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(54) **Title:** USE OF THE MAIZE X112 MUTANT AHAS 2 GENE AND IMIDAZOLINONE HERBICIDES FOR SELECTION OF TRANSGENIC MONOCOTS, MAIZE, RICE AND WHEAT PLANTS RESISTANT TO THE IMIDAZOLINONE HERBICIDES

(57) **Abstract:** A system for transformation of maize X112 mutant *ahas*2 gene into monocot (rice, corn, and wheat) cells, selection of transformed cells with the imidazolinone class of herbicide compound, and production of transgenic maize, rice and wheat plants resistant to the imidazolinone herbicides is described. The mutant *ahas*2 gene can be used as an effective selectable marker in transformation, useful in selection for stacked gene traits, useful as a selectable marker in breeding or hybrid seed production, and useful as a quality control tool.

USE OF THE MAIZE X112 MUTANT AHAS 2 GENE AND IMIDAZOLINONE HERBICIDES FOR SELECTION OF TRANSGENIC MONOCOTS, MAIZE, RICE AND WHEAT PLANTS RESISTANT TO THE IMIDAZOLINONE HERBICIDES.

The present application claims priority from U. S. provisional application Serial No. 60/200,658, filed April 28, 2000.

FIELD OF THE INVENTION

The present invention relates, specifically, to the transformation of maize X112 mutant *ahas* 2 gene into monocots such as maize (corn), wheat and rice, selection of transformed maize (corn), rice and wheat cells with imidazolinone, production of transgenic maize (corn), rice and wheat materials and plants resistant to the imidazolinone herbicides, in vitro characterization of the transformed plants, and greenhouse performances of imidazolinone resistant transgenic maize (corn), rice and wheat plants treated with various herbicides.

BACKGROUND OF THE INVENTION

The existence of the branch chain amino acid (valine, leucine and isoleucine) biosynthetic pathway in plants, and its absence in animals is one of the major differences of plant and animal biochemistry. Therefore, inhibition of the branch chain amino acid biosynthesis is detrimental to plants but has no effect on animals. Imidazolinone and sulfonylurea herbicides inhibit, acetohydroxyacid synthase (AHAS, or acetolactate synthase--ALS; E.C.4.1.3.18), the key enzyme in the biosynthesis of branch chain amino acids (Chaleff and Mauvais, 1984; Shaner et al. 1984). Consequently, because imidazolinone and sulfonylurea herbicides are effective at very low application rates, and relatively non-toxic to animals, they are widely used in modern agriculture.

The differential sensitivity to the imidazolinone herbicides is dependent on the chemical nature of the particular herbicide and differential metabolism of the compound from toxic to non-toxic form in the plants (Shaner et al. 1984; Brown et al. 1987). Other plant physiological differences such as absorption and translocation also play an important role in selectivity (Shaner and Robinson 1985). Computer-based modeling of the three dimensional conformation of the

AHAS-inhibitor complex predicts several amino acids in the proposed inhibitor binding pocket as sites where introduced mutations would likely confer selective resistance to imidazolinones (Ott et al. 1996). Transgenic plants produced with these rationally designed mutations in the proposed biding sites of the AHAS enzyme exhibited specific resistance to a single class of herbicides (Ott et al. 1996).

Application of imidazolinone herbicides in field production of major crops enables more effective weed control and less environmental impact than other chemicals. Among the major agricultural crops, only soybean is naturally resistant to imidazolinone herbicides due to its ability to rapidly metabolize the compounds (Shaner and Robinson 1985) while others such as corn (Newhouse et al. 1991), wheat (Newhouse et al. 1992) and rice (Barrette et al. 1989) are somewhat susceptible. In order to extend the application of imidazolinone and sulfonylurea herbicides to more crops, it is necessary to enhance plant resistance to these compounds. To date, three major approaches have been used to enhance plant resistance: 1) screening for spontaneous resistant mutations in cell culture (Chaleff and Ray 1984; Anderson and Georgeson 1989; Sebastian et al. 1989; Magha et al. 1993), 2) artificially inducing mutations in seeds or microspores (Swanson et al. 1989; Newhouse et al. 1992; Croughan 1996), and 3) transferring resistance genes between different species by genetic engineering (Haughn et al. 1988; Charest et al. 1990; Odell et al. 1990; Li et al. 1992; Tourneur et al. 1993). Thus far spontaneous herbicide resistant mutants have been identified and characterized in tobacco (Chaleff and Ray 1984), soybean (Sebastian et al. 1989), corn (Anderson and Georgeson 1989) and rapeseed (Magha et al. 1993). Chemical mutagenesis successfully produced resistant mutants in wheat (Newhouse et al. 1992), canola (Swanson et al. 1989) and rice (Croughan 1996). Studies on tobacco (Haughn et al. 1988; Odell et al. 1990; Charest et al. 1990) and rice (Li et al. 1992) suggested the potential of transferring genes from one species to another for the production of resistant crops.

