AgrEvo

98-349-01p

Ms. Rebecca Bech
Director, Scientific Services
USDA, APHIS, PPQ, SS
4700 River Road, Unit 147
Riverdale, MD 20737-1237

Re: **REQUEST FOR EXTENSION OF A DETERMINATION OF NONREGULATED STATUS**

AgrEvo USA is submitting an Application for an Extension of the Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for a hybrid seed production system in corn based on male sterility and glufosinate tolerance as a marker previously granted under 95-228-01p. The new event is called **MS6**. The event was transformed in the same manner as the event in the previous petition. The data submitted supports the contention that this event exhibits the same properties as the previously approved event. The enclosed request does not contain any confidential business information.

Enclosed are five copies of the petition extension for MS6. I have also enclosed a checklist for the molecular characterization data as defined by the Canada and United States Bilateral on Agricultural Biotechnology. All literature cited in the petition is not included in the reprints since the excluded literature is either a book, a government document, or a manuscript that is readily available to the public at many libraries in the United States. If these reprints are required for you to consider this petition complete, please contact me by January 4, 1999.

Please contact me at (302) 892-5951 if you have any questions concerning our petition.

Best Regards,

Penny L. Hunst

Penny L. Hunst, Ph.D.
Registration Manager-Biotechnology

enclosures (5)

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12/15/98

98-349-01p

Application for an Extension of the Determination of Nonregulated Status for a Hybrid Seed Production System in Corn Based on Male Sterility and Glufosinate Tolerance as a Marker (95-228-01p): Event MS6

The undersigned submits this petition under 7 CFR 340.6 to request that the Director, SS, make a determination that the article should not be regulated under 7 CFR 340.

Submitted by:

Penny L. Hunst

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December 15, 1998

Contains No Confidential Business Information

Summary

AgrEvo USA Company is submitting an Extension Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for male sterile corn, event MS6. To provide a more reliable pollination control system, Plant Genetic Systems N.V. (PGS), wholly owned by AgrEvo GmbH in Frankfurt Germany, has developed a new hybridization system, designated SeedLink™. This type of male sterility is linked to an efficient field selection system resulting in the transformation event MS6. A prior male sterile transformation event, known as MS3 (petition number 95-228-01), received a determination of nonregulated status on February 22, 1996 from APHIS.

The chimeric *barnase* gene construct induces male sterility of the plants. The *barnase* gene, isolated from *Bacillus amyloliquefaciens*, encodes the barnase enzyme, a ribonuclease that degrades RNA. Under the control of the PCA55 promoter, cloned from *Zea mays*, the *barnase* gene is expressed in the tapetal cell layer of the anther, a cell layer that plays a vital nutritive role during pollen formation. Introduction of the chimeric *barnase* gene construct therefore inhibits pollen formation and results in male sterility of the transformed plants. The protein does not contain pesticidal activity and does not have any adverse environmental or toxicological effect.

Linkage of the *barnase* gene to a marker gene - a glufosinate-ammonium herbicide tolerance gene, called *bar* - provides a useful means for integration of the system in breeding schemes and for seed production. The chimeric *bar* gene encodes the enzyme phosphinothricin acetyltransferase. The *bar* gene was isolated from *Streptomyces hygroscopicus*, a non-pathogenic bacterium. The integration of the *bar* gene enables the selection of the male sterile line independent of the plant stage, which is a prerequisite for efficient rouging of fertile plants in a segregating population, the basis of quality assurance in hybrid seed production. The protein does not contain pesticidal activity and does not have any adverse environmental or toxicological effect.

Event MS6 has been field tested by AgrEvo USA Company since 1997 in the primary corn growing regions of the Midwestern United States and in Hawaii and Puerto Rico. These tests have occurred at approximately 26 sites under field release authorizations granted by APHIS (USDA authorizations: 97-125-01n, 97-279-02n, 97-092-10N, 97-121-03N, 97-125-01N, 97-121-04n, 98-071-04n, 98-089-51n, 98-083-02n, 98-089-52n, 98-114-14n)). Data collected from these field trials, laboratory analyses, reports and literature references presented herein demonstrate that corn containing the transformation event MS6 presents no indications to anticipate: 1) the introduction of new types of plant pests via event MS6; 2) altered weediness and/or invasiveness in natural and agricultural environments; 3) significant exposure to new proteins; 4) any influence on commercial agricultural practices or biotic organisms in another way than nontransgenic corn; and 5) horizontal and vertical gene transfer between event

MS6 and respective microorganisms and corn relatives native to the United States.

Primary transformation event MS6 has been self-pollinated and backcrossed into elite lines.

AgrEvo USA Company requests a determination from APHIS that transformation event MS6 and any progeny derived from crosses of this event with traditional corn lines, and any progeny derived from crosses of this event with transgenic corn lines that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR 340.

ACRONYMS AND SCIENTIFIC TERMS

bar	gene isolated from *Streptomyces hygroscopicus;* encodes the enzyme phosphinothricin acetyltransferase which confers tolerance to glufosinate-ammonium; enables the selection of the male sterile line independent of the plant stage
barnase	gene isolated from *Bacillus amyloliquefaciens;* encodes the barnase enzyme, a ribonuclease, which degrades RNA; introduction into the tapetal layer of the anther results in male sterility of the transformed plants
bla	β-lactamase gene; ampicillin resistance gene
ELISA	Enzyme Linked Immunosorbent Assay
GS	glutamine synthetase
ori	origin of replication required for replication of the plasmid in *Escherichia coli;* functions in bacterial host only
PAT	Phosphinothricin acetyltransferase
PT	Phosphinothricin

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I. Rationale for Submission of Request for Extension

There are no changes in rationale from the previously approved petition number 95-228-01p. The specific differences between event MS6 and the event in the previous petition are discussed in the appropriate sections. The new event to be considered under this extension is MS6. MS6 is a regulated article since it contains DNA sequences from the plant pathogens *Agrobacterium tumefaciens* and cauliflower mosaic virus (CaMV).

II. The Corn Family

There are no changes from the previously approved petition submission.

III. Description of the Transformation System

The MS6 event was transformed in the same manner (via electroporation) as stated in the previous petition except plasmid pVE136 was used (Fromm, et al., 1990; Dekeyser, et al., 1990; D'Halluin, et al., 1992). Plasmid pVE136 contains two chimeric gene constructs, designed to be functional in plants, i.e.:

- the PCA55-*barnase*-3' nos gene construct, conferring male sterility, and
- the P35S-*bar*-3'nos gene construct, conferring tolerance to herbicides with glufosinate-ammonium as active ingredient.

Both gene constructs are cloned on a small plasmid, containing an origin of replication (*ori*) required for replication of the plasmid in *Escherichia coli*, and the β-lactamase gene (*bla*) which confers resistance to ampicillin. Both of these sequences have a function in the bacterial host only. It was concluded that only a small part of the ori & *bla* sequences are inserted in MS6 therefore this inserted sequence will not produce an intact protein in any host. Corn inbred line H99 was used as the recipient line for transformation. This is the same inbred line used for primary transformation event MS3. Prior to transformation the vector was digested with *Hind*III to linearize it. As was the strategy for the previous male sterile event, the commercialization strategy for MS6 is to use traditional crossing and breeding to transfer the *barnase* locus from the transformation event to a wide range of varieties with a wide range of maturities.

IV. Donor Genes and Regulatory Sequences

Event MS6 was transformed with the plasmid pVE136 which differs from the MS3 plasmid pVE108 in several respects. Transformation event MS6 does not require the helper plasmid pMc5 barstar and as deduced from mRNA analyses, does not contain an intact copy of the *bla* gene (Section V, part B, subpart 3). A summary of the genetic elements of pVE136 (6287bp) is given below:

1. pUC19 sequence (1-421bp). Sequence derived from pUC19 (Yannisch-Perron, et al., 1985).

2. Synthetic polylinker derived sequences (422-425bp).

3. 3'nos (426-686bp). A 3' nontranslated region of the nopaline synthase gene from *Agrobacterium* involved in transcription termination and polyadenylation (Depicker, et al., 1982).

4. Synthetic polylinker derived sequences (687-702bp).

5. Residual sequence from *Bacillus amyloliquefaciens* (703-798bp). Situated downstream of the *barnase* gene (Hartley, 1988).

6. *barnase* (1134-799bp) The gene, isolated from *Bacillus amyloliquefaciens*, encodes the barnase enzyme, a ribonuclease that degrades RNA (Hartley, 1988). The *barnase* gene is expressed in the tapetal cell layer of the anther, a cell layer that plays a vital nutritive role during pollen formation. Introduction in a tissue-specific manner and expression of the chimeric *barnase* gene construct inhibits pollen formation, resulting in male sterility of the transformed plants (Mariani et.al., 1990).

7. Synthetic polylinker derived sequence (1135bp).

8. . PCA55 promoter (2313-1136bp) The promoter region of the anther specific gene CA55 from *Zea mays* L. (PCT Patent Application WO92 13957).

9. Synthetic polylinker derived sequences (2314-2350bp).

10. CaMV 35S promoter (2351-3183bp) The 35S promoter sequence is derived from CaMV (Odell et al., 1985). The promoter controls transcription initiation of the *bar* gene. CaMV is a doublestranded DNA caulimovirus with a host range restricted primarily to cruciferous plants. The 35S promoter directs high level constitutive expression and is widely used as a promoter for high expression of genes (Harpster, et al., 1988). The CaMV sequence, as used in the MS6 event, does not cause corn to become a plant pest.

11. *bar* (3184-3735bp) The coding region of the bialaphos resistance gene from *Streptomyces hygroscopicus*. The *bar* gene encodes tolerance to herbicides with glufosinate-ammonium as the active ingredient. As an analogue of glutamate, phophinothricin (PT) inhibits glutamine synthase (GS) in plants. The inhibition of GS by PT results in an accumulation of ammonium. In addition, a process in connection with photorespiration

plays a central role on photosynthesis inhibition by PT (Wild et al., 1984; Manderscheid et al., 1985; Wild et al., 1987; Sauer et al., 1987; Wendler et al., 1990). To protect corn plants against the toxic effects of the glufosinate-ammonium compound, the *bar* gene incorporated into the plant genome can be expressed, leading to the production of the enzyme phosphinothricin acetyltransferase. This enzyme acetylates PT and inactivates the molecule, thereby preventing the death of the plant cell (De Block et al., 1987). When linked to the CaMV 35S promoter, the *bar* gene is expressed in a constitutive manner (Odell et al., 1985; Fromm et al., 1990; Gordon-Kamm et al., 1990).

The integration of the *bar* gene enables the use of glufosinate as a selective agent at the *in vitro* stage. Since the *barnase* gene construct is physically linked with the *bar* gene, these genes will co-segregate as a single locus. Therefore, the male sterile line can be maintained through crossing with wild type plants followed by the application of the herbicide (Mariani et al., 1990). Furthermore, the integration of the *bar* gene enables identification of the male sterile line independent of the stage of the plant, which is a prerequisite for efficient rouging of fertile plants in a segregating population, as such efficient rouging is the basis of quality assurance in the hybrid seed production.

12. Synthetic polylinker derived sequences (3736-3754bp).

13. 3'nos (3755-4015bp). A 3' nontranslated region of the nopaline synthase gene from *Agrobacterium* involved in transcription termination and polyadenylation (Depicker, et al., 1982).

14. Synthetic polylinker derived sequences (4016-4019bp).

15. pUC19 sequence (4019-6287bp). Nucleotide sequence derived from pUC19 (Yanisch-Perron, et al., 1985); the coding sequence for the β-lactamase gene (*bla* gene) is localized from nucleotides 6087-5227. The β-lactamase gene was isolated from pBR322, a plasmid of *E. coli* (Sutcliffe, 1978). It encodes β-lactamase. β-lactamase genes are found throughout nature (Sykes and Smith, 1979). The gene is expressed in bacteria where it is used in the selection of transformed bacteria which are then used to amplify the plasmid vector.

Event MS6 contains one copy of the *barnase* gene and two copies of the *bar* gene. In contrast, Event MS3, the subject of the previous submission, contained three copies of the *barnase* gene, one copy of the *bar* gene and two copies of the *bla* gene.

V. Genetic Characterization of Event MS6

A. Southern Gel Analysis

Southern hybridization was used to determine the nature and number of *barnase, bar* and *bla* gene insertions which occur in transformation event MS6. When transforming a plant with restriction digested or intact, circular vector DNA there is no way to predict at which site(s) on the vector recombination will initiate. Therefore, we have used Southern blot analyses to examine the integrity of the inserted vector in MS6 transformation event. These analyses also serve to determine the copy number of the inserted genes. Event MS6 differs in copy number of integrated DNA from the event MS3 that was the subject of petition 95-228-01.

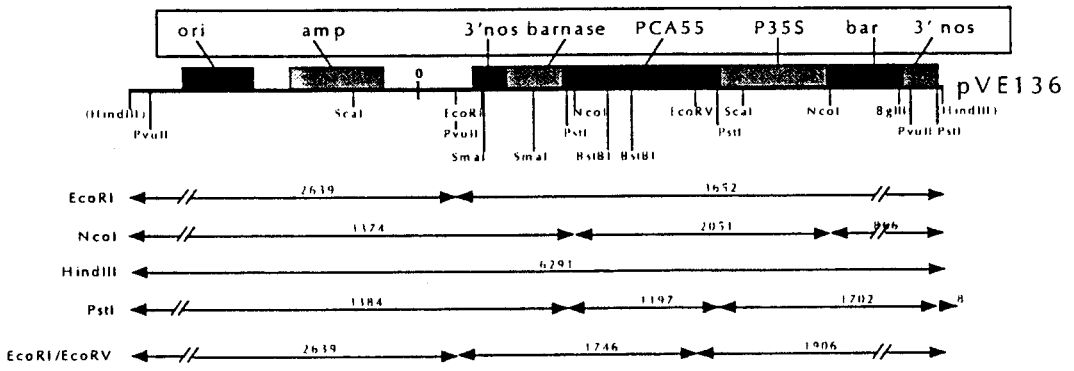
In the experiments, restriction digested genomic DNA from transgenic plants homozygous for the integrated DNA were run in parallel with a digested genomic DNA from a nontransgenic H99 plant, supplemented with approximately one copy of digested transforming plasmid. After blotting and hybridization with *barnase, bar* and *bla* probes, the number of copies (intact and/or partial) of the genes in the corn genome were quantified by comparing hybridization intensity of the corn DNA with the hybridization intensity of the diluted transformation vector. The dilution of the transformation vector can only give a rough estimate of the copy number since the parameters for calculation (mass of corn genome, spectrophotometric quantification of vector and plant DNA, dilution of DNAs, visual comparison of band intensity) are not absolutely precise. The determination of the integrated copies is deduced from analyzing all obtained Southern blot data.

Several aliquots of event MS6 DNA were digested with restriction enzymes. See Figure 1 and pVE136 plasmid map in the Appendix to locate restriction sites on pVE136. Following separation of the DNA by gel electrophoresis, the DNA was transferred to a nylon membrane and hybridized with the ^{32}P-labeled probes which had been isolated from different plasmids by restriction enzyme digestion (Table 1; plasmid maps are contained in the Appendix). Hybridization of digested genomic DNA of *Zea mays* transformation event MS6 with the complete pVE136 plasmid yielded an inventory of all hybridizing fragments (Figure 2). Lanes contain approximately 10ug of digested DNA. The amount of restricted pVE136 plasmid in positive control lanes is equivalent to 1 copy of the plasmid integrated in 10ug corn DNA. Assignment of the hybridizing fragments to specific elements of the transforming plasmid was established by stripping the blots and rehybridizing with the different elements of the plasmid (Table 2 and Figures 3, 4, 5 and 6). Electronic scans of the autoradiographs are presented in this document.

Table 1. DNA probes used in Southern hybridizations.

Probe	Plasmid – Restriction Fragment
barnase	pVE136 – EcoRI/XbaI 459bp fragment
PCA55	pVE136 – PstI 1197bp fragment
bar	pDE110 – NcoI/BglII 549bp fragment
P35S	pVE136 – NcoI/PstI 844bp fragment
ori + *bla*	pVE136 – PvuII 2364bp fragment
3'nos	pNOS2 – EcoRI/HindIII 320bp fragment
pVE136	pVE136 - linearized with HindIII

Figure 1: Schematic drawing of pVE136



All but one probe used for event MS6 characterization was specific for the transgenic sequences introduced into *Zea mays* L. because they did not hybridize to DNA isolated from the nontransgenic H99 line. The PCA55 probe does hybridize to the endogenous *Zea mays* L. PCA55 promoter (Fig. 4, lanes 3, 5, 7, 9). Every probed used hybridized with the linearized pVE136 plasmid control DNA. This indicated that all hybridizations were performed under conditions allowing hybridization of the probes with the target sequences.

Assignment of the hybridizing fragments to specific elements of the transforming plasmid was established by hybridizing blots with different elements of pVE136. The elements were isolated from plasmids pVE136, pDE110 and pNOS2 (Table

1; see Appendix for plasmid maps). The results of the hybridizations are summarized in Table 2 and Figures 2-6.

The transforming DNA of pVE136 was found to reside on one HindIII fragment (Fig. 2, lane 6). As shown in Figure 1, the HindIII fragment encompasses the pVE136 DNA. (The other band shown in Figure 2, lane 6 is the endogenous PCA55 promoter; the ~5077bp band in lane 4 is the result of a partial digest.) This results suggests that DNA derived from pVE136 plasmid preparation has inserted at a single site in the genome of corn. This is comparable to event MS3, the subject of the previous petition.

Two hybridizing fragments, 5200 and 2050 bp, in the Nco1 digested DNA were observed when the P35S probe was used (Fig. 3, lane 4). Both fragments also hybridized with the PCA55 probe (Fig. 4, lane 4) which is expected since PCA55 resides within the 2051 bp Nco1 fragment (Fig. 1). A 2051bp band is expected if both NcoI sites are intact (Fig. 1, Table 2). Since the 5200bp band was not expected, these results suggest that two copies of P35S and PCA55 are present in MS6. The 5200 bp fragment contains part of a second plasmid copy and represents the junction between transgenic and plant DNA sequences.

The results with the *barnase* probe (Fig. 5, lanes 2, 4, 6, 8) indicate that one copy of the gene was integrated (based on comparison with pVE136 control in lane 10). However, the obtained 1650 bp EcoR1-EcoRV fragment is approximately 100 bp too short (based on the map of pVE136, Fig. 1) to represent an intact copy of the PCA55-*barnase*-3' nos cassette (1746 bp expected, Fig. 1). In results not presented, the PCA55 probe hybridized to the expected 1200 bp fragment (Pst1) (Fig. 1). This indicated that the 100 bp deletion is situated at the 3' nos terminator.

The hybridization results with the ori and *bla* probe yielded very faint hybridization signals (Fig. 6, lanes 2, 4, 6, 8) as compared with the pVE136 control (Fig. 6, lane 10). These weakly hybridizing fragments were also observed when hybridizing with the pVE136 probe (Fig. 2, lanes 2, 8). These results suggest that only small parts of the ori and *bla* sequences are inserted in event MS6.

Combining the results of all the hybridizations, the data indicate that one copy of PCA55-*barnase*-3' nos cassette is integrated and two copies (complete and/or partial) of P35S-*bar*-3' nos cassette are integrated (Table 2).

Table 2. Summary of Molecular Characterization Data for Event MS6

Gene	Complete copy present in plant?	# of complete copies?	Is protein expressed in plant?	Reference for gene expression
ori & bla	no	0	no	Deduced from mRNA analysis, p. 22.
barnase	yes	1 (100bp too short for intact copy)	yes	Deduced from plant phenotype; limited to tapetal cell layer, p. 22
bar	yes	2	yes	ELISA results, p. 22.

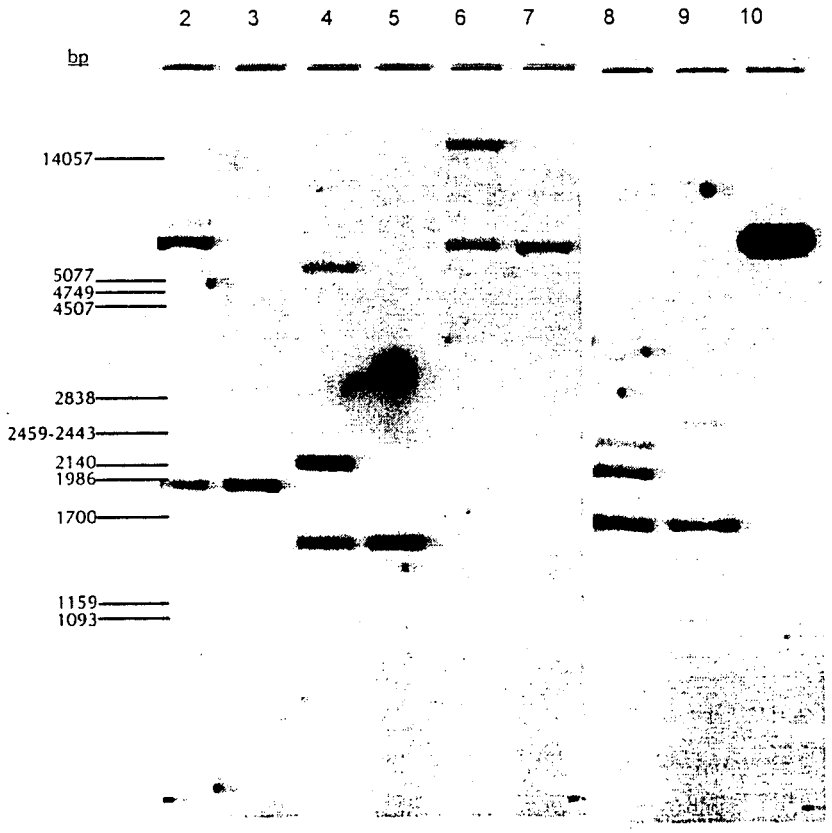


Figure 2. Southern Blot Analysis of corn Event MS6 – pVE136 probe. DNAs were isolated from corn containing Event Ms6 and the nontransgenic H99 corn line. DNAs (10ug) were digested with the indicated restriction enzymes. The pVE136 plasmid (see Fig. 1) was used as the probe. The lanes contain:

1. Phage lambda DNA – Pst1 (not shown)
2. MS6 – EcoR1
3. H99 – EcoR1
4. MS6 – Nco1
5. H99 – Nco1
6. MS6 – HindIII
7. H99 – HindIII
8. MS6 – EcoR1/EcoRV
9. H99 – EcoR1/EcoRV
10. H99 – HindIII + 1 copy pVE136 – HindIII

MW marker (lambda DNA digested Pst1) sizes given in base pairs.

Table 3. Observed and Expected Hybridizing Fragments in Southern Blots of event MS6 probed with pVE136.

	Digest							
Probe	EcoRI		NcoI		HindIII		EcorI/EcoRV	
	O[a]	E[b]	O	E	O	E	O	E
pVE136	6400 6000 **1800**	>3652 >2639	5200 2050 **1500**	>3374 2051 >866	>14000 **6400**	>6291	2400 2000 1850 1650 **1650**	>2639 >1906 1746
barnase	6000	>3652	2050	>3374	>14000	>6291	1650	1746
PCA55	6000 **1800**	>3652	5200 2050 **1500**	2051	>14000 **6400**	>6291	2000 1850 1650 **1650**	>1906 1746
bar	6000	>3652	5200	>866	>14000	>6291	2000	>1906
P35S	6000	>3652	5200 2050	2051	>14000	>6291	2000	>1906
ori&*bla*	6400	>2639	2050	>3374	>14000	>6291	2000	>1906

[a]Observed fragments.
[b]Expected fragments based on 1 copy of inserted vector.
Bold numbers represent endogenous PCA55 fragments.

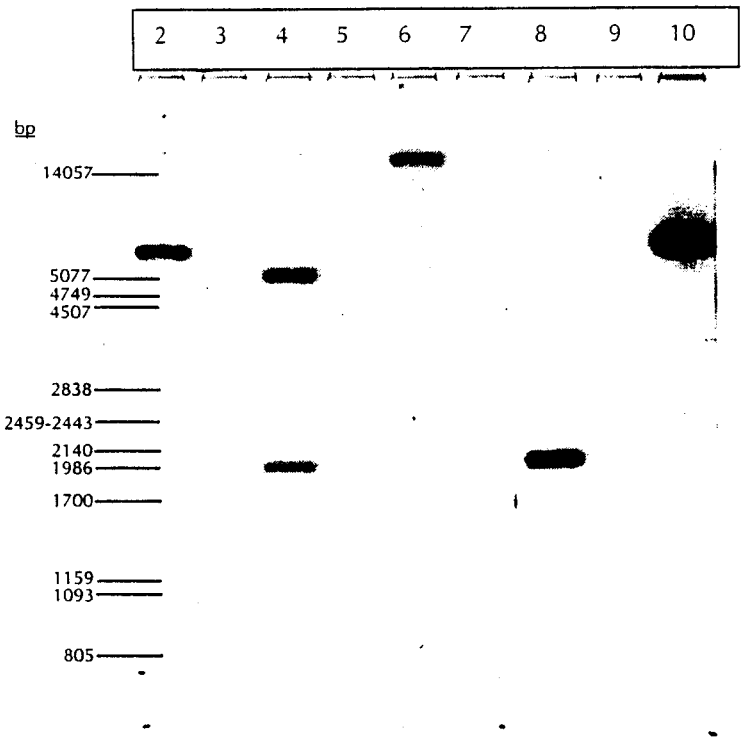


Figure 3. Southern Blot Analysis of corn Event MS6 – P35S probe. DNAs were isolated from corn containing Event Ms6 and from the H99 nontrangenic corn line. DNAs (10ug) were digested with the restriction enzymes shown. The lanes contain:

1. Phage lambda DNA – Pst1 (not shown)
2. MS6 – EcoR1
3. H99 – EcoR1
4. MS6 – Nco1
5. H99 – Nco1
6. MS6 – HindIII
7. H99 – HindIII
8. MS6 – EcoR1/EcoRV
9. H99 – EcoR1/EcoRV
10. H99 – HindIII + 1 copy pVE136 – HindIII

MW marker DNA (lambda DNA digested with Pst1) is given in base pairs.

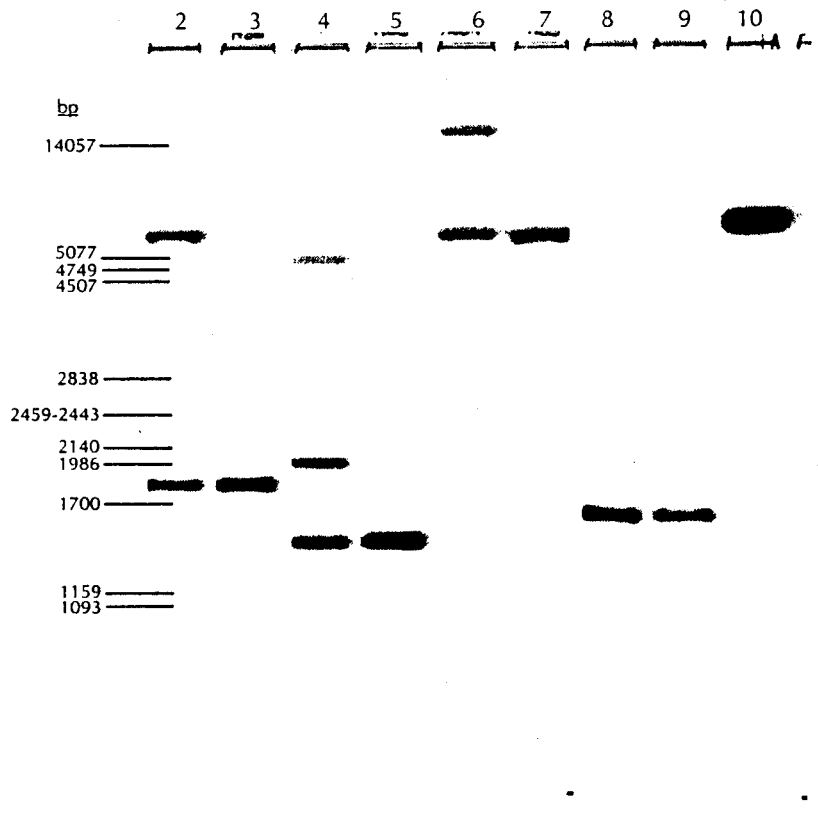


Figure 4. Southern Blot Analysis of corn event MS6 – PCA55 probe. DNA was isolated from corn containing Event Ms6 and from H99 nontransgenic corn line. DNAs (10ug) were digested with the indicated restriction enzymes. The PCA55 was used as the probe. The lanes contain:

1. Phage lambda DNA – Pst1 (not shown)
2. MS6 – EcoR1
3. H99 – EcoR1
4. MS6 – Nco1
5. H99 – Nco1
6. MS6 – HindIII
7. H99 – HindIII
8. MS6 – EcoR1/EcoRV
9. H99 – EcoR1/EcoRV
10. H99 - HindIII + 1 copy pVE136 – HindIII

MW of lambda DNA is given in base pairs.

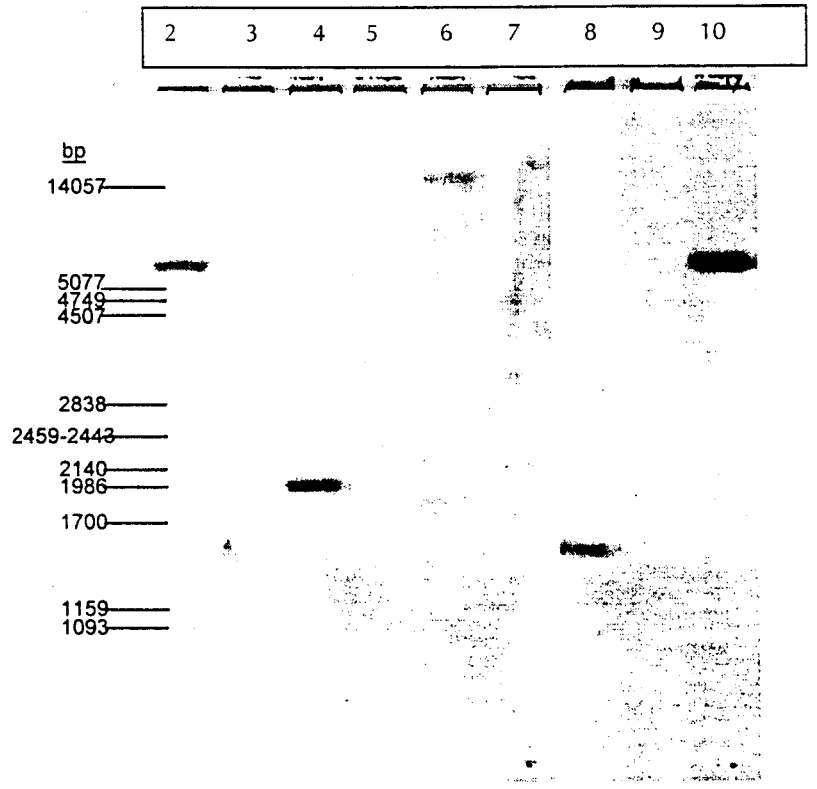


Figure 5. Southern blot analysis of corn event MS6 – *barnase* probe. DNAs were isolated from corn containing Event Ms6 and from H99 nontransgenic corn line. DNAs (10ug) were digested with the enzymes indicated. The lanes contain:

1. Phage lambda DNA – Pst1 (not shown)
2. MS6 – EcoR1
3. H99 – EcoR1
4. MS6 – Nco1
5. H99 – Nco1
6. MS6 – HindIII
7. H99 – HindIII
8. MS6 – EcoR1/EcoRV
9. H99 – EcoR1/EcoRV
10. H99 – HindIII + 1 copy pVE136 – HindIII

MW of lambda DNA is given in base pairs.

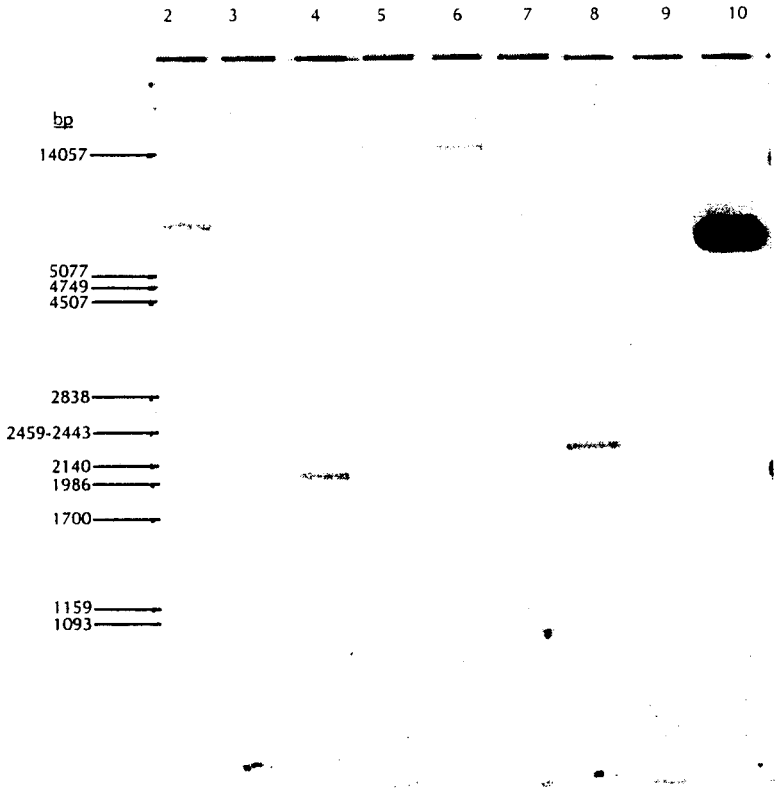


Figure 6. Southern blot analysis of corn event MS6 – ori & *bla* probe. DNAs were isolated from corn containing Event Ms6 and from H99 nontransgenic corn line. DNAs (10ug) were digested with the enzymes indicated. The lanes contain:

1. Phage lambda DNA – Pst1 (not shown)
2. MS6 – EcoR1
3. H99 – EcoR1
4. MS6 – Nco1
5. H99 – Nco1
6. MS6 – HindIII
7. H99 – HindIII
8. MS6 – EcoR1/EcoRV
9. H99 – EcoR1/EcoRV
10. H99 – HindIII + 1 copy pVE136 – HindIII

MW of lambda DNA is given in base pairs.

B. Expression of Inserted Genes

The content of phosphinothricin acetyltransferase (PAT) protein in the transformation event MS6 was determined in forage, fodder and grain of transgenic plants treated with Liberty® herbicide by the use of Enzyme Linked Immunosorbent Assay (ELISA). The PAT ELISA is a sandwich immunoassay in which PAT-specific polyclonal antibodies are used. The antiserum detects both degraded and intact PAT protein. Therefore, the amount of PAT protein detected is likely to be an overestimate of the amount of enzymatically active protein present. The expression of the *barnase* gene was deduced from the plant phenotype. To determine whether any of the *bla* gene fragments were expressed, RNA expression of cryptic genes was determined by Northern blot analysis.

1. PAT Expression.

The PAT ELISA is an Enzyme Linked Immunosorbent Assay (ELISA) based on the specific interaction between antibody and PAT protein. It was used to determine the concentration of the PAT protein (*bar* gene product) in the crop. Samples consisting of extracts from transformed plants, non-transformed plants (controls), and purified PAT protein as a standard were applied. PAT protein that was present in the samples was captured by the coated antibody. Unbound material was removed by rinsing with a wash solution.

The plate was subsequently incubated under the same conditions with the second antibody, which recognizes another epitope of PAT protein, followed by removal of unbound material with a wash solution. The binding of the second PAT antibody to the PAT protein was detected by the addition of a third antibody labeled with horseradish peroxidase.

The peroxidase substrate, tetramethylbenzidine, was converted by the peroxidase to a blue product in proportion to the amount of PAT protein present in the sample. The reaction was stopped with 0.5 M H_2SO_4 and the color changed to yellow. The intensity of yellow color was measured in a microplate reader at 450nm. The resultant color development is proportional to the concentration of PAT protein in each well. Three dilutions of each extract were tested and the value nearest to the midpoint of the standard curve is used to determine the PAT content. Results from the ELISA are shown in Table 4.

Table 4. PAT Content[a] in Grain, Forage and Fodder of Plants Containing Event MS6 as Detected by ELISA.

Plant	mg TEP[c]/ g sample	ug PAT/ g sample	% PAT/ TEP
MS6-G[b]	8.73	3.54	0.04
MS6-For	1.31	2.01	0.15
MS6-Fod	1.26	2.15	0.17

[a] Values reported are the average of two samples (2 subsamples/sample were analyzed).
[b] G = grain; For = forage; Fod = fodder.
[c] TEP = total extractable protein

ELISA detects PAT in event MS6 since the ELISA detects both intact and degraded enzyme protein. However, it is not known whether any of the PAT protein detected is active. For this reason, the PAT protein content in grain, forage and fodder from event MS6 corn plants is likely to be an overestimate of the level of active PAT protein.

2. *barnase* Expression.

The expression of the *barnase* gene was deduced from the plant phenotype as stated in the previous petition. The *barnase* gene is expressed in the tapetal cell layer of the anther. The tapetal cell layer plays a nutritive role during pollen cell formation. Therefore, introduction of the chimeric *barnase* gene construct inhibits pollen formation and results in male sterility of the transformed plants. Expression of the *barnase* gene in any other cells would lead to disruption of normal cell function and result in abnormal plant growth. With the exception of the male sterility trait, transgenic plants containing event MS6 developed in a comparable manner to non-transgenic plants. It was concluded, therefore, that the expression of the *barnase* gene is limited to the tapetal cell layer.

3. *bla* Expression.

Transformation event MS6 contains small portions of the *bla* sequence as evidenced by the weakly hybridizing bands observed in the Southern analyses (Fig. 6). However, no expression of the *bla* is expected since this gene is under the control of bacterial expression signals and should only be expressed in bacteria. Even so, Northern analysis of RNA extracted from several tissues and hybridized with a single stranded RNA probe was conducted. The single stranded probe was prepared using the PCR generated template (MDB402-VDS41 913bp fragment; see pVE136 plasmid in Appendix) and the SP6/T7 Transcription Kit of Boehringer Mannheim.

Primers MDB402 – VDS41 amplify a 913bp fragment (867bp of *bla* sequences and 2 x 23 bp of T7 and SP6 promoter and 5' end sequences). The first 23 nucleotides of primer MDB402 are the 17 nucleotides from the T7 promoter and 6 nucleotides, which form the first 6 bases of in vitro, transcribed RNA. The position of this primer in pVE136 is 5214 – 5236bp. The first 23 nucleotides of primer VDS41 are the 17 nucleotides from the SP6 promoter and 6 nucleotides which form the first 6 bases of *in vitro* transcribed RNA. The position of this primer in pVE136 is 6081 – 6063bp (See pVE136 plasmid map in Appendix).

The *bla* anti-sense RNA probe used to examine cryptic *bla* gene expression hybridized with the reference substance dilution series (Figure 7, Lanes 10 – 17). The sense *bla* RNA fragment obtained showed the expected size of 913 nucleotides. This demonstrated that the Northern blot analysis was performed under conditions allowing hybridization to specific target sequences. The lowest visible amount of the reference substance is 2 pg/5 ug total RNA. In none of the tissues tested (leaf, root and seed) from the test and control substances were any positive signals observed using the *bla* anti-sense RNA probe (Figure 7, Lanes 3 – 8). These results indicate that the piece of the *bla* gene present in event MS6 is not expressed.

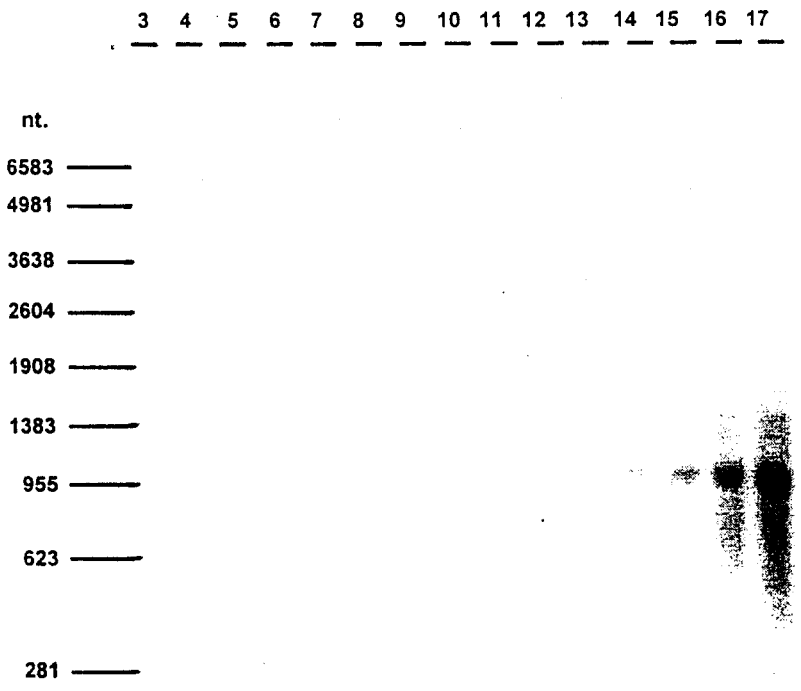


Figure 7: Northern blot analysis of event MS6 - probe: *bla* anti-sense RNA

Total RNA was isolated from different tissues of event MS6 and H99 wild type. Five µg MS6 or H99 wild type RNA was loaded in each lane. The 913 nucleotide *in vitro* synthesized *bla* anti-sense RNA was used as a probe (MDB402-VDS41 T7 transcript).
Lane 1. RNA MW marker. Lane 2. Blank. (Lanes 1 and 2 were removed).
Lane 3. MS6 leaf RNA. Lane 4. H99 wild type leaf RNA. Lane 5. MS6 root RNA. Lane 6. H99 wild type root RNA. Lane 7. MS6 seed RNA. Lane 8. H99 wild type seed RNA. Lane 9. Blank. Lanes 10 to 17: calibration RNA series of *in vitro* synthesized *bla* sense RNA (MDB402-VDS41 SP6 transcript, size: 913 nucleotides) respectively 0.25, 0.5, 1, 2, 4, 8, 16 and 32 pg. These samples were complemented with 5 µg H99 wild type leaf RNA. RNA MW marker (G319, Promega Corporation) sizes are given in nucleotides.

C. Stability of Gene Insert

1. Mendelian Inheritance

The analysis for segregation of one or more traits in offspring from a cross with a particular parental line can evaluate Mendelian inheritance of each trait and the linkage of the respective traits. Since the *barnase* and *bar* genes are physically linked, the genes will segregate in genetic offspring as a single locus in a 1:1 segregation ratio (dominant functions): 50% of the progeny plants are herbicide tolerant and male sterile; the other 50% of the progeny plants are herbicide susceptible and male fertile. The latter phenotype is eliminated in each generation of a multiplication or backcrossing program by treating the plants with glufosinate-ammonium. Thus, at each generation the segregation of the transgenes can be monitored and the linkage between the two genes can be confirmed.

In Table 5, an example of the segregation of the *barnase* gene in a field experiment is presented. The experiment utilized two locations: Iowa and Wisconsin. There were two replications at each location. Seed from BC10 (backcross) hybrids were planted at each location. The plants were identified as either sterile or fertile at the flowering stage (based on the absence or reduction in the amount of pollen in the anthers = sterile phenotype). According to chi-square analysis, the Mendelian inheritance is normal and the *barnase* gene shows no evidence of instability.

Table 5. Segregation Data for Event MS6.

Location	# Sterile Plants	# Fertile Plants	Chi-Square
Iowa	583	562	0.38*
Wisconsin	577	528	2.17*

*Not significant at p=0.05 (chi-square = 3.84, 1 df).

2. Molecular Characterization of Insert Stability

Southern blot analyses of genomic DNA from transgenic plants using the PCA55 sequence as the probe (Table 1 and plasmid map of pVE136 in Appendix) were performed to demonstrate the stability of *Zea mays* L. transformation event MS6 over multiple generations and different genetic backgrounds.

In the analysis, restriction digested genomic DNA from transgenic plants were run in parallel with a digested genomic DNA from a nontransgenic H99 plant, supplemented with approximately one copy of digested transforming plasmid.

After blotting, the digests were probed with the PCA55 probe (see Fig. 1, NcoI digest). A summary of the hybridization results obtained is given in Table 6 and the hybridization results are shown in Figures 8 and 9.

The Nco1 restriction enzyme has two recognition sites at positions 1132 and 3183 in plasmid pVE136. The excised 2051 bp fragment contains P35S and PCA55 promoters (Fig. 1). The MS6 insert also contains part of a second plasmid copy, the *bar*, P35S and PCA55 sequences. This second copy resides on a 5200 bp Nco1 fragment, representing the junction between transgenic DNA and plant DNA.

The PCA55 probe hybridizes to the endogenous *Zea mays* L. PCA55 promoter, therefore, hybridization signals (1500bp bands) were also observed in the nontransgenic H99 DNA samples (see Fig. 8, lane 5, and Fig. 9, lanes 2 and 5).

When hybridizing Nco1 digested DNA from different generations (Fig. 8, lanes 1-4) and different backgrounds (Fig. 9, lanes 1-4) of *Zea mays* L. transformation event MS6, three fragments were observed: 5200 bp, 2050 bp and 1500 bp fragments. The 2050 bp fragment represents the internal fragment and the 1500 bp fragment represents the fragment carrying the endogenous promoter sequences. The 5200 bp fragment contains part of a second plasmid copy and represents the junction between transgenic and plant DNA sequences.

The three fragments were observed in the four different generations and the two different backgrounds of *Zea mays* L. transformation event MS6, thus showing the stability of the MS6 event at the genomic level over multiple generations and backgrounds.

Table 6. Summary of Hybridization Results: Demonstration of the Stability of *Zea mays* L. Event MS6 Over Multiple Generations and Backgrounds.

Probe PCA55	Digest	
	Nco1	
DNA Probed	O[a]	E[b]
MS6	5200	
	2050	2051
	1500	
Control Wild-Type	**1500**	
Control Wild-Type + 1 Copy pVE136	2051	2051
	1500	

[a]Observed fragments.
[b]Expected fragments based on 1 copy of inserted vector.
Bold fragments represent endogenous PCA55 fragments.

98-349-01p

Molecular Genetic Characterization of Transgenic Plants Checklist

Application: Application for an Extension of the Determination of Nonregulated Status for a Hybrid Seed Production System in Corn Based on Male Sterility and Glufosinate Tolerance as a Marker (95-228-01p): Event Ms6

Document Date: December 15, 1998

Submitted by: Penny L. Hunst, Ph.D.
AgrEvo USA

Checklist Elements:		**Page # in Document**
1.0	**The Transformation System**	
1.1	Description of transformation method	8
1.1.1	Describe and provide references for the method	8
1.1.2	For direct transformation methods, describe the nature and source of any carrier DNA used	none used
1.1.3	For *Agrobacterium*-mediated transformation, provide the strain designation, disarming of the Ti plasmid-based vector, and whether *Agrobacterium* was cleared from the transformed tissue	N/A
1.1.4	For transformation systems other than *Agrobacterium,* provide the following information:	
1.1.4.1	Does the system utilize a pathogenic organism or nucleic acid sequences from a pathogen?	9-10
1.1.4.2	How were pathogenesis-related sequences removed prior to transformation?	9-10
1.1.4.3.	Did the transformation process involve the use of helper plasmids or a mixture of plasmids?	N/A
1.2	Description of the genetic material potentially delivered to the recipient plant material (modifications/constructs)	8-10, 12
1.2.1	Provide a summary of all genetic components which comprise the vector including coding regions, and non-coding sequences of known function. For each genetic component provide a citation where these functional sequences were described, isolated, and characterized and indicate:	
1.2.1.1	The portion and size of the sequence inserted.	8-10
1.2.1.2	The location, order, and orientation in the vector.	8-10, 12
1.2.1.3	The function in the plant.	8-10

1.2.1.4	The source of the donor organism.	8-10
1.2.1.5	If the genetic component is responsible for disease or injury to plants or other organisms, and is a known toxicant, allergen, pathogenicity factor, or irritant.	8-10
1.2.1.6.	If the donor organism is responsible for any disease or injury to plants or other organisms, produces toxicants, allergens or irritants or is related to organisms that do.	8-10
1.2.1.7	If there is a history of safe use of the source organism or components thereof.	8-10
1.2.2	If there has been a significant modification that affects the amino acid sequence of genes designed to be expressed in the plant, provide the citation.	N/A
1.2.3	Provide a detailed map of the vector.	12, Appendix
2.0	**Inheritance and stability of introduced traits which are functional in the plant**	10-29
2.1	Provide data that demonstrates the pattern and stability of inheritance and expression of the new transgene traits.	10-29
2.2	Provide data to demonstrate that the transgene trait is stably maintained and expressed during vegetative propagation for those plants for which it is difficult to produce seed.	N/A
3.0	**Characterization of the DNA Inserted in the Plant**	10-19
4.0	**Protein and RNA Characterization and Expression**	21-24

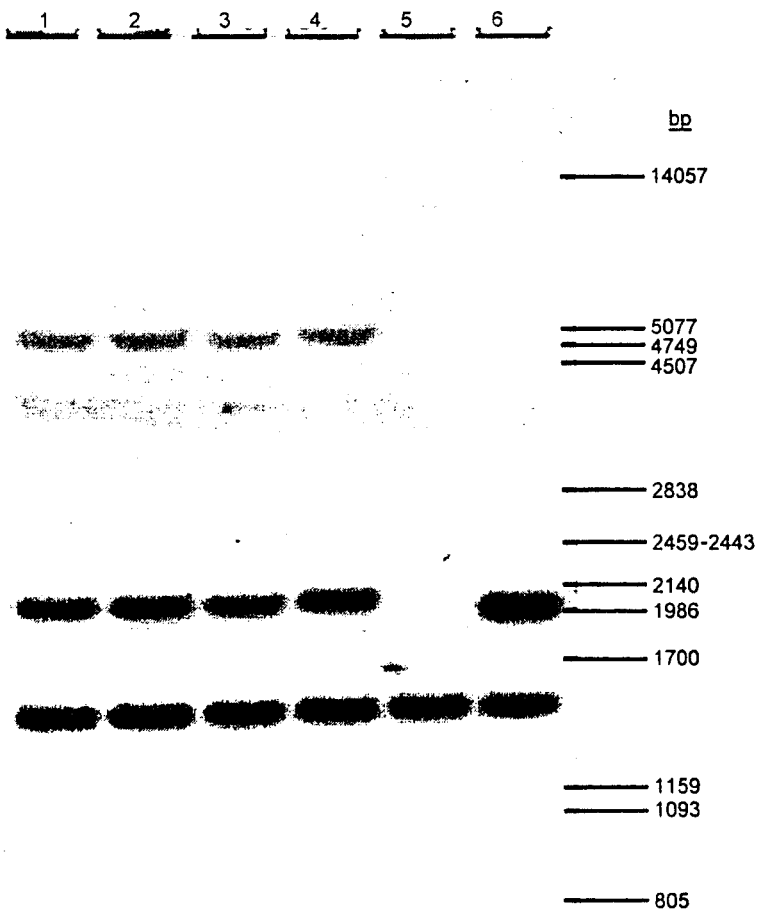


Figure 8. Southern blot analysis of event MS6 over several generations – PCA55 probe.
DNAs were isolated from corn containing event Ms6 and the nontransgenic H99 corn line. DNAs (10ug) were digested with Nco1 restriction enzyme. The lanes contain:

1. BC (=back cross)1 (MS6 x H99) – Nco1
2. BC2 (MS6 x H99) – Nco1
3. BC3 (MS6 x H99) – Nco1
4. BC4 (MS6 x H99) – Nco1
5. Control wild type – Nco1
6. Control wild type-Nco1 + 1 copy pVE136 –Nco1
7. Phage lambda DNA – Pst1 (not shown). MW marker DNA sizes given in base pairs (bp).

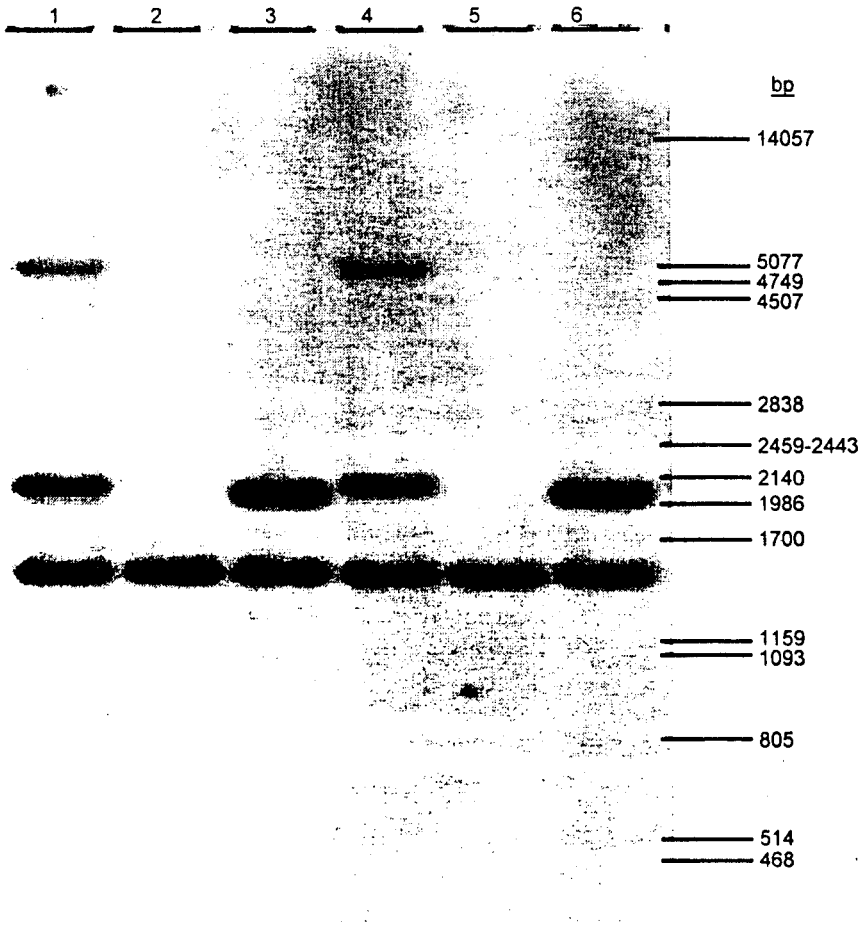


Figure 9. Southern blot analysis of event MS6 in different genetic backgrounds – PCA55 probe.
DNAs were isolated from corn containing event MS6 and the nontransgenic H99 corn line. DNAs (10ug) were digested with Nco1 restriction enzyme. The lanes contain:

1. F1 ((MS6 x H99) x A632) – Nco1
2. Control wild type – Nco1
3. Control wild type – Nco1 + 1 copy of pVE136 – Nco1
4. BC2 ((MS6 x H99) x B73) – Nco1
5. Control Wild Type – Nco1
6. Control Wild Type – Nco1 + 1 copy pVE136 – Nco1
7. Phage lambda DNA – Pst1 (not shown). MW marker DNA sizes are given as base pairs (bp).

VI. Agronomic, Disease and Pest Characteristics

Event MS6 was evaluated in the field in 1997 and 1998 at 26 sites (Iowa, Illinois, Indiana, Idaho, Hawaii, Puerto Rico) under authorizations granted by APHIS (USDA authorizations: 97-125-01n, 97-279-02n, 97-092-10N, 97-121-03N, 97-125-01N, 97-121-04n, 98-071-04n, 98-089-51n, 98-083-02n, 98-089-52n, 98-114-14n). The purpose of the trials was to increase seed, advance generations, demonstrate the agronomic performance, and/or to evaluate segregation ratios of the event. In the field trials, there was no evidence of any unexpected disease or pest resistance or unusual weediness. The Appendix contains termination reports submitted to the USDA for the environmental releases that have been completed in the United States and Puerto Rico. The field trials were observed and monitored by personnel familiar with corn cultivation practices (plant breeders, station managers, field agronomists, or associates).

Agronomic data which was recorded for the field trials included seed germination rates, plant stand, plant vigor, deleterious effects, disease and pest resistance/susceptibility (Table 7 and termination reports in Appendix). With regard to all of the aforementioned agronomic traits, there was no difference in the transgenic MS6 corn as compared with the nontransgenic parent corn variety. Seed germinated well and the plants developed normally. The only difference noted between MS6 corn and the nontransgenic corn was that the peduncle and tassel of MS6 corn was smaller and never filled with pollen. This was expected since MS6 is a male sterile line. MS6 corn demonstrated no greater potential to become a weed than its nontransgenic counterpart based on stand counts.

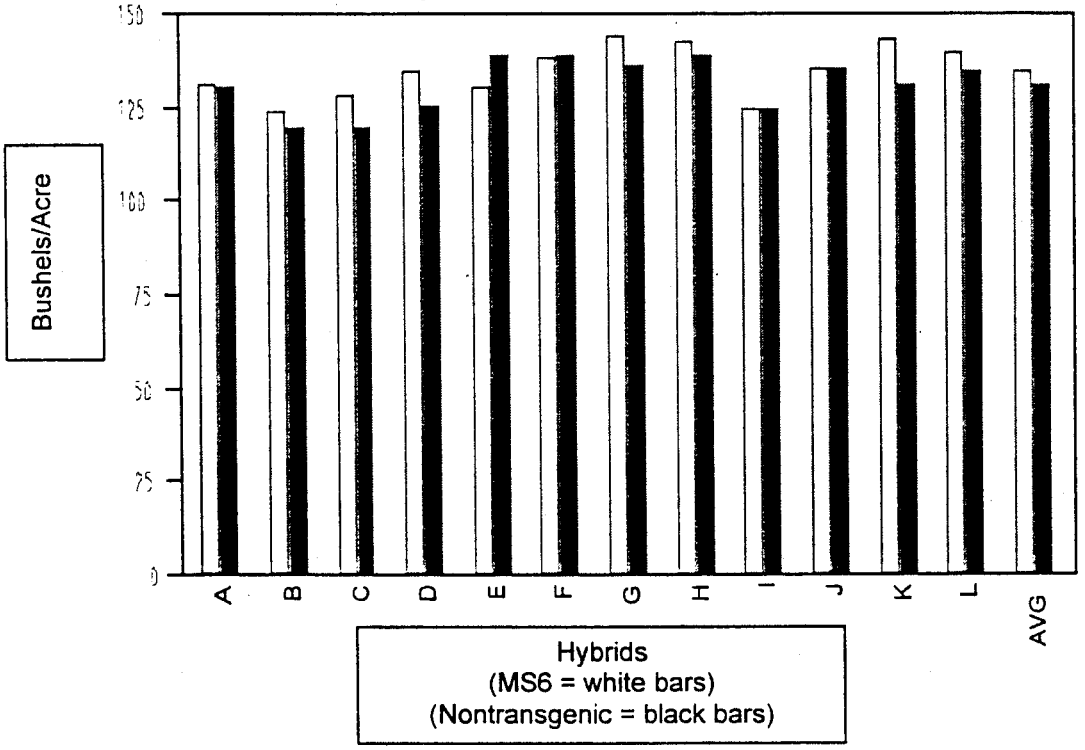
Table 7. Disease and Pest Observations on event MS6 (H99 background) and Control Line H99.

Locations	Examples of Observed Disease Infestations	Examples of Observed Pest Infestations	Remarks
Iowa	grey leaf spot smut	European corn borer Western corn rootworm Northern corn rootworm aphids	No differences in susceptibility observed
Illinois	grey leaf spot smut Northern leaf blight		No differences in susceptibility observed
Indiana	grey leaf spot Stewart's wilt		No differences in susceptibility observed
Wisconsin		Western corn rootworm Northern corn rootworm	No differences in susceptibility observed

Quantitative agronomic data was collected from four locations in Iowa and Illinois in 1998. MS6 corn hybrids and their corresponding check hybrids were planted.

Grain yield, % grain moisture and test weight were recorded. The data was analyzed using the least significant difference (LSD) at 0.05 alpha. The results are presented in Figures 10 and 11.

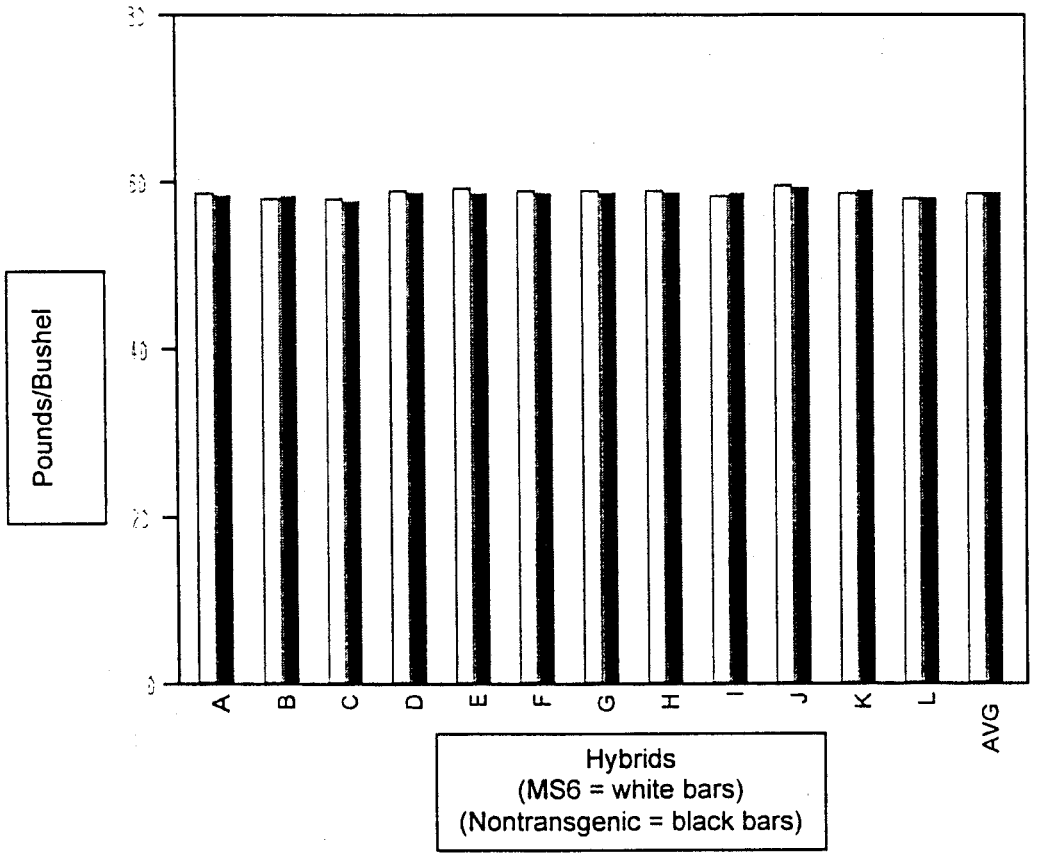
Figure 10. Grain Yield of MS6 Hybrids and Nontransgenic Counterpart Hybrids at Four Locations



*The data are combined from the 4 locations: 3 replications at two locations and 2 replications at two locations. The data are from 12 transgenic hybrids and their corresponding nontransgenic check hybrids—all planted at each location.

For grain yield (Fig. 10), all but four of the hybrids tested exhibited a trend of increased grain yield with event MS6, although none of the differences were significant. The overall average (columns designated "AVG" in Fig. 10) exhibited an increase of 3.6 bushels/acre with event MS6 (this comparison cannot be tested for significance).

Figure 11. Test Weight of Grain from MS6 Hybrids and Nontransgenic Hybrids at Four Locations.



*The data are combined from the 4 locations: 3 replications at two locations and 2 replications at two locations. The data are from 12 transgenic hybrids and their corresponding nontransgenic check hybrids—all planted at each location.

For the test weight (Fig. 11), all but four hybrids exhibited a trend of increased test weight with event MS6. None of the differences were significant, however. The overall average (columns designated "AVG" in Fig. 11) exhibited an increase of 0.1 pounds/bushel with event MS6 (this comparison cannot be tested for significance).

Although no significant differences were found, clear trends of increased yield with event MS6 were observed. This indicates that event MS6 hybrids are equal to or possibly better in agronomic performance attributes important to the grower than the nontransgenic hybrids.

VII. Environmental Consequences of Introduction

Field tests of MS6 in trials with nontransgenic corn lines demonstrated no significant differences apart from the intended change. No morphological, beneficial organism, disease, or pest differences between MS6 and previously

considered male sterility events were noted. There is no reason to think cultivation of event MS6 and its progeny will have environmental effects different from cultivation of other male sterile events, which have already been considered by APHIS. No adverse consequences from the introduction of MS6 are expected.

VIII. Statement of Grounds Unfavorable

No unfavorable information and data has been demonstrated for male sterility event MS6.

IX. References

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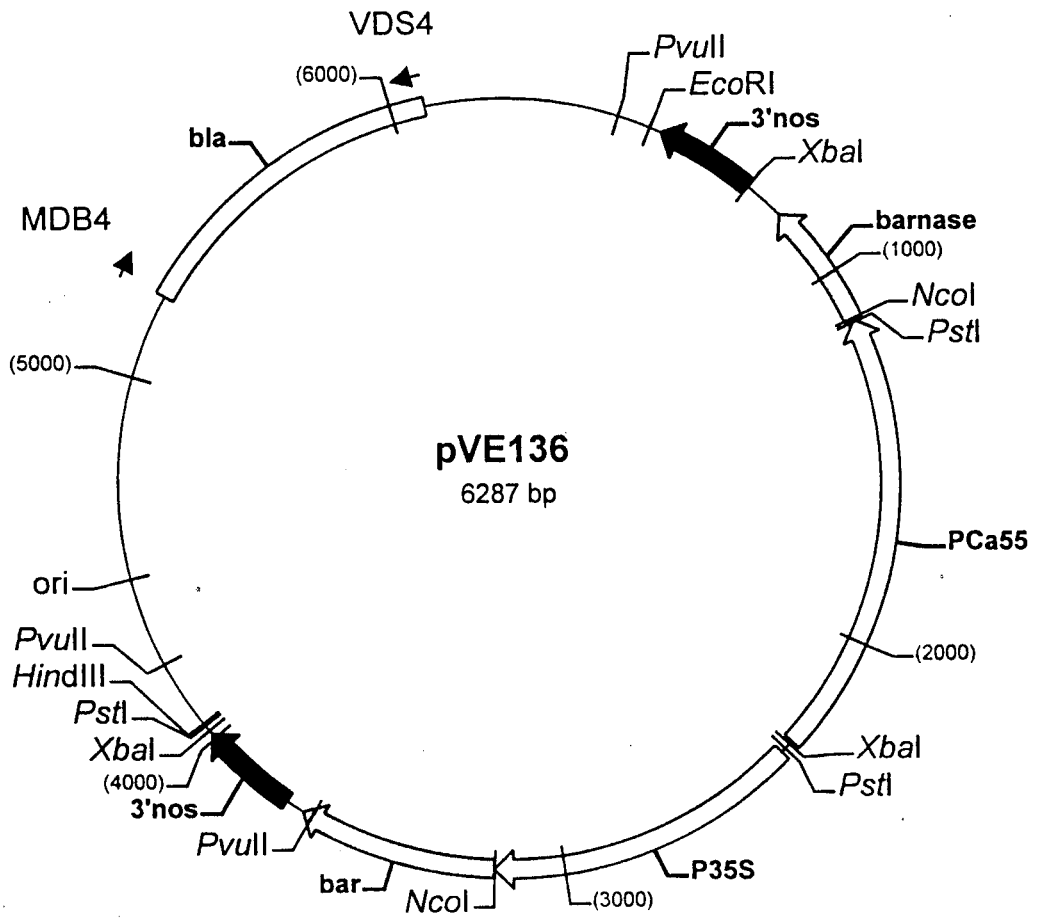
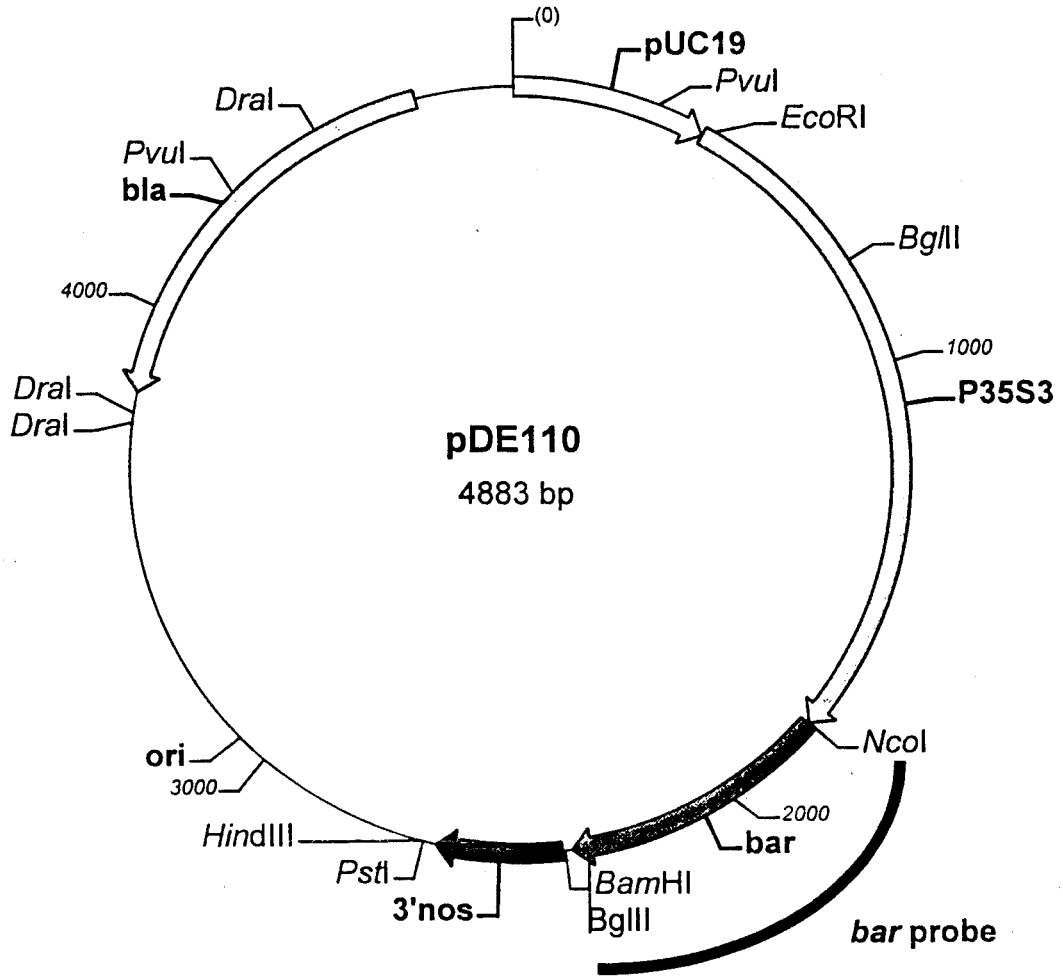
X. APPENDIX

Plasmid Map of pVE136
Plasmid Map of pDE110
Plasmid Map of pNOS2
List of Release Authorizations
USDA Field Trial Termination Reports

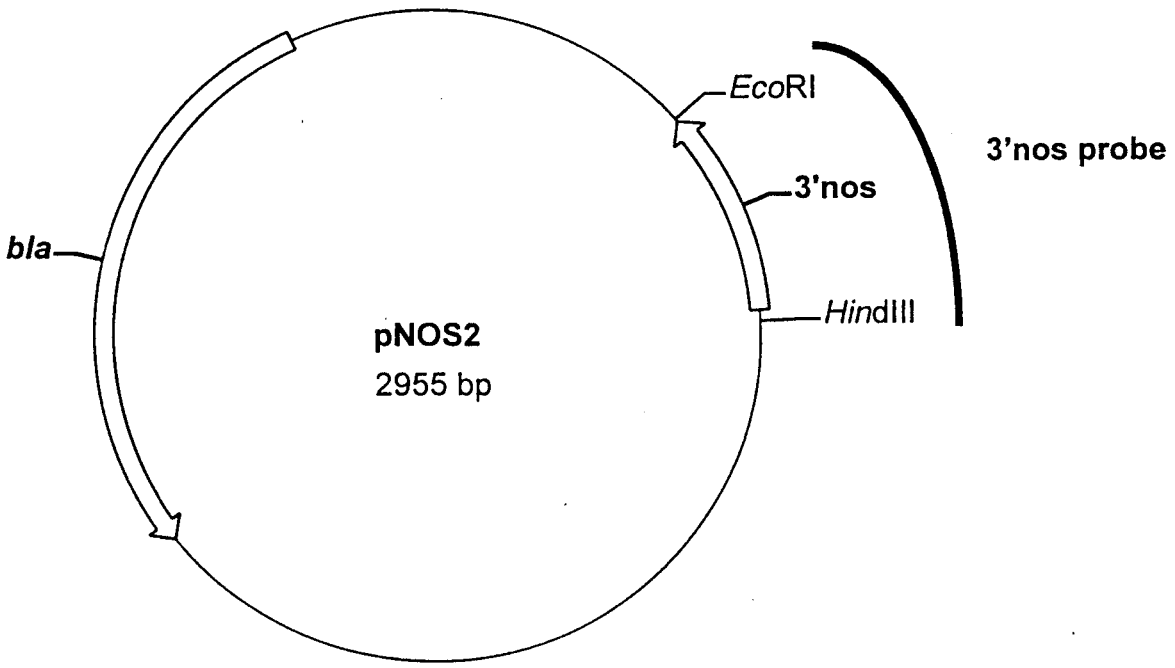
Plasmid Map of pVE136.



Plasmid Map of pDE110.



Plasmid Map of pNOS2.



List of Release Authorizations

Authorization Number	States and Sites
97-092-10N	PR (1)
97-121-03N	IA--not planted
97-121-04n	IN (1)
97-125-01n	IL (2)
97-279-02n	HI (6), NE (1)
98-071-04n	IA (2), WI (1), IL (1)
98-089-51n	ID (1)
98-083-02n	ID (1)
98-089-52n	IA (2), IL (3)
98-089-53n	HI (1)
98-089-57n	HI (1)
98-089-55n	IN (1)
98-114-14n	IL (1)

PLANT GENETIC SYSTEMS

APHIS ENVIRONMENTAL RELEASE REPORT

USDA Permit Number: 97-092-10N

PGS Reference: PGS-97-26

Permittee: Sue MacIntosh, Plant Genetic Systems (America), Inc., 7200 Hickman Rd, Suite 202, Des Moines, IA, 50322 [515-276-6642]

Line identification: MS6, MS14, MS15, MS9, MS10, MS12, MS11, MS13

Location(s) of Release: Isabela, Puerto Rico

Responsible person: Luz Velazques/Zonyli Ortic Silva

Transgenic Acreage: a) 0.051, b) 0/10, c) 0.024

Date(s) of Release: a) 5/19/97, b) 9/23/97, c) 2/18/98

Isolation Method: crossing block, detasseled or tassel bag to prevent unwanted pollinations

Date of Harvest: a) 9/19/97, b) 1/22/98, c) 6/19/98

Purpose of Release: backcrossing into elite corn lines

Special Management Practices: normal practices for male sterile corn

Observations - Were any obvious differences noted between the transgenic and non-transgenic lines?

Plant Phenoptype: 50% tolerance to Liberty herbicide, as expected
Insect Susceptibility: None noted
Stand / Germination, Seedling Vigor: None noted
Weediness: None noted
Disease Susceptibility: None noted
Volunteers?: Will monitor.
Other Comments: Performed as expected

Signed: Susan MacIntosh Date: 10/7/98

Susan MacIntosh

PLANT GENETIC SYSTEMS (AMERICA) INC.
7200 Hickman Road • Des Moines, Iowa • 50322 U.S.A.
Phone (515) 276-6642 • Fax (515) 278-8054

PLANT GENETIC SYSTEMS

APHIS ENVIRONMENTAL RELEASE REPORT

USDA Permit Number: 97-121-03N

PGS Reference: PGS-97-52

Permittee: Sue MacIntosh, Plant Genetic Systems (America), Inc., 7200 Hickman Rd, Suite 202, Des Moines, IA, 50322 [515-276-6642]

Line identification: MS6, MS14, MS15, MS9, MS10, MS12, MS11, MS13

Location(s) of Release: a) Williams, IA; b) Piper City, IA

Responsible person: a) Daryl Hexum; b) Robert Steidl

Transgenic Acreage: None planted

Date(s) of Release: NA

Isolation Method: NA

Date of Harvest: NA

Purpose of Release: NA

Special Management Practices: NA

Observations - Were any obvious differences noted between the transgenic and non-transgenic lines?

Plant Phenoptype: NA
Insect Susceptibility: NA
Stand / Germination, Seedling Vigor: NA
Weediness: NA
Disease Susceptibility: NA
Volunteers?: NA

Other Comments: NA

Signed: Susan MacIntosh Date: 10/7/98

Susan MacIntosh

PLANT GENETIC SYSTEMS (AMERICA) INC.
7200 Hickman Road • Des Moines, Iowa • 50322 U.S.A.
Phone (515) 276-6642 • Fax (515) 278-8054

PLANT GENETIC SYSTEMS

APHIS ENVIRONMENTAL RELEASE REPORT

USDA Permit Number: 97-125-01N

PGS Reference: PGS-97-44

Permittee: Sue MacIntosh, Plant Genetic Systems (America), Inc., 7200 Hickman Rd, Suite 202, Des Moines, IA, 50322 [515-276-6642]

Line identification: MS6, MS14, MS15, MS9, MS10, MS12, MS11, MS13

Location(s) of Release: Mt. Pulaski, IL

Responsible person: Robert Foley

Transgenic Acreage: less than 0.1 acre

Date(s) of Release: 6/4/97

Isolation Method: time and distance – 660 ft from all other corn

Date of Harvest: 9/17/97

Purpose of Release: breeding, hand pollination and hand harvested

Special Management Practices: Sprayed Liberty herbicide on each MS event for culling our off-types prior to pollination.

Observations - Were any obvious differences noted between the transgenic and non-transgenic lines?

Plant Phenoptype: Uniform within the event
Insect Susceptibility: None noted
Stand / Germination, Seedling Vigor: Fine
Weediness: All events appeared uniform
Disease Susceptibility: None noted
Volunteers?: Will monitor.
Other Comments: Site in Sugar Grove, IL not planted.

Signed: Susan MacIntosh Date: 10/7/98

Susan MacIntosh

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Phone (515) 276-6642 • Fax (515) 278-8054

PLANT GENETIC SYSTEMS

APHIS ENVIRONMENTAL RELEASE REPORT

USDA Permit Number:	97-121-04n
PGS Reference:	PGS-97-53
Permittee:	Sue MacIntosh, Plant Genetic Systems (America), Inc., 7200 Hickman Rd, Suite 202, Des Moines, IA, 50322 [515-276-6642]
Line identification:	MS6 and other Male sterile research lines (RZM#s)
Location(s) of Release:	Remington, Indiana
Responsible person:	Ron Castleberry
Transgenic Acreage:	0.1 acres
Date(s) of Release:	6/11/97
Isolation Method:	By time - planted late - all other corn planted May 7. Plant material was plowed under after selected ears harvested.
Date of Harvest:	10/12/97
Purpose of Release:	Breeding and testing
Special Management Practices:	Used standard nursery planting and care methods. Hand harvested ears under pollination bag.

Observations - Were any obvious differences noted between the transgenic and non-transgenic lines?

Plant Phenotype:	Segregation as expected.
Insect Susceptibility:	None, rootworm control using insecticide treatment/
Stand / Germination, Seedling Vigor:	OK, none noted.
Weediness:	None
Disease Susceptibility:	None, fungicide used
Other Comments:	
Volunteers?:	Will watch field next year (1998)

Signed: Susan MacIntosh Date: Feb 15, 1998

Susan MacIntosh February 15, 1998

PLANT GENETIC SYSTEMS (AMERICA) INC.
7200 Hickman Road • Des Moines, Iowa • 50322 U.S.A.
Phone (515) 276-6642 • Fax (515) 278-8054

PLANT GENETIC SYSTEMS

APHIS ENVIRONMENTAL RELEASE REPORT

USDA Permit Number:	97-125-01n
PGS Reference:	PGS-97-44
Permittee:	Sue MacIntosh, Plant Genetic Systems (America), Inc., 7200 Hickman Rd, Suite 202, Des Moines, IA, 50322 [515-276-6642]
Line identification:	MS6 and other Male sterile research lines (RZM#s)
Location(s) of Release:	4 mi. N of Tolono, IL, 2 mi. W. of St. Rt. 45 (IFSI Research Farms)
Responsible person:	David Deutscher, Illinois Foundation Seeds, Inc.
Transgenic Acreage:	0.39 acres
Date(s) of Release:	6/6/97 and 6/16/97
Isolation Method:	By time - planted late, hand pollinated ears harvested for lab shelling and use for research purposes. All other plants destroyed by shredding and discing.
Date of Harvest:	9/26/97
Purpose of Release:	Breeding and testing
Special Management Practices:	None

Observations - Were any obvious differences noted between the transgenic and non-transgenic lines?

Plant Phenotype:	None
Insect Susceptibility:	None
Stand / Germination, Seedling Vigor:	None
Weediness:	None
Disease Susceptibility:	None
Other Comments:	
Volunteers?:	Will watch field next year (1998)

Signed: Susan MacIntosh Date: Feb 15 1998

Susan MacIntosh February 15, 1998

PLANT GENETIC SYSTEMS (AMERICA) INC.
7200 Hickman Road • Des Moines, Iowa • 50322 U.S.A.
Phone (515) 276-6642 • Fax (515) 278-8054

1998 STATUS REPORT
Regulated Transgenic Corn
Hastings, NE Clay County

Submitted To:

USDA - Aphis
4700 River Road
Riverdale, MD 20737
Attn: Diane Hatmaker

Nebraska Department of Agriculture
Bureau of Plant Industry
P.O. Box 94756
Lincoln, NE 68509
Attn: Steve Johnson

Regulated Article:

Transgenic inbred corn lines containing regulated genes conferring insect resistance and/or herbicide resistance and/or male sterility.

Interstate Movement and/or Release Notifications:

NC+ number AGRBT98; USDA-Aphis number 97-279-01N
NC+ number AGRMS98; USDA-Aphis number 97-279-02N
*Monsanto number 97-042XRAB; USDA-Aphis number 97-024-02N

*This event was deregulated while this release was in progress.

Responsible Parties:

Primary:
NC+ Hybrids Research and Conditioning
RR #2, Box 190
Hastings, NE 68901
Attn: Dr. Alonzo Hester
Doug Volkmer
PH: 800-365-9804
FAX: 402-463-6549

Trial Specifics:

Type: Nursery

Dimension: 2200 rows; 20 feet X 30 inches (2.53 acres)

Genotypes: 97-279-01N 895 rows (1.03 acres)
97-279-02N 69 rows (.08 acres)
97-024-02N 200 rows (.23 acres)
non transgenic rows 1036 (1.19 acres)

Location: NC+ Hybrids, Clay County, 5 miles East and 1 mile North of Hastings, NE Field 304

Gene Containment: temporal isolation; bagging and hand harvest; on site crop destruction.

Planting Date: May 29,1998

Pollinating Dates: July 1, 1998 thru August 19, 1998

Harvest Date: October 1998

Progeny Management:

Hand pollinations will be maintained by NC+ in a seed vault. Unused plants were detasseled and ear shoots covered to prevent unwanted seed set.

Fallow Management:

To be done in spring of 1999.

Comments and Observations:

Homozygous lines from event 97-279-01N very chlorotic and lacked vigor

Responsible Party:

Name: Douglas Volkmer

Signature: _____

Date: _____

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1997 FINAL REPORT
Regulated Transgenic Corn
Hawaii Nursery #6

Submitted To:
USDA - Aphis
4700 River Road
Riverdale, MD 20737
Attn: Diane Hatmaker

Hawaii Department of Agriculture
635 Mua Street
Kahului, Hawaii 96732
Attn: Mr. Dennis Tokuoka

Hawaii Dept. of Agriculture
Plant Quarantine Branch
701 Ilalo Street
Honolulu, HI 96813
Attn: Larry Nakahara

Regulated Article:
Transgenic inbred corn lines containing regulated genes conferring insect resistance and/or herbicide resistance, and/or male sterility.

Interstate Movement and/or Release Notifications:
Monsanto number 97-042XRAB; USDA-Aphis number 97-024-02n
NC+ number AGRBt98; USDA- Aphis number 97-279-01n
NC+ number AGRMS98; USDA-Aphis number 97-279-02n
NC+ number MS98RRR; USDA-Aphis number 97-288-02n

Responsible Parties:
Primary:
NC+ Hybrids Research and Conditioning
RR #2, Box 190
Hastings, NE 68901
Attn: Dr. Alonzo Hester
Doug Volkmer
PH: 800-365-9804
FAX: 402-463-6549

Sub-contractor:
Hawaiian Research
P.O. Box 40
Mauna Loa Hwy. West
Kuanakakai, Molokai, Hawaii
96748-0040
Attn: Peter Eickhorn
Barry O'Keefe
PH: 808-553-5070
FAX: 808-553-5436

Trial Specifics:
Type: Nursery
Dimension: 1050 rows; 15 feet x 40 inches (1.22 acres)
Genotypes: 395 transgenic rows (.46 acres)
655non-transgenic rows (.75 acres)
Location: Hawaiian Research field #9B11A
Kuanakakai, Molokai, Hawaii
Gene Containment: Isolation, controlled pollinations, on site crop destruction and 30 days or more of fallow management.
Seed Source: NC+ Hybrids and Associates, Hawaii Transgenic Nursery #5

Planting Date: November 14, 1997
Pollinating Dates: January 12 to February 3, 1998
Harvest Date: March 13, 1998

Progeny Management: Transgenic Seed will be turned around for another nursery in Hawaii and the remainder sent to NC+ Hybrids and Associates.

Fallow Management:

All regulated plant debris and discarded seed will be left in the field and then alternately disked and irrigated until totally decomposed.

Volunteer Report

7/22/98	15 plts/sq. meter
8/05/98	16 plts/sq. meter
8/19/98	6 plts/sq. meter
9/02/98	0 plts/sq. meter

Sub-Contractor Comments and Observations:

Responsible Party:
Name: Douglas Volkmer
Signature: Douglas Volkmer
Date: 12-2-98

Sub-contractor:
Name: Peter H. Eichhorn
Signature: Peter H. Eichhorn
Date: 12/19/98

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1997 FINAL REPORT
Regulated Transgenic Corn
Hawaii Nursery #7

Submitted To:

USDA - Aphis
4700 River Road
Riverdale, MD 20737
Attn: Diane Hatmaker

Hawaii Department of Agriculture
635 Mua Street
Kahului, Hawaii 96732
Attn: Mr. Dennis Tokuoka

Hawaii Dept. of Agriculture
Plant Quarantine Branch
701 Ilalo Street
Honolulu, HI 96813
Attn: Larry Nakahara

Regulated Article:

Transgenic inbred corn lines containing regulated genes conferring insect resistance and/or herbicide resistance.

Interstate Movement and/or Release Notifications:

NC+ number AGRBT98; USDA-Aphis number 97-279-01n

Responsible Parties:

Primary:
NC+ Hybrids Research and Conditioning
RR #2, Box 190
Hastings, NE 68901
Attn: Dr. Alonzo Hester
Doug Volkmer
PH: 800-365-9804
FAX: 402-463-6549

Sub-contractor:
Hawaiian Research
P.O. Box 40
Mauna Loa Hwy. West
Kuanakakai, Molokai, Hawaii
96748-0040
Attn: Peter Eickhorn
Barry O'Keefe
PH: 808-553-5070
FAX: 808-553-5436

Trial Specifics:
Type: Nursery
Dimension: 1000 rows; 15 feet x 40 inches (1.15 acres)
Genotypes: 333 transgenic rows (.38 acres)
667non-transgenic rows (.77 acres)
Location: Hawaiian Research field #9B11B (1-600) #9A7A (601-1000)
Kuanakakai, Molokai, Hawaii
Gene Containment: Isolation, controlled pollinations, on site crop destruction and 30 days or more of fallow management.
Seed Source: NC+ Hybrids and Hawaii Transgenic Nursery #5

Planting Date: November 28-29, 1997
Pollinating Dates: February 4-16, 1998
Harvest Date: March 24, 1998

Progeny Management: A small portion will remain in Hawaii for another nursery. The bulk will return to NC+ Hybrids – Hastings, NE.

Fallow Management:

All regulated plant debris and discarded seed will be left in the field and then alternately disked and irrigated unitl totally composed.

Volunteer Report

7/22/98	15 plts/sq. meter
8/05/98	16 plts/sq. meter
8/19/98	6 plts/sq. meter
9/22/98	0 plts/sq. meter

Sub-Contractor Comments and Observations:

Responsible Party:
Name: Douglas Volkmer
Signature: Douglas Volkmer
Date: 12-2-98

Sub-contractor:
Name: Peter H. Eichhorn
Signature: Peter H. Eichhorn
Date: 10/19/98

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1998 FINAL REPORT
Regulated Transgenic Corn
Hawaii Nursery #8

Submitted To:

USDA - Aphis
4700 River Road
Riverdale, MD 20737
Attn: Diane Hatmaker

Hawaii Department of Agriculture
635 Mua Street
Kahului, Hawaii 96732
Attn: Mr. Dennis Tokuoka

Hawaii Dept. of Agriculture
Plant Quarantine Branch
701 Ilalo Street
Honolulu, HI 96813
Attn: Larry Nakahara

Regulated Article:

Transgenic inbred corn lines containing regulated genes conferring insect resistance and/or herbicide resistance, and/or male sterility.

Interstate Movement and/or Release Notifications:

*Monsanto number 97-042XRAB; USDA-Aphis number 97-024-02n
*NC+ number AGRBT98; Aphis number 97-279-01N
NC+ number AGRMS98; Aphis number 97-279-02N

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* Both events deregulated while this release was in progress

Responsible Parties:

Primary:
NC+ Hybrids Research and Conditioning
RR #2, Box 190
Hastings, NE 68901
Attn: Dr. Alonzo Hester
Doug Volkmer
PH: 800-365-9804
FAX: 402-463-6549

Sub-contractor:
Hawaiian Research
P.O. Box 40
Mauna Loa Hwy. West
Kuanakakai, Molokai, Hawaii
96748-0040
Attn: Peter Eickhorn
Barry O'Keefe
PH: 808-553-5070
FAX: 808-553-5436

Trial Specifics:
Type: Nursery
Dimension: 1086 rows; 15 feet x 40 inches (1.25 acres)
Genotypes: 540 transgenic rows (.62 acres)
546 non-transgenic rows (.63 acres)
Location: Hawaiian Research field #15C1B
Kuanakakai, Molokai, Hawaii
Gene Containment: Isolation, on site crop destruction

Seed Source: NC+ Hybrids and Associates; Hawaii Transgenic Nursery #6

Planting Date: March 25, 1998
Pollinating Dates: May 15, 1998 thru June 12, 1998
Harvest Date: June 5 & 6, 1998

Progeny Management: Transgenic Seed was turned around for Nursery #9 and/or sent to NC+ Hybrids and Associates

Fallow Management:

All regulated plant debris and discarded seed will be left in the field and then alternately disked and irrigated until totally decomposed.

Volunteer Report

8/19/98	28 plts/sq. meter
9/02/98	10 plts/sq. meter
9/16/98	12 plts/sq. meter
9/30/98	4 plts/sq. meter
10/14/98	0 plts/sq. meter

Sub-Contractor Comments and Observations:

Responsible Party:
Name: Douglas Volkmer
Signature: Douglas Volkmer
Date: 12-2-98

Sub-contractor:
Name: Peter H. Eichhorn
Signature: Peter H. Eichhorn
Date:

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1998 STATUS REPORT
Regulated Transgenic Corn
Hawaii Nursery #9

Submitted To:
USDA - Aphis
4700 River Road
Riverdale, MD 20737
Attn: Diane Hatmaker

Hawaii Department of Agriculture
635 Mua Street
Kahului, Hawaii 96732
Attn: Mr. Dennis Tokuoka

Hawaii Dept. of Agriculture
Plant Quarantine Branch
701 Ilalo Street
Honolulu, HI 96813
Attn: Larry Nakahara

Regulated Article:
Transgenic inbred corn lines containing regulated genes conferring male sterility.

Interstate Movement and/or Release Notifications:
NC+ number AGRMS98; USDA-Aphis number 97-279-02n

Responsible Parties:
Primary:
NC+ Hybrids Research and Conditioning
RR #2, Box 190
Hastings, NE 68901
Attn: Dr. Alonzo Hester
Doug Volkmer
PH: 800-365-9804
FAX: 402-463-6549

Sub-contractor:
Hawaiian Research
P.O. Box 40
Mauna Loa Hwy. West
Kuanakakai, Molokai, Hawaii
96748-0040
Attn: Peter Eickhorn
Barry O'Keefe
PH: 808-553-5070
FAX: 808-553-5436

Trial Specifics:
Type: Nursery
Dimension: 2171 rows; 15 feet x 40 inches (2.50 acres)
Genotypes: 38 transgenic rows (.04 acres)
2133 non-transgenic rows (2.46 acres)
Location: Hawaiian Research field #11C2A, 11C3, 11C3A, 11C4A
Kuanakakai, Molokai, Hawaii
Gene Containment: Isolation, on site crop destruction

Seed Source: Hawaii Transgenic Nursery #8, NC+ Hybrids and Associates

Planting Date:	Field 11C2A, 11C3	July 23, 1998
	Field 11C3A,	July 30,1998
	Field 11C4A,	August 19,1998
Pollinating Dates:	Field 11C2A, 11C3	September 9, 1998 to September 24,1998
	Field 11C3A	September 15, 1998 to September 30,1998
	Field 11C4A	October 15, 1998 to October 30, 1998
Harvest Date:	Field 11C2A, 11C3	October 21, 22 and November 4,5,6, 1998
	Field 11C3A	October 28,29,30, 1998
	Field 11C4A	December 3 & 4, 1998

Progeny Management: Seed will be turned around for Nursery #10 in Hawaii or sent back to NC+ Hybrids for storage.

Fallow Management: Uncompleted

Sub-Contractor Comments and Observations:

Responsible Party:
Name: Douglas Volkmer
Signature:_____
Date:_____

Sub-contractor:
Name: _____
Signature:_____
Date:_____

1998 FINAL REPORT
Regulated Transgenic Corn
Hawaii
Line increase EJ4006B2

Submitted To:
USDA - Aphis
4700 River Road
Riverdale, MD 20737
Attn: Diane Hatmaker

Hawaii Department of Agriculture
635 Mua Street
Kahului, Hawaii 96732
Attn: Mr. Dennis Tokuoka

Hawaii Dept. of Agriculture
Plant Quarantine Branch
701 Ilalo Street
Honolulu, HI 96813
Attn: Larry Nakahara

Regulated Article:
Transgenic inbred corn line containing regulated gene conferring insect resistance and herbicide resistance.

Interstate Movement and/or Release Notifications:
NC+ number AGRBT98; USDA - Aphis number 97-279-01n

Responsible Parties:
Primary:
NC+ Hybrids Research and Conditioning
RR #2, Box 190
Hastings, NE 68901
Attn: Dr. Alonzo Hester, Doug Volkmer
PH: 800-365-9804
FAX: 402-463-6549

Sub-contractor:
Hawaiian Research
P.O. Box 40
Mauna Loa Hwy. West
Kuanakakai, Molokai, Hawaii
96748-0040
Attn: Peter Eickhorn
Barry O'Keefe
PH: 808-553-5070
FAX: 808-553-5436

Trial Specifics:
Type: Line increase
Dimension: .5 acre

Genotypes: .5 acre transgenic
No non-transgenic acres
Location: Hawaiian Research
Kuanakakai, Molokai, Hawaii
Gene Containment: 660 ft. isolation

Seed Source: NC+ Hawaii nursery #6

Planting Date: March 30, 1998
Pollinating Dates: June 1998
Harvest Date: July 1998

Progeny Management: 150 lbs. of seed. Some was turned around in Hawaii and the rest sent to a Seed Associate.

Fallow Management:
Does not apply. Event was deregulated in May 1998.

Sub-Contractor Comments and Observations:

Responsible Party:
Name: DOUGLAS VOLKMER
Signature: Douglas Volkmer
Date: 12-2-98

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10/12/98

1998 FINAL REPORT
Regulated Transgenic Corn
Hawaii
Line increase EJ4009B2

Submitted To:
USDA - Aphis
4700 River Road
Riverdale, MD 20737
Attn: Diane Hatmaker

Hawaii Department of Agriculture
635 Mua Street
Kahului, Hawaii 96732
Attn: Mr. Dennis Tokuoka

Hawaii Dept. of Agriculture
Plant Quarantine Branch
701 Ilalo Street
Honolulu, HI 96813
Attn: Larry Nakahara

Regulated Article:
Transgenic inbred corn line containing regulated gene conferring insect resistance and herbicide resistance.

Interstate Movement and/or Release Notifications:
NC+ number AGRBT98; USDA - Aphis number 97-279-01n

Responsible Parties:
Primary:
NC+ Hybrids Research and Conditioning
RR #2, Box 190
Hastings, NE 68901
Attn: Dr. Alonzo Hester, Doug Volkmer
PH: 800-365-9804
FAX: 402-463-6549

Sub-contractor:
Hawaiian Research
P.O. Box 40
Mauna Loa Hwy. West
Kuanakakai, Molokai, Hawaii
96748-0040
Attn: Peter Eickhorn
Barry O'Keefe
PH: 808-553-5070
FAX: 808-553-5436

Trial Specifics:
Type: Increase
Dimension: .5 acre

Genotypes: .5 acre transgenic
No non-transgenic acres
Location: Hawaiian Research
Kuanakakai, Molokai, Hawaii
Gene Containment: 660 ft. isolation

Seed Source: NC+ Hawaii nursery #6

Planting Date: March 25, 1998
Pollinating Dates: May 27,1998 50% Shed
Harvest Date: July 1998

Progeny Management: 1500 lbs. of Seed sent to NC+ Hybrids, Hastings

Fallow Management:
Does not apply. Event was deregulated in May 1998.

Sub-Contractor Comments and Observations:

Responsible Party:
Name: DOUGLAS VOLKMER
Signature: Douglas Volkmer
Date: 12-2-98

MS6 USDA Termination Report, 1998

MS6 corn and its nontransgenic parent line PA91 x H99 (H99) were field tested at approximately fifteen (15) locations in the U.S. during the 1998 growing season. MS6 corn is a male sterile corn plant, and it also contains the bar gene whose expression product is the PAT enzyme. The PAT enzyme confers resistance to the broad-spectrum herbicide glufosinate-ammonium.

USDA Notification Numbers and Locations: **Polk and Boone Counties, IA, Jefferson County WI, and, Will County, IL (98-071-04n); Canyon County, ID (98-089-51n) and (98-083-02n); Shelby and Dallas Counties, IA, Iroquois, Champaign and Henry Counties, IL (98-089-52n); Maui County HI (98-089-53n); Kauai County, HI (98-089-57n); Jasper County, IN (98-089-55n); and, Henry County, IL (98-114-14n)**

Planting Conditions: Planting occurred on sites ranging in size from 0.2 acres to 5 acres from mid to late May 1998, on all sites in the Midwest U.S. Planting at the Hawaiian sites occurred in February, March, July and August, 1998. Hawaii's climate is suitable for a year-round growing season. Therefore planting times vary considerably from those in the corn belt. Midwest sites were planted when weather conditions were warm enough to allow for seed germination in a time frame comparable to commercial fields.

Frequency of Observations: The sites were observed by plant breeders, station managers, field agronomists, or associates several times (6+) throughout the growing season. Data was recorded at several different growing stages from emergence through the V1, V2, V4, V6, V8, tasseling and harvest stages.

Agronomic Data Recorded: Seed germination rates, plant stand, plant vigor, deleterious effects, disease and pest resistance/susceptibility were monitored throughout the growing season. With regard to all of the aforementioned agronomic traits, there was no difference in the transgenic MS6 corn as compared with the nontransgenic parent corn variety PA91 x H99 (H99). Seed germinated well and the plant grew healthily. Plant stand was good. The only agronomic difference noted between MS6 corn and the parent variety PA91 x H99 (H99) is that the peduncle and tassel for MS6 corn was smaller and never filled with pollen. This is expected because MS6 is a male sterile line. MS6 corn demonstrated no greater potential to become a weed than its nontransgenic parent.

Susceptibility to Disease and Insect Pressure: Disease pressure was low at most sites. The recorded observations of disease infestation were as follows. Mild infestation of grey leaf spot was observed in Will County, IL, Polk County, IA, and Jasper County, IN. In all cases infestation was identical for transgenic and nontransgenic plants. Minor damage from smut was recorded for transgenic and nontransgenic plants in Iroquois County, IL, and Boone County, IA. Other diseases reported were Stewart's disease in Jasper County, IN, and northern leaf blight in Iroquois, IL. In these cases disease levels were the same for both the transgenic MS6 plants and the parental PA91 x H99(H99), nontransgenic plants. Western and northern corn rootworm pressure was observed in Jefferson County, WI and in Polk County, IA. European corn borer and fall armyworm worm were also observed in Polk County, IA. Aphids were observed in Shelby

and Polk Counties, IA. In all cases the insect damage and observed populations were the same for transgenic and nontransgenic plants.

Resistance to Glufosinate-Ammonium: In breeding trials treatment with glufosinate-ammonium revealed that approximately ½ of the transgenic plants were susceptible to the herbicide and approximately ½ were resistant. This result demonstrates segregation was occurring in the expected 1:1 ratio. The nontransgenic parent PA91 x H99(H99) plants were all susceptible to treatment with glufosinate-ammonium. No other herbicides were used on these particular plots.

Effects on Beneficial Organisms: Healthy populations of ladybugs and earthworms were observed at all sites. Populations were the same in the MS6 transgenic plots and in the nontransgenic parent PA91 x H99 (H99) plots.

Volunteer Monitoring and Mitigation Measures: Since harvest for the 1998 season has just occurred in the past few weeks, volunteer monitoring has not been necessary as of date. Proper mitigation methods such as the discing under of fields two–to-three times will be carried out in the next few weeks. Monitoring for volunteers will occur in early spring of 1999.