requirements. This environmental assessment was prepared in accordance with: (1) The National Environmental Policy Act of 1969 (NEPA), as amended (42 U.C § 4321 et seq.); (2) regulations of the Council on Environmental Quality for implementing the procedural provisions of NEPA (40 CFR §§ 1500-1508); (3) USDA regulations and implementing NEPA (7 CFR § 1b); and (4) APHIS NEPA Implementing Procedures (7 CFR § 372).

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Figure 1

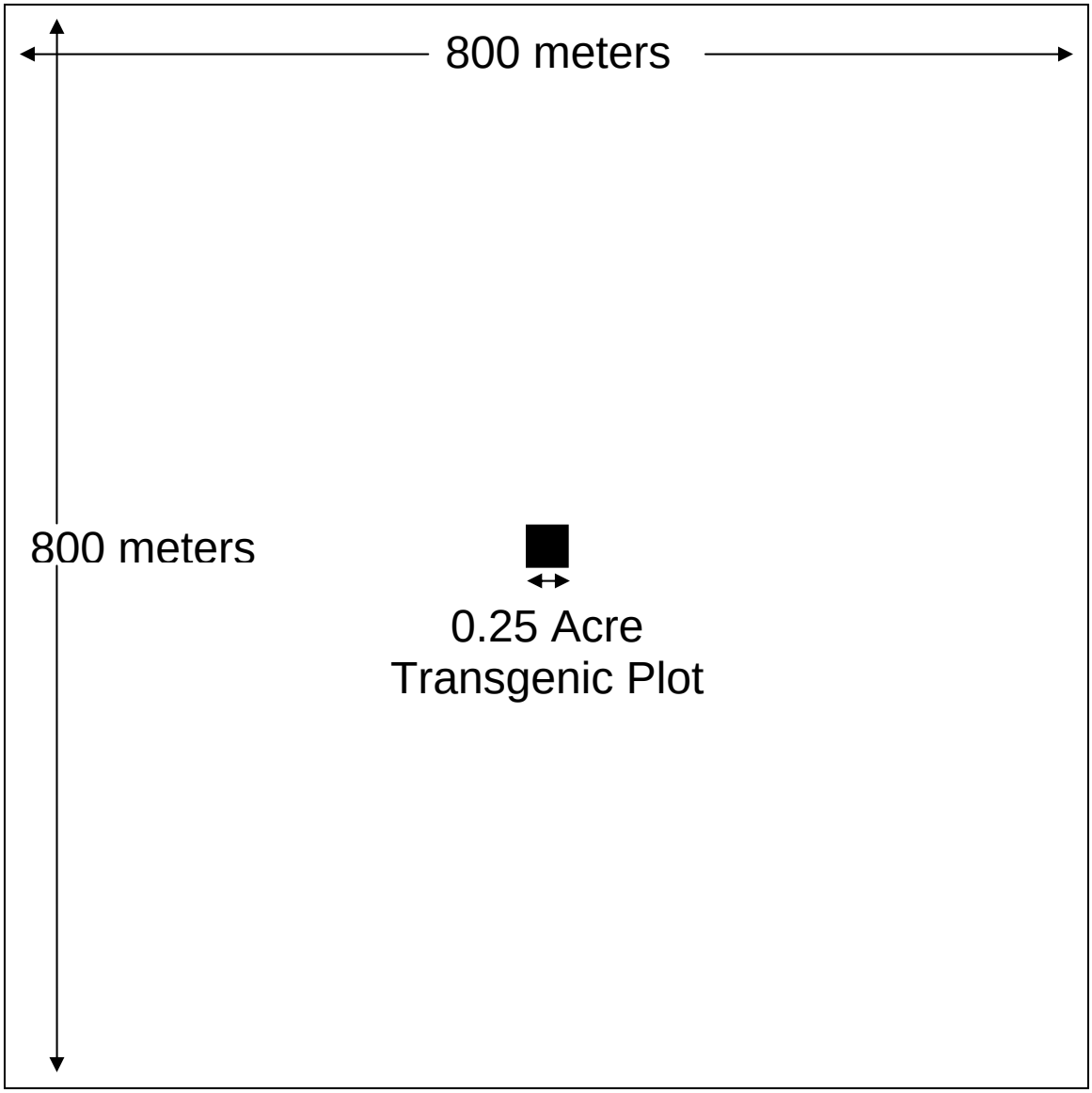

Figure 1. Size and dimensions of the field site and the inner 0.25 acre plot containing the transgenic plants. Drawn to scale.

Figure 2

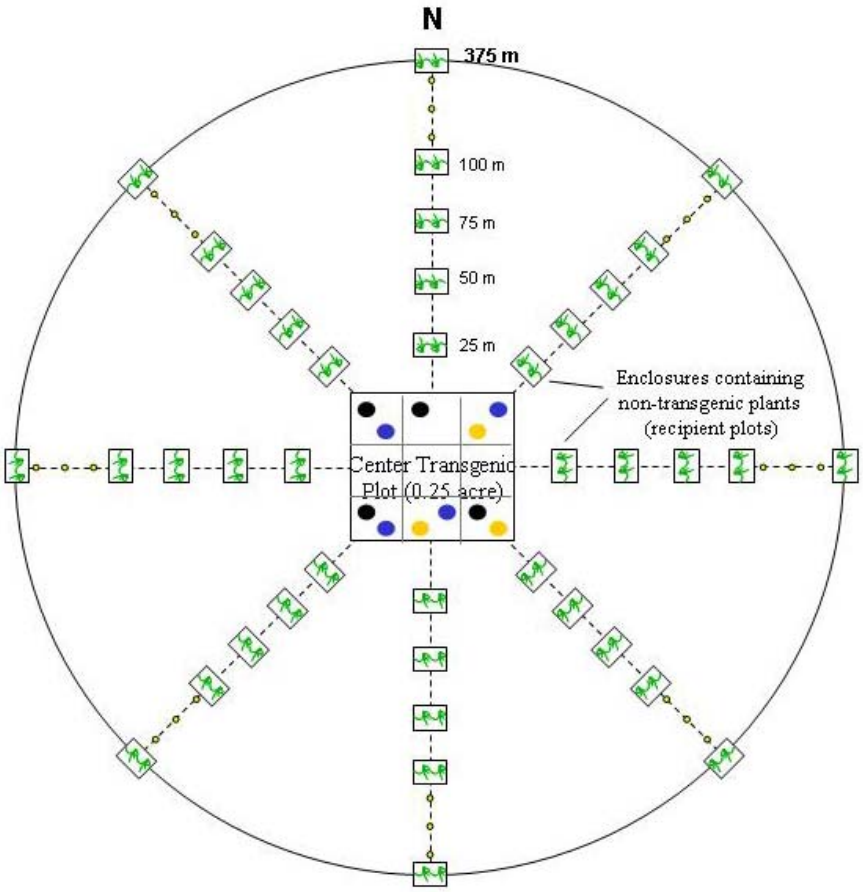

Figure 2. Experimental design of the field study. The center 0.25 acre plot contains the transgenic tall fescue (one line) and the transgenic Italian ryegrass (2 lines) plants. See Figure 3 for a detailed description of the 0.25 acre transgenic plot. Eight transects radiate from the center transgenic plot. Each transect contains 8 recipient plots containing 4 non-transgenic tall fescue and 4 non-transgenic Italian ryegrass plants. The 8 recipient plots on each transect are spaced every 25m from the center transgenic plot, from 25m to 375m.

Figure 3

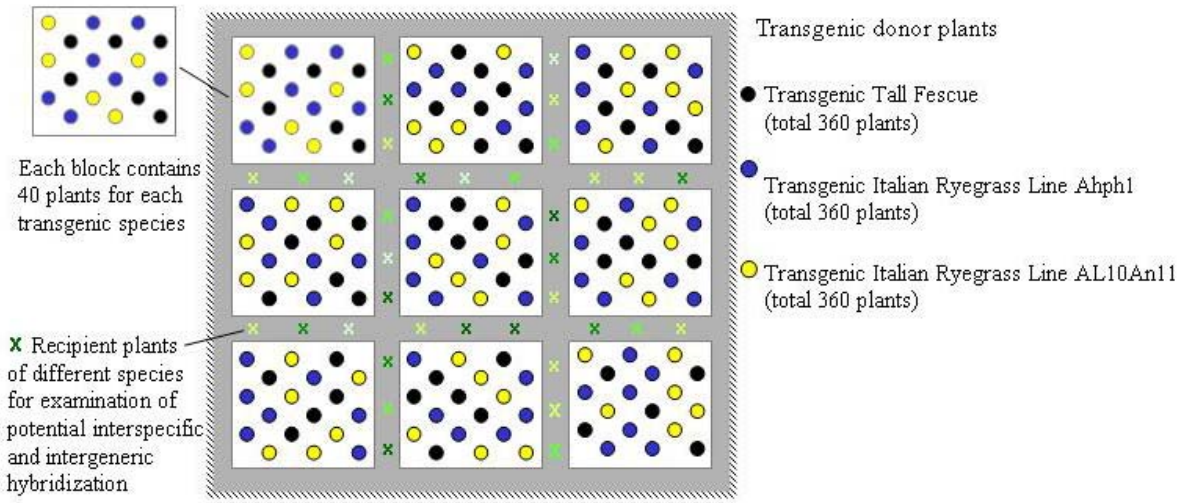

Figure 4: Experimental design for the 0.25 acre transgenic plot

Figure 3. Experimental design of the 0.25 acre transgenic plot containing the transgenic tall fescue (one line) and the transgenic Italian ryegrass (two lines) plants.

APPENDIX 1: Description of the Field Experiment

A single transgenic field plot will be located at the Dixon Road Property (Coffey Ranch) of the Noble Foundation in Love County, Oklahoma. Transgenic tall fescue and Italian ryegrass plants will occupy a 0.25 acre plot in the center of a 170 acre field (Figure 1). The 0.25 acre transgenic plot will consist of 9 blocks, with each block containing 40 transgenic tall fescue plants conferring hygromycin resistance and expression of beta-glucuronidase, 40 transgenic Italian ryegrass plants conferring hygromycin resistance, and 40 transgenic Italian ryegrass plants expressing the down regulation of the pollen allergen gene *Lol p1*, hygromycin resistance and beta-glucuronidase (Figure 3).

The transgenic plants in each block will be established with a 3 ft planting distance. Within each block the position of the transgenic donor plants will be randomized. Several non-transgenic species will be planted in a checker-board pattern among the transgenic plants in order to effectively assess hybridization potential, namely interspecific and intergeneric gene flow. The species included for assessment will include *Festuca pratensis*, *F. rubra*. *F. ovina*, *F. idahoensis*, *F. gigantea*, *F. mairei*, *F. versuta*, *Lolium temulentum* and *L. rigidum*. These species were chosen because they are commercially grown or they are native species of North America. A preliminary field experiment by the applicant suggests minimal cross-pollination between these species.

To examine the distance transgenic pollen is able to flow and remain viable, and the frequency of pollen contamination at different distances from the pollen source, the central 0.25 acre plot of transgenic tall fescue and Italian ryegrass will be surrounded by 2m x 2m enclosures containing 4 non-transgenic tall fescue and 4 non-transgenic Italian ryegrass plants (Figure 2). Eight transects will be established from the central plot. Each transect will be spaced 45 degrees apart in a circle radiating from the 0.25 acre transgenic plot in a 'wagon-wheel' design. Along each transect, enclosures will be placed every 25m, from 25m to 375m. Total land requirement for this field experiment is 155 acres and is wholly contained within the 170 acre field site boundary.

During the experiment, agronomic characteristics including number of tillers, flowering date, plant height and seed yield will be measured for the transgenic and non-transgenic plants. This data will be used to examine the performance of transgenic plants compared to non-transgenic plants.

APPENDIX 2: Biology of Tall Fescue and Italian Ryegrass

Tall fescue (*Festuca arundinacea* Schreb.) and Italian ryegrass (*Lolium multiflorum* Lam.) are closely related grasses of significant value in temperate grasslands. These two species (both in the tribe Poeae, subfamily Pooideae) are part of the *Festuca-Lolium* species complex containing 80 *Festuca* and 8 *Lolium* species of highly related, well-adapted, productive grasses distributed in temperate and cool climates world-wide (Jauhar 1993, Spangenberg et al. 1998). Because the mechanism by which genes are moved from one flowering plant to another is through cross-pollination of sexually compatible plants, the plants with which tall fescue and Italian ryegrass can cross-pollinate are described. However, the taxonomy and systematics of the *Festuca* and *Lolium* genera are exceedingly complex (Schoth and Weihing 1962, Buckner et al. 1967, Buckner et al. 1979, Hanson 1979, Terrell 1979, Bulinska-Radomska and Lester 1986, 1988, Barnes 1990, Jauhar 1993, Loos 1993, Evers et al. 1995, Sleper and Buckner 1995, Jung et al. 1996, Moser and Hoveland 1996, Sleper and West 1996, Spangenberg et al. 1998) and thus the analysis below focuses on fescues and ryegrasses used in the experimental design, are able to form hybrids, and are found in the area of the proposed field site.

A. Tall Fescue

Tall fescue (*Festuca arundinacea*) is a wind-pollinated, perennial cool-season grass and forms the forage basis for beef cow and calf production (Wang et al. 2004a) and is also widely used as turf in lawns, parks, sports fields, highway medians and roadsides (Sleper and Buckner 1995). It is indigenous to Europe where it is well adapted. It was introduced to North America in the early to mid-1800's and has become the predominant cool-season pasture grass in the USA (Buckner et al. 1979, Barnes 1990). Its widespread use in the US is due to its adaptation to a wide range of soil conditions, tolerance of continuous grazing, high yields of forage and seed, long grazing season, compatibility with varied management practices and low incidence of pest problems (Hanson 1979, Sleper and West 1996).

There are 20 *Festuca* species in the United States. In Oklahoma, the following species are found (with synonyms in parentheses): *F. arundinacea*, *F. ovina*, *F. rubra*, *F. trachyphylla* (syn. *F. brevipila*, *F. cinerea*, *F. duriuscala*, *F. longifolia)*, *F. arizonica*, *F. filiformis* (syn. *Leptochola panacea* spp. Brachiata), *F. ligulata*, *F. versuta*, *F. paradoxa* (syn, *F. nutans* and *F. shortii*), *F. subverticillata* (syn. *F. obtuse*), *F. cristata* (syn. *Rostraria cristata*), *F. barbata* (syn. *Schismus barbatus*), *F. bromoides* (syn. *F. dertonensis*, *Vulpia bromoides*), *F. megalura* (syn. *F. myuros*, *Vulpia myuros*), *F. octoflora*, *F. gracilenta* (syn. *F. tenella*, *Vulpia octoflora* var glauca) and *F. sciurea* (syn. *Vulpia sciurea*), and *F.* pratensis (USDA 2005).

Of these, *F. arundinacea*, *F.ovina*, *F rubra*, and *F. versuta* are proposed to be used to examine out-crossing between species. The applicant will also use *F. idahoensis*, *F. gigantean*, and *F. mairei*, which are native to the United States, in the proposed experiment. Hybrids are only known to readily occur between *F. arundincacea*, *F. gigantean*, and *F. pratensis* (section *Bovinae*) as well as between the out-crossing *Lolium* species (see below) (Bulinska-Radomska and Lester 1986, 1988,

Jauhar 1993). In fact the members of the *Bovinae* and *Lolium* genera show higher relatedness with each other then with other members of their own genus (Bulinska-Radomska and Lester 1986, 1988, Jauhar 1993). However, for each hybrid pairing, the pollen viability in the resulting offspring is low, indicating lower fitness than intraspecific reproduction (Bulinska-Radomska and Lester 1988).

B. Italian Ryegrass

Italian ryegrass (*Lolium multiflorum*) is an annual or biennial highly palatable nutritious grass which shows a rapid establishment from seed, good production in the seedling year and rapid recovery after defoliation (Buckner et al. 1967, Spangenberg et al. 1998). *Lolium multiforum* is indigenous to the temperate regions of Asia, Europe and North Africa and was introduced for cultivation in the Americas (Jauhar 1993, Spangenberg et al. 1998). *L. multiflorum* is an out-breeding species and readily outcrosses with other out-crossing members of the genus, particularly with *L. perenne*, and *L. rigidum* (Jauhar 1993). The relatedness between these species is to such an extent that some authors conclude that these out-crossing species should not be considered distinct species (Naylor 1960, Bulinska-Radomska and Lester 1985). The inbreeding species of this genus, *L. temulentum*, *L. persicum*, *L. remotum* and *L. subulatum* are reproductively isolation through self-fertilization (Jauhar 1993). Of these species, only *L. multiflorum*, *L. rigidum* and *L. temulentum* are proposed to be used in the permit applications.

In Oklahoma and Texas, the following species in the genus *Lolium* are found: *L. multiflorum*, *L. perenne*, and *L. temulentum* (syn. *L. arvense*) (USDA 2005). Again, as stated above, *L. multiflorum* forms interspecific hybrids with *L. perenne* (perennial ryegrass). Perennial ryegrass is grown mainly as a pasture or turf grass, and northern expansion of the grass tends to be limited by low temperature and low precipitation. Hybrids between Italian ryegrass and perennial ryegrass are not known to be more invasive or weedy than offspring of *L. multflorum* plants.

APPENDIX 3: Description of the Regulated Grass Plants

1. Tall fescue

Transformed tall fescue plants (cultivar Kentucky 31) were developed at the Genetic Transformation Laboratory of Forage Improvement Division, the Samuel Robert Noble Foundation, Admore, OK. The plasmids were constructed in Swiss Federal Institute of Technology, Zurich Switzerland (Bilang et al. 1991, Spangenberg et al. 1995).

The transgenic tall fescue was created using microprojectile bombardment, which is a well-characterized transformation system which integrates the donor genes into the chromosome of the recipient plant cell (Batty and Evans 1992, Wang et al. 2003, Wang et al. 2004a). The system does not require the use of the plant pathogen, *Agrobacterium tumefaciens*, or other transformation vectors. The donor DNA sequences are stably and irreversibly integrated into the plant's chromosomal or organellar DNA, where they are maintained and inherited as any other genes of the plant cell. Copy number of the transgene is estimated to vary from one to four copies in the transgenic tall fescue plants.

Sterilized seeds/caryopses of tall fescue were used as explants for callus induction. Embryogenic calluses were transferred to liquid culture medium to establish cell suspension cultures (Wang et al. 1994, Spangenberg et al. 1995). Cell clusters from the suspension cultures were used as direct targets for biolistic transformation to generate transgenic plants. A PDS-1000/He biolistic device (Bio-Rad #165-2257) was used to deliver DNA-coated gold particles to target cells.

Inserted into the tall fescue plants were two plasmids, pAch1 which carried the hygromycin phosphotransferase gene (*hph*) and pCintG which bears a chimeric β-glucuronidase gene (*gusA*). The hygromycin phosphotransferase coding sequence on plasmid pAch1 is under control of the rice actin-1 5' regulatory signals (promoter and intron) from pCOR117 (McElroy et al. 1991). The chimeric β-glucuronidase gene on plasmid pCintG is driven by the cauliflower mosaic virus 35S promoter with a rice tungro virus intron. Both plasmids contain the 35S terminator from cauliflower mosaic virus. For the transgenic tall fescue plants, hygromycin phosphotransferase renders transformed cells resistant to hygromycin and was used as a selectable marker and β-glucuronidase is used as a reporter gene.

Hygromycin resistance calluses were obtained after biolistic transformation of suspension cells and subsequent selection in the presence of hygromycin. Transgenic tall fescue plants were regenerated from the hygromycin resistance calluses and later transferred to the greenhouse.

Southern blot analysis confirms the transgenic nature of the tall fescue plants (Wang et al. 2003). Hybridization signals corresponding to the high-molecular weight bands and the full-length hygromycin phosphotransferase gene (*hph*) gene were observed in the Southern analysis using digested and undigested genomic DNA samples, respectively (Wang et al. 2003). Single-copy integration of transgenes, different hybridization patterns, including additional *hph*-hybridizing bands were also observed for some samples, indicating that multiple insertions of rearranged or partial copies of the chimeric *hph* gene occurred (Wang et al. 2003).

2. Italian Ryegrass

The original Italian ryegrass plants (cultivar Andy) engineered to express hygromycin resistance, beta-glucuronidase, and the down-regulation of pollen allergen *Lol p1* were developed at the Plant biotechnology Centre, Primary Industries Research Victoria, located at La Trobe University, Australia. Transgenic Italian ryegrass plants were imported form Australia to the Noble Foundation under USDA permit. Seeds were produced from these transgenic plants in the greenhouse of the Noble Foundation. F1 transgenic plants have been established from the seeds and will be used for the proposed experiment. The original imported transgenic plants were autoclaved.

Two transgenic lines of Italian ryegrass were developed: line Ahph1 expresses hygromycin resistance, and line AL10An11 expresses hygromycin resistance, beta-glucuronidase, and the down regulation of pollen allergen *Lol p1*.

The lines of transgenic Italian ryegrass were created using the same microprojectile bombardment as for the transgenic tall fescue plants, which is a well-characterized transformation system which integrates the donor genes into the chromosome of the recipient plant cell (Batty and Evans 1992, Wang et al. 2003, Wang et al. 2004a). The system does not require the use of the plant pathogen, *Agrobacterium tumefaciens*, or other transformation vectors. The donor DNA sequences are stably and irreversibly integrated into the plant's chromosomal or organellar DNA, where they are maintained and inherited as any other genes of the plant cell. Copy number of the transgene is estimated to vary from one to four copies in the transgenic Italian ryegrass plants.

Sterilized seeds/caryopses of Italian ryegrass were used as explants for callus induction. Embryogenic calluses were transferred to liquid culture medium to establish cell suspension cultures (Wang et al. 1994, Spangenberg et al. 1995). Cell clusters from the suspension cultures were used as direct targets for biolistic transformation to generate transgenic plants. A PDS-1000/He biolistic device (Bio-Rad #165-2257) was used to deliver DNA-coated gold particles to target cells.

For transgenic Italian ryegrass line Ahph1, plasmid pAch1 (described above) was used to insert the gene for hygromycin resistance to be used as a selectable marker. The hygromycin phosphotransferase coding sequence on plasmid pAch1 is under control of the rice actin-1 5' regulatory signals (promoter and intron) from pCOR117 (McElroy et al. 1991) and the 35S terminator from cauliflower mosaic virus was also used.

Plasmid pAch1 was also inserted into Italian ryegrass line AL10An11 to be used as a selectable marker. This grass line also contained plasmid pZIGSAR which bears the perennial ryegrass (*Lolium perenne*) pollen allergen gene (*Lol p1*) in antisense orientation under control of the maize (*Zea mays*) pollen-specific *Zm13* promoter and the nos (nopaline synthase) terminator sequence from *Agrobacterium tumefaciens*. A chimeric β-glucuronidase (*gusA*) gene driven by an enhanced 35S cauliflower mosaic virus promoter and terminated by the 35S terminator from cauliflower mosaic virus was also included as a reporter gene in the transformation vector.

Hygromycin resistance calluses were obtained after biolistic transformation of suspension cells and subsequent selection in the presence of hygromycin. Transgenic tall fescue plants were regenerated from the hygromycin resistance calluses and later transferred to the greenhouse.

The transgenic nature of Italian ryegrass was demonstrated with Southern hybridization analysis (Petrovska et al. 2004). For most samples, complex hybridization patterns indicated the integration of multiple and rearranged copies of the transgene (Petrovska et al. 2004). β-glucuronidase activity was detected exclusively in the pollen grains (Petrovska et al. 2004, Wang et al. 2004a) , confirming the pollen-specificity of the *Zm13* promoter. Accumulation of *Lol p1* pollen allergens in transgenic Italian ryegrass was significantly reduced compared to non transgenic plants (Petrovska et al. 2004). The hypo-allergenic character of pollen from transgenic antisense *Lol p1* containing Italian ryegrass plants was further confirmed by immunoblots using IgE antibodies from sera of ryegrass pollen-sensitized allergic patients (Petrovska et al. 2004).

APPENDIX 4: Threatened and Endangered Species Analysis

According to the Fish and Wildlife Service (http://ecos.fws.gov/tess_public/servlet/gov.doi.tess_public.servlets.UsaLists?usMap=1&status=listed&state=OK) (accessed on 12/22/ 2005) there are 22 federally listed threatened and endangered animals and 2 threatened plant species in the state of Oklahoma. These are:

Mammals

- Gray Bat (*Myotis grisescens*)
- Indiana Bat (*Myotis sodalis*)
- Ozark big-eared Bat (*Corynorhinus* (=*Plecotus*) *townsendii ingens*)
- Lynx (*Lynx canadensis*)
- Grizzly Bear (*Ursus arctos horribilis*)
- Gray Wolf (*Canis lupus*)

Birds

- Whooping Crane (*Grus americana*)
- Interior Least Tern (*Sterna antillarum*)
- Black-capped Vireo (*Vireo atricapillus*)
- Red-cockaded Woodpecker (*Picoides borealis*)
- Eskimo Curlew (*Numenius borealis*)
- Piping Plover (*Charadrium melodus*)
- Bald Eagle (*Haliaeetus leucocephalus*)

Fish

- Ozark Cavefish (*Amblyopsis rosae*)
- Neosho Madtom (*Noturus placidus*)
- Arkansas River Shiner (*Notropis girardi*)
- Leopard Darter (*Percina pantherina*)
- Bull Trout (*Salvelinus confluentus*)

Invertebrates

- American Burying Beetle (*Nicrophorus americanus*)
- Ouachita Rock Pocketbook (*Arkansia wheeleri*)
- Scaleshell (*Leptodea leptodon*)
- Winged Entire Mapleleaf (*Quadrula fragosa*)

Plants

- Eastern Prairie Fringed Orchid (*Platanthera leucophaea*)
- Western Prairie Fringed Orchid (*Platanthera praeclara*)

None are known to reside in Love County, Oklahoma. Whooping cranes of the Aransas/Wood Buffalo population migrate through west-central Oklahoma, but the proposed field site does not contain a suitable wetland for overnight roosting during migration. The Eskimo curlew uses the Central Flyway during its spring migration, however, little is known about stopovers during migration for this species and only isolated unconfirmed reports exist for the state of Oklahoma. The two threatened orchid species,

although historically known to inhabit grasslands in Oklahoma, have not recently been found in this state.

APPENDIX 5: Standard Permit Conditions

1. APHIS' Biotechnology Regulatory Services (BRS) or a Regional Biotechnologist may conduct an inspection of the test site at the beginning of the test. If there are additional movements or releases to sites not listed in this permit, submit the site specific information in the same format as in the permit, the phenotype of the plants and the permit number to the State's Regulatory Official (addresses found on our website at http://www.aphis.usda.gov/brs/lt_sta.html), the Regional Biotechnologists (addresses enclosed), and Mrs. Linda Lightle, USDA APHIS BRS, Biotechnology Permit Services, 4700 River Road, Unit 147, Riverdale, Maryland 20737. This information should be transmitted via next day mail service (e.g., FedEx). The action can take place 10 days after notification of all parties and all the actions must be under the stated permit conditions.

2. Additional inspections may be conducted by a Plant Protection and Quarantine Officer.

3. Notify APHIS BRS within 14 days of any proposed changes to the protocol referenced in the permit application.

4. This approved Biotechnology Permit (APHIS form #2000) does not eliminate the permittee's legal responsibility to obtain all necessary Federal and State approvals, including: (1) for the use of any non-genetically engineered plant pest or pathogens as challenge inoculum; (2) plants, plant parts or seeds which are under existing Federal or State quarantine or restricted use; (3) experimental use of unregistered chemical; and (4) food or feed use of genetically engineered crops harvested from the field experiment.

5. Harvested plant material may not be used for food or animal feed unless it is first devitalized and approved for such use by the U.S. Food and Drug Administration; and for plant-incorporated protectants, a tolerance for the pesticide must first be established by the U.S. Environmental Protection Agency (EPA).

6. APHIS shall be notified orally within 24 hours followed by a written notification within 5 days upon discovery in the event of any accidental or unauthorized release of the regulated article.

    A. For immediate oral notification, contact APHIS BRS Compliance Staff at (301) 734-7324 and ask to speak to Compliance and Inspection staff member.
    B. In the event of an emergency and you are unable to reach the BRS Compliance Staff at the above number, you may call:

        The APHIS/BRS Regional Biotechnology Coordinator assigned to the state, where the field test occurs:

            For Western Region, contact Ralph D. Stoaks by phone at (970) 494-7573 or e-mail Ralph.D.Stoaks@aphis.usda.gov

For Eastern Region, contact Ashima Sengupta by phone at (919) 855-7623 or e-mail Ashima.Sengupta@aphis.usda.gov

Or

The APHIS PPQ Regional Biotechnology Coordinator assigned to the state where the field test occurs

For Western Region, contact Stuart W. Kuehn by phone at (970) 494-7563 or e-mail Stuart.W.Kuehn@aphis.usda.gov

For Eastern Region, contact Susan Dublinski by phone at (919) 855-7324 or e-mail Susan.G.Dublinski@aphis.usda.gov

Or

The APHIS State Plant Health Director assigned of the state where the field test occurs. The list of APHIS State Plant Health Directors is available at http://ceris.purdue.edu/napis/names/sphdXstate.html

C. Written notification should be sent:

By e-mail:
BRSCompliance@aphis.usda.gov

By mail:
Animal and Plant Health Inspection Service (APHIS)
Biotechnology Regulatory Services (BRS)
Compliance and Inspection Branch
4700 River Rd. Unit 147
Riverdale, MD 20737

7. Send notices and all reports (CBI and CBI deleted or non CBI copies) to BRS by e-mail, mail, or fax. Confidential Business Information (CBI) will be handled according to the APHIS policy statement at 50 FR 38561-63.

BRS E-mail:
BRSCompliance@aphis.usda.gov

BRS Mail:
Animal and Plant Health Inspection Service (APHIS)
Biotechnology Regulatory Services (BRS)
Compliance and Inspection Branch
4700 River Rd. Unit 147

Riverdale, MD 20737

BRS Fax:
Compliance and Inspection Branch
(301) 734-8669

A. Planting Report

Within 28 calendar days after planting, submit a Planting Report that includes the following information for each field test site:

i. A map of the site, with sufficient information to locate it, that includes: the state, county, address, GPS coordinates for each corner of the plot (inclusive of the border rows of any sexually compatible plants);
ii. The location and the approximate number and/or acres of transgenic plants which were actually planted at the test site for each of the target proteins;
iii. The total acreage of the test plot (exclude border rows, if any);
iv. The distance from the genetically engineered plants to the nearest plants of the same crop which will be used for food, feed, or seed production. A survey should be done within the distance specified in the permit.
v. The actual planting date.

B. Field Test Report

Within 6 months after the expiration date of the permit, the permittee is required to submit a Field Test Report. Field Test Reports shall include:

i. Constructs and specific transformed lines (event) planted
ii. Planting and harvest dates
iii. Total acreage of the test
iv. The methods of observation.
v. The resulting data and analysis regarding all deleterious effects on plants, non-target organisms, or the environment. This should include, but not be limited to, data on insect damage, disease susceptibility, gross morphology and any other indications of weediness.

C. Monitoring Report

Within 3 months after the end of the monitoring period, submit a Volunteer Monitoring Report. The report must include:

i. Dates when the field site and perimeter fallow zone were inspected for volunteers.
ii. Number of volunteers observed.
iii. Any actions taken to remove or destroy volunteers.

Under the Plant Protection Act, individuals or corporations who fail to comply with these conditions and authorizations, or who forge, counterfeit, or deface permits or shipping labels may receive civil or criminal penalties, and may have all current permits cancelled and future permit applications denied.

The permit holder is responsible for the disposition of the organisms throughout the duration of the permit. If the permit holder leaves the institution where the organisms are kept, all organisms must be destroyed, unless a new individual who assumes responsibility for continued maintenance submits an APHIS form 2000 application and obtains a permit prior to the permittee's departure.

This permit does not authorize movement or use of plant pathogens listed in the Public Health Security and Bioterrorism Preparedness and Response Act of 2002.