Advances in transformation technologies of monocots, especially of rice have made possible the transfer of genes between species for development of transgenic plants with improved characteristics. Transgenic rice plants have been produced by transformation of protoplasts (Shimamoto et al. 1989; Peng et al. 1990), bombardment of cells (Christou et al. 1991; Li et al. 1993), and more recently, *Agrobacterium*-mediated transformation of immature embryos (Chan et al. 1992; Hiei et al. 1994; Aldemita et al. 1996). Critical in all the transformation processes is the ability to select for the cells that have been transformed over the rest of the population of cells. Typically a combination of an antibiotic and a gene conferring resistance to the antibiotic has been used. Examples include the neomycin phosphotransferase (*neo*) gene for resistance to kanamycin or genetic (G-418), hygromycin B transferase (*hyh*) for

hygromycin B resistance (Shimamoto et al. 1989; Hayashimoto et al. 1990), and the *bar* gene for phophinothricin resistance (Christou et al. 1991; Rathore et al. 1993). All of these selectable genes (*neo, hyh* and *bar*) are of bacterial origin. In one report, use of a mutant *als* gene from *Arabidopsis* coupled with selection on sulfonylurea herbicide was demonstrated for production of transgenic rice plants (Li et al. 1992). An increase in *in vitro* resistance to chlorsulfuron of similar magnitude (200-fold) was demonstrated in transgenic rice containing 35S/*als* transgene (Li et al. 1992), and imidazolinone-resistant growth of transgenic tobacco was reported to be 100-fold greater than nontransformed control plants (Sathasivan et al.1991). In the literature, expression of the introduced AHAS (or ALS) gene at different magnitudes was achieved by manipulating several aspects of the transformation that included the use of different promoters and screening larger populations of transformants (Odell et al. 1990; Sathasivan et al. 1991; Li et al. 1992). Studies showed that replacing the *Arabidopsis* ALS promoter with the CaMV35S promoter resulted in 40-fold differences in *in vitro* resistance to chlorsulfuron (Li et al. 1992). In tobacco, increase in resistance to imazethapyr in individual calli transformed with mutant *als* gene from *Arabidopsis* ranged from 10- to 1000-fold, most likely reflecting the differences in gene copy numbers or in chromosomal positions of the transgenes (Sathasivan et al. 1991).

Imidazolinone-specific resistance has been reported in a number of patents. U.S. Pat. No. 4,761,373 described in general terms an altered *ahas* as a basis of herbicide resistance in plants, and specifically disclosed certain imidazolinone resistant corn lines. U.S. Pat. No. 5,013,659 disclosed that mutants exhibiting herbicide resistance possess mutations in at least one amino acid in one or more conserved regions. The mutations described therein encode either cross-resistance for imidazolinones and sulfonylureas or sulfonylurea-specific resistance but no imidazolinone-specific resistance. Additionally, U.S. Pat. No. 5,731,180 and continuation-in-part 5,767,361 isolated a gene encoding imidazolinone-specific resistance in a monocot and determined it to be associated with a single amino acid substitution in a wild-type moncot AHAS amino acid sequence. U.S. Patents Nos. 5,731,180 and 5,767,361, as well as U.S. Patent No. 5,750,866 and 6,025,541, are incorporated herein by reference. However, while the referenced patents generally allude to the use of the gene as a selectable marker for selection on imidazolinone, the present invention describes the specific application of the maize X112 mutant *ahas* 2 gene to monocots such as maize (corn), rice and wheat varieties, or use of the mutant XI12 *ahas* 2 gene as a selectable marker coupled with a imidazolinone compound as a selection system for resistance to the imidazolinone herbicides.

The AHAS gene codes for acetohydroxyacid synthase (AHAS, E.C.4.1.3.18; also called acetolactate synthase; ALS) which is the first common enzyme in the biosynthetic pathway of

branch chain amino acids (Shaner et al. 1984). The imidazolinone herbicides are a class of herbicides that inhibit AHAS activity thus, preventing further growth and development of susceptible plants such as rice and many weed species. In biochemical studies, selectivity of the imidazolinone herbicides has been shown to be based on differences in nature and rate of metabolism of the herbicides (Shaner and Robinson 1985; Brown et al. 1987). In genetic studies, mutations in the *ahas* gene have been linked for resistance to the imidazolinone herbicides in canola (Swanson et al. 1989) and corn (Newhouse et al. 1991). Analysis of the mutant *ahas* 2 gene, isolated from maize (XI12) plants revealed that a single base mutation from G to A at nucleotide 621 relative to the initiation codon resulted in a one amino acid change in the AHAS enzyme from Ser to Asn (Dietrich, 1998). The term mutant *ahas* 2 gene as used herein includes any additions, deletions, or substitutions in the nucleic acid sequence as described in Dietrich, U.S. Patent 5,731,180, that do not change the function of the mutant *ahas* 2 gene. The function of the mutant *ahas* 2 gene is to confer resistance to imidazolinone herbicides. The maize XI12 mutant *ahas* 2 gene presents a number of advantages for use in plant transformation. It is a plant gene and even this mutant form is known to exist in plant populations. A combination of the maize XI12 mutant *ahas* 2 gene and imidazolinone chemistry provides a useful system for selection of transformed cells, plants and progeny. Use of a plant mutant *ahas* gene for selection in plant transformation circumvents the problems associated with transformation of antibiotic gene of bacterial origin in transgenic plants. The term mutant *ahas* 2 gene as used herein includes any additions, deletions, or substitutions in the nucleic acid sequence as described in Dietrich, U.S. Patent 5,731,180, that do not change the function of the mutant *ahas* 2 gene. The function of the mutant *ahas* 2 gene is to confer resistance to imidazolinone herbicides.

BRIEF SUMMARY OF THE INVENTION

In this invention we report the transformation of maize X112 mutant *ahas* 2 gene into maize and wheat embryos and rice protoplasts, selection of transformed cells with an imidazolinone compound, production of transgenic maize, rice and wheat plants resistant to the imidazolinone herbicides, *in vitro* characterization of the transformed plants, and greenhouse performances of imidazolinone resistant transgenic plants treated with various herbicides.

In rice, resistant calli were recovered from transformations following selection on imazethapyr and regenerated into fertile plants. Genetic study on two transgenic rice lines (26

and 29) showed that the introduced maize *ahas2* gene was stably transmitted to progeny plants and conferred a single dominant trait inherited in a Mendelian fashion. Homozygous imidazolinone resistant lines were readily identified and isolated based on seed germination and greenhouse screen tests. A 100-fold increase in *in vitro* resistance to imazethapyr in cell lines and immature embryos derived from transgenic homozygous resistant plants was demonstrated. Also the invention can be used to deliver a second gene in co-transformation. Also, the invention identifies the production of transgenic maize and wheat plants by *Agrobacterium*-mediated transformation using the maize X112 mutant *ahas*2 gene as a selectable marker coupled with selection of callus material and regeneration of plants on media supplemented with the imidazolinone herbicides. Transformation efficiency averaged about 2% and reached to as high as 16%-20% in some experiments for corn and ranged from 0.4 to 3.1% for wheat.

We evaluated performances of transgenic maize, rice and wheat plants containing the maize XI12 *ahas* 2 gene in response to applications of various herbicides in greenhouse. A total of 9 herbicides including five imidazolinones (PURSUIT® imazethapyr, CADRE® imazameth, RAPTOR® imazamox, SCEPTER® imazaquin, and ARSENAL® imazapyr, two rice herbicides, AC322,140 cyclosufamuron and LONDAX® bensulfuron-methyl, ACCENT® nicosulfuron and CLASSIC® clorimuron-ethyl applied at 4 different rate were used in a study of transgenic rice plants. The results showed that transgenic plants were resistant to the five imidazolinone herbicides at rates as high as 6x typical use rates and retained sensitivity to the sulfonylurea herbicides as compared to untransformed control plants. When treated with herbicides, transgenic plants produced yields comparable with their untreated counterparts. Untransformed control plants, however, had 10 to 20% higher yields than transgenic plants in the absence of herbicide treatments. All herbicides at all rates tested had no detrimental effects on seed set, except higher rates of ARSENAL® imazapyr caused severe sterility in transgenic plants. Enzyme assays provided evidence that the introduced XI12 maize *ahas 2* gene conferred selective resistant AHAS enzyme in the transgenic plants.

Evaluation of effects of different imidazoline herbicides on transgenic corn plants showed there was no injury up to 16x ARSENAL® imazapyr (384 g/ha), 8x PURSUIT® imazethapyr (500 g/ha), 4x CADRE® imazameth (800 g/ha). At 4x imazamox (160 g/ha), no or slight injury to the plants was observed. An increase of 5x tolerance to imazamox was demonstrated in transgenic wheat plants.

The mutant *ahas*2 gene can be used as an effective selectable marker in transformation, useful in selection for stacked gene traits, useful as a selectable marker in breeding or hybrid seed production, and useful as a quality control tool.

BRIEF DESCRIPTION OF THE FIGURES/DRAWINGS

Figure legends

Fig. 1. Fresh weight of suspension cells (**A**) and calli (**B**) derived from immature embryos of transformed (26-4) and untransformed control (Nt) plants on media amended with various concentrations of imazethapyr.

Fig . 2A-C. Enzyme assay for AHAS activity. Nt is untransformed control and Lines 26 and 29 are transgenic rice plants.

Fig . 3A-I. Effect of different herbicides on fresh weight of rice plants. Herbicides were sprayed at three-leaf stage (post-emergence) at 1, 2, and 4x rates indicated in each graph. Lines 26 and 29 are transgenic plants and Nt is untransformed control.

Fig .4A-B. Plasmid constructs used in transformation.

Fig. 5. Crossing scheme for transgenic corn

Fig. 6. Effect of imazamox on plant height of transformed and non-transformed wheat plants. Plant height of non-transformed Ciccio and Closseo plants (Fig. 6A) when treated with 0 to 50 g/ha imazamox. Transgenic line 040701-10 and non-transformed Ciccio treated with 0 to 50 g/ha imazamox at three-leaf stage (Fig. 6B).

DESCRIPTION OF THE INVENTION

In a preferred embodiment, transgenic rice plants are produced by transformation with the maize XI12 mutant *ahas 2* gene, which is then utilized as a selectable marker. Rice protoplasts isolated from rice varieties Nortai (Nt) and Radon (Rd) were transformed with pCD220, a plasmid construct containing the maize X112 *ahas* promoter and mutant X112 *ahas* 2 gene (Ser621Asn), via PEG mediated transformation. The transformation efficiency in the present study was slightly lower than but similar with those reported for other resistant gene/selection systems used for rice (Peng et al. 1990, Li et al. 1992). In tobacco, recovery of transformants from chlorsulfuron (a herbicide with mode of action similar to that of imidazolinone herbicides) was also reported to be much lower than recovery of transformants from kanamycin (Charest et al. 1990). Unlike antibiotics such as kanamycin (Peng et al. 1991), the imidazolinone herbicide did not have a detrimental effect on the plant regeneration potential of the transformed rice calli. Therefore, coupling of the maize mutant *ahas*2 gene with selection on imidazolinone herbicides presents another valuable selectable gene and selection system for

genetic engineering of rice and other monocots.

Also, in further embodiments, the invention identifies transgenic maize and wheat plants produced by *Agrobacterium*-mediated transformation using the maize X112 mutant *ahas* 2 gene as a selectable marker. In particular, immature embryos, 0.8 to 1.5 mm., were isolated at 10 to 16 days after pollination and co-cultivated with *Agrobacterium* cells harboring the maize X112 mutant *ahas 2* gene for 3-7 days. Explants were then transferred to selection medium containing the imidazolinone herbicides for 7-10 weeks for wheat and 5-8 weeks for maize and subcultured every 2-3 weeks. In our selection scheme, the initial concentration of the imidazolonone herbicides was very low, only 0.05 to 0.1 μM imazethapyr or imazamox was added to medium. We found it was critical to keep the selection pressure low in the beginning to assure formation of regenerable callus cells. When high concentration of the imidazolinone compounds was used in the initial selection stage, only slimy callus that could not regenerate into plants was formed. During the second and third selection stages, concentrations of imazethapyr or imazamox were increased to 0.5 or 0.3 μM, respectively. Most cells that grew normally at these concentrations were transformed with the maize mutant XI12 *ahas* 2 gene. Concentration of imazethapyr was lowered to 0.25 μM during maize shoot regeneration and no imidazolinoe compound was added during root formation. During wheat regeneration, concentration of imazamox was 0.1 μM and no selection reagent during root formation either.

Putative transgenic plants were then sprayed with imidazolinone herbicides. Wheat plantlets were sprayed with 25-50 g/ha RAPTOR® at 10-14 days after transplanting and corn plants were sprayed with 125 to 250 g/ha PURSUIT® imazethapyr about two weeks after transplanting. Plants survived the herbicide treatments were transplanted to larger pots and grown to maturity.

Transgenic maize and wheat plants produced from these transformation systems contained the introduced maize X112 mutant *ahas* 2 gene. PCR (Polymerase Chain Reaction) and/or Southern blot analysis was used to confirm presence of the transgene. Southern blot analysis of DNA extracted from plants (putative transformants) regenerated from imazethapyr resistant calli showed that all the plants assayed had the introduced maize *ahas* 2 gene. Some of the plants received multiple copies with multiple insertions and others were transformed with one to two copies of the gene integrated into a single locus. Plants that had single integration patterns, with one to two copies of the intact transgenes were carried to subsequent generations for further evaluation in vitro and in vivo. Transmission of the transgene to rice T1 plants were studied by PCR analysis and spray tests.

Three tests were performed to evaluate herbicide resistance of T2 progeny of transgenic

rice. T2 progeny were first evaluated by a seed germination test followed by the greenhouse spray test and PCR analysis. As a prelude to testing the transgenic plants, an experiment was conducted to germinate untransformed rice seeds at imazethapyr concentrations ranging from 0.1 to 10 μM. Untransformed seeds were completely inhibited at 1 μM; therefore 5 μM imazethapyr, equivalent to 5 times the threshold, was used for subsequent seed germination tests of the T2 generation. The greenhouse spray test and PCR analysis were used to further confirm the results of the seed germination test with consistent identification of lines as resistant homozygous, susceptible homozygous or segregating hemizygous.

Data obtained from characterization of the transformed cells and plants as shown the examples show that transgenic maize, wheat and rice produced according to the invention have sufficient resistance at plant level. Rice is naturally susceptible to the imidazolinone herbicides *in vitro*. Very low concentrations (0.1 μM and higher) of imazethapyr inhibit the growth of untransformed rice cells. Transgenic cells with the introduced maize XI12 mutant *ahas 2* gene exhibited a 100-fold increase in resistance to imazethapyr *in vitro* in both fine suspension cells or freshly isolated immature embryos derived from the transgenic plants.

In vitro AHAS assays were performed to demonstrate that the resistant AHAS enzyme was the basis of selective herbicide tolerance in the transgenic plants. Previously published procedures were used for the extraction and in vitro assay for AHAS activity (Singh et al. 1988). Lower portions of the shoots were used as the source of plant tissue for the assay. The desalted crude extracts were used for the *in vitro* enzyme assays. Acetolactate produced by the enzyme was converted to acetoin, which was measured by the Westerfield assay system.

The present invention further provides for fertile transgenic plants, which were morphologically normal and transmitted and inherited the transgenes. The regenerated plants had morphological traits that closely resembled their seed-grown counterparts and were mostly self-fertile although some plants had very low seed set. Production of viable seed and inheritance of the transgene permits transmission of the herbicide resistance from transgenic lines to other elite breeding lines.

Finally, the examples show the greenhouse performances of imidazolinone resistant transgenic plants treated with various herbicides. The results of greenhouse evaluation on performances of transgenic plants containing the maize XI12 mutant *ahas 2* gene presented here is very encouraging. Transgenic rice and maize plants so produced can be used in field production to sustain application of herbicide with minimum damage to the crop. The two varieties of wheat *Ciccio* and *Colosseo* used for our transformations are extremely sensitive to the imidazolinone herbicides, especially to RAPTOR® imazamox. When treated with 10 g/ha

(~1/3 field application rate), plant heights of Ciccio and Colosseo were reduced to 50% less than those of transformed plants having the mutant maize X112 *ahas* gene. Plants were completely killed when sprayed with more than 10g/ha RAPTOR® imazamox. The present invention further provides for transgenic wheat plants with elevated resistance to the imidazolinone herbicide.

Examples of Preferred Embodiments

Example 1: Plasmid constructs used for transformation:

The following plasmids are used for monocot transformation (see Fig.4).

pCD220: The plasmid pCD220 contains the maize *ahas* promoter driving the maize XI12 mutant *ahas* 2 gene and its native terminator. The pCD220 plasmid was constructed by subcloning the XI12 *ahas* 2 gene as a *Pst* I fragment into pBluescript II (pKS-) (pKS- was purchased from Stratagene, 11011 North Torrey Pines Rd. La Jolla, California 92037).

pAC1558: This plasmid is made by insertion of an *Xba*I fragment of pCD220 into JT vector pSB12 and integrated into pSB1 (pSB12 and pSB1 are vectors from Japan Tobacco Inc, see US patent 5591616, American Cyanamid has a license to use these vectors). It contains the maize *ahas* promoter driving the maize XI12 mutant *ahas*2 gene and its native terminator.

Example 2: Rice transformation and selection, and characterization of transgenic rice for resistance to the imidazolinone herbicides

Protoplasts were isolated from rice varieties Nortai (Nt) and Radon (Rd) suspension cells that are gift of Dr, Thomas K. Hodges (Department of Botany and Plant Pathology, Purdue University, W. Lafayette, IN 47907) and transformed according to procedures described by Peng et al (1990, 1992). The construct (pCD220) used for the transformation, as described in Example 1, contained the mutant maize XI12 *ahas2* gene (Ser621Asn) driven by its own promoter. After transformation, the protoplasts were either cultured on Millipore filters placed on top of solid agarose medium containing feeder cells (Lee et al. 1989). The agarose used for rice culture had gelling temperature 36-42°C, and was purchased from GIBCOBRL (Grand Island, NY 14702). Alternately, the protoplasts were embedded in alginate films by mixing 1 volume of protoplast with 1 volume of 3% alginate in 7% glucose solution and cultured in the same protoplast culture medium as described above (Peng et al., 1990, 1992) with feeder cells but in liquid form. The alginate cultures were maintained in the dark on a slow shaker (40-50 rpm). The media are not critical to the process and can be varied according to the knowledge of those skilled in the field. In the agarose culture, selection for transformed cells started three weeks after transformation on

0.5 μM imazethapyr (AC263,499) and continued for 6 to 8 weeks with one or two subcultures in-between. When the alginate/liquid culture method was used, selection began 3-5 days after transformation on 0.5 μM imazethapyr and the cultures were transferred to fresh liquid medium containing 0.5 to 1 μM imazethapyr every 7 to 10 days. Seven to nine weeks after transformation, resistant colonies (about 0.5 mm in size) were picked up and placed onto LS medium (Linsmaier and Skoog, 1965) supplemented with 0.5 mg/l 2,4-D, 2% sucrose , and 0.6% agarose) (Peng et al., 1990) with the same concentration of imazethapyr and proliferated for another three weeks after which time they were transferred to fresh medium without selection reagent for another three weeks. Resistant calli were transferred to MSKT medium (MS (Murashige and Skoog, 1962) basal medium supplemented with 5 mg/l kinetin, 5 mg/l zeatin, 0.1 mg/l naphthaleneacetic acid, 3% sucrose and 0.6% agarose) for 2-3 weeks for shoot induction. Small shoots were transferred to MSO Medium (MS medium supplemented with 3% sucrose and 0.6% agarose with no plant growth regulators, Peng et al., 1992) for root formation. Two to three weeks later. plantlets were transplanted to a mixture of half soil and half Metromix 360 (The Scotts Company, Marysville, OH 43040) in 6 inch pots in the greenhouse. Plants were bagged to assure self-pollination and grown to maturity to produce seeds.

In rice, the apparent transformation efficiency based on resistant colonies recovered after selection ranged from 1 to 14 per million protoplasts treated. Three to 10 percent, with one exception, of the selected calli regenerated into plants (Table 1).

Example 3: PCR analysis of putative transgenic rice plants

DNA was isolated from individual rice plants using the well known procedure of Wang et al (1993). PCR conditions were as follows: 50 μl reaction volume containing 1X PCR Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 200 mM each deoxynucleoside triphosphate, 1.25 units of AmpliTaq DNA Polymerase (all from Perkins Elmer Applied Biosystems, Foster City, CA), and 7.5 pmoles of each primer. The reaction mixture was heated to 95^0C for 3 min, amplified using 40 cycles of 95^0C for 1 min, 55^0C for 2 min, 72^0C for 2 min, followed by incubation at 72^0C for 5 min. To identify the maize XI12 AHAS gene a forward primer 5'-AGCAGGGAGGCGGTGCTTGC-3', identified as Seq. I.D. No. 1, and reverse primer 5'-AAGGGTCAACATTCCAGCGGT-3', identified as Seq. I.D. No. 2, the primers were designed to amplify a 251 bp fragment from the 3' end of the gene. The primers were obtained from Genosys Biotechnologies, Inc., The Woodlands, Texas 77380-3600. PCR analysis verified the presence of the introduced maize *ahas* 2 gene in the T0, T1 and all T2 plants derived from a resistant line. In one example, of the 15 T1 plants assayed for the PCR reaction product, 12 and 13 were positive for the transgene in Line 26 and Line 29, respectively,

indicating that the transgene was indeed stably transmitted to some of the T1 progeny plants (Table 2). The absence of the transgene from some of the T1 plants was expected due to the hemizygous status of the transgene in the original T0 plants.

Example 4: Germination tests

T2 seeds harvested from individual T1 plants were sterilized in 50% Clorox® bleach (2.3% sodium hyperchlorite) for 30 min followed by a thorough rinse with autoclaved water and pre-germinated in water for 2-3 days. Thirty to 35 pre-germinated seeds were transferred to the surface of folded paper towels placed perpendicular to the bottom of a Magenta box (Sigma, St. Louis, MO) to which 20 ml of H2O containing 5 μM imazethapyr (AC263,499, tech grade) was added. The seedlings were grown under 12 hr light at 26^0C for about a month. Seedlings that survived the imazethapyr treatment were scored as resistant and those that died as susceptible. 5.0 μM imazethapyr, equivalent to 5 times the threshold, was used for subsequent seed germination tests of the T2 generation. T2 seeds harvested from 16 T1 lines derived from T0 plant No. 26 and those from 15 T1 lines derived from T0 plant No. 29 were screened in the seed germination tests. After 1 month treatment with 5 μM imazethapyr, all control seeds died, and resistant transgenic plants survived and continued to grow with no apparent differences from control plants treated with water. Results from the seed germination tests indicated that there were a total of 10 T1 lines producing all resistant T2 seeds; 5 were all susceptible and 14 of them had T2 seeds segregating as resistant or susceptible to the imazethapyr treatment (Table 2). Statistic analysis of data obtained from the seed germination test indicated that in Lines 26 and 29, the imazethapyr resistant trait conferred by the introduced maize XI12 *ahas* 2 gene was probably a single dominant trait inherited in a Mendelian fashion (Table 2).

Example 5: *In vitro* growth analysis of transgenic rice cells

Immature embryos (12-14 days after fertilization) were dissected from transgenic homozygous T2 plants, identified as 26-4 in Figure 1, or seed-grown Nortai plants and placed in N6 basal medium (Chiu et al., Sci. Sin 18:659-68, 1975) supplemented with 2 mg/l 2,4-D, 3% sucrose, 0.6% agarose (3SN6d2) amended with 0, 0.01, 0.033, 0.1, 0.3, 1.0, 3.3, 10, 25, or 50 μM imazethapyr (AC263,499). Five embryos were placed on one 10 x 60 mm Petri dish with two to three plates for each concentration as replications. Three weeks later, *in vitro* cellular response was monitored by measuring the fresh weight of callus derived from individual embryos.

Cell suspension cultures were initiated from immature embryo-derived calli from either transgenic homozygous T2 plant 26-4-10 or Nortai plants in 3SN6d2 liquid medium and cultured for two to three months prior to treatment with various concentrations of imazethapyr.

The suspension cells were filtered through a 40 μ nylon mesh, collected, and resuspended in 3SN6d2 liquid medium 0.2 ml of the suspended cells were loaded onto a Millipore filter, then placed on top of agarose solidified medium containing 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10, 25, or 50 μM imazethapyr (AC263,499). Three weeks later, all cultures were transferred once to fresh media with the same concentrations of imazethapyr and grown for another 5 weeks. Fresh weight was measured at the end of an 8-week culture period.

Transgenic and control embryos responded well to callus initiation on media without imazethapyr. When placed on media amended with imazethapyr, callus induction from immature embryos isolated from Nortai plants (control) was totally inhibited at 0.1 μM and growth was reduced to less than 50% at 0.03 μM with response to callus initiation. For transgenic embryos, normal callus induction and growth on media amended with imazethapyr up to 1 μM was observed, with about 50% reduction in growth at 3.3 μM and no callus induction at 10 μM (Fig. 1A). It is, therefore, apparent that the transgenic embryos had a 100-fold increase in resistance to imazethapyr *in vitro*.

Fast growing suspension cultures with cell clusters less than 40 μ in diameter were evaluated for their *in vitro* response to imazethapyr. In the absence of imazethapyr, there was no visible difference in the growth of transgenic and control cells. In the presence of imazethapyr, the growth of control cells was halted at 0.1 μm, the lowest concentration used in the experiment, and growth of transgenic suspension cells was normal up to 1.0 μm followed by a sharp decrease at 2 μm with no growth at 5 μm (Fig. 1B).

Example 6: AHAS activity in transgenic plants

In vitro AHAS assays were performed to understand the basis of herbicide tolerance in the transgenic plants. Previously published procedure was used for the extraction and *in vitro* assay for AHAS activity (Singh et al. 1988). The lower portions of the shoots were used as the source of plant tissue for the assay. The desalted crude extracts were used for the *in vitro* enzyme assays. Acetolactate produced by the enzyme was converted to acetoin, which was measured by the Westerfield assay system (Westerfield 1945). The specific activity of AHAS was about 30% higher in Line 26 (67 nmoles/mg protein/h) and Line 29 (64 nmoles/mg protein/h) compared to the activity in Nortai (Nt) (50 nmoles/mg protein/h). A significant portion of the enzyme activity from the tolerant Line 26 and Line 29 were insensitive to inhibition by imazethapyr and imazapyr (Fig. 2) when compared with the inhibition curve for the normal enzyme from Nortai plants. Interestingly, AHAS activity from all three lines was inhibited in a similar manner by chlorsulfuron (Fig. 2). This result illustrates that the maize XI12 mutant *ahas* 2 gene is selectively resistant the imidazolinone herbicides only.

Example 7: Greenhouse performances of rice plants sprayed with PURSUIT® imazethapyr

We evaluated the response of transgenic rice to PURSUIT® imazethapyr application in the greenhouse. In this experiment, we included 2 transgenic lines and their untransformed counterpart (Nt). The transgenic lines, designated as Line 26 and 29, were T3 progeny derived from previously identified homozygous resistant transgenic plants containing the maize XI12 *ahas2* gene driven by its own promoter. All seeds were pre-germinated in water for 2-3 days then grown in a planting mixture consisting of 1:1 soil and MetroMix 360 (The Scotts Company, Marysville, OH 43040) in 6-inch pots (2 seeds per pot) in the greenhouse and sprayed with PURSUIT® imazethapyr at 63, 125, or 250 g ai/ha. For post-emergence application, the plants were sprayed 15 days after seeding, at which time most plants had 3 leaves. For pre-emergence treatment, PURSUIT® imazethapyr was sprayed one day after seeding. Each treatment contained 20 plants and, and 3 replicates per treatment. One set of materials was not sprayed and used as control. The plants were grown in the greenhouse and harvested at maturity. The amount of PURSUIT® imazethapyr applied in this study (63, 125, or 250 g ai/ha) was close to 1, 2 or 4x field application rate for imi-resistant corn (refer to Table 3) since PURSUIT® imazethapyr is not currently labeled for use in rice fields. A custom designed belt sprayer was operated as described (Newhouse et al. 1992) for all herbicide applications. Five plants from each treatment were randomly chosen and individually measured for morphological traits. Means and standard deviations were calculated over three replications. Plant heights were measured from the base of the plant to the tip of the flag leaf 93 days after herbicide applications (DAH) and number of tillers per plant were counted 102-103 DAH. Data on number of panicles per plant and yield were collected after plants were harvested.

Results of this experiment, which are summarized in Tables 4 & 5, demonstrate that: (1) In the pre-emergence application, transgenic lines were resistant to all rates of PURSUIT® imazethapyr used. Control (Nortai) plants were completely killed at 125 and 250 g/ha PURSUIT® imazethapyr, and had about 20% reduction in plant height and 40% reduction in yield at 63 g/ha. Transgenic plants of Lines 26 and 29 survived at all rates applied, grew very well, and produced yields comparable to their untreated counterparts (Table 4). (2) In the post-emergence application, transgenic plants again performed well, and comparable yields were obtained from all treatments (Table 5). For the controls, 63g ai/ha PURSUIT® imazethapyr had some effect, higher rate 125 g ai/ha) caused a dramatic reduction in all the traits measured, and 250 g ai/ha killed all plants (Table 5). (3) In general, without herbicide treatment, untreated transgenic plants were shorter and had more tillers and panicles than untransformed control

plants. Untransformed Nortai plants had approximately 10% (in postemergence experiment) to 20% (in preemergence experiment) higher yields than transgenic plants.

Example 8: Response of transgenic rice plants to application of various herbicides

A total of 9 herbicides, 5 imidazolinones, two rice herbicides and ACCENT® (nicosulfuron) and CLASSIC® (clorimuron-ethyl) were applied in this study. Materials and methods were basically the same as described above, except that instead of 6" pots, flats with 18 (3" x 3" per cell) cells were used, and 10-12 seeds were planted to one 3" x 3" cell. Ten plants were maintained in one cell in the post-emergence application. Each herbicide was sprayed at 4 different rates, 1, 2, 4, and 6x, as either pre or post-emergence applications with three replications per treatment. The name and amount of herbicide applied in this experiment and information relevant to their commercial applications are listed in Table 3. For simplicity and convenience, the lowest concentration used is referred as 1x rate, 2, 4 and 6x rates can be calculated as multiples of 1x.

To monitor plant response to various herbicides, data on fresh weight was collected. For pre- emergence applications, all plants from one cell were harvested 32 days after spraying (36-day old plants) and weighed. Twenty-nine days after herbicide application, 5 plants (44-day old plants) from each cell in the post-emergence application, were cut and weighed individually. Mean fresh weight for each treatment was calculated over three replications. The remaining 5 plants were grown in the greenhouse to maturity at which time, panicles of 3 plants were collected, and number of filled seeds and number of total kernels were counted to determine percentage of seed set.

The results from visual observation and from fresh weight measurements show that overall, transgenic plants performed better in post-emergence applications than they did in pre-emergence applications, and the two rice herbicides (AC322,140 cyclosufamuron and LONDAX® bensulfuron-methyl) had a minimal effect on growth of all plants treated. In post-emergence applications, growth of transgenic plants was not affected by any of the imidazolinone herbicides -- CADRE® imazameth, ARSENAL® imazapyr, PURSUIT® imazethapyr, RAPTOR® imazamox and SCEPTER® imazaquin up to 4x and was slightly affected at 6x. ACCENT® nicosulfan and CLASSIC® clorimuron-ethyl greatly affected plant growth even at 1x rates. A similar trend was observed in the pre-emergence applications, except that growth of both transgenic lines was affected by application of 2x or higher SCEPTER® imazaquin and 6x RAPTOR® imazamox. Low rates of ACCENT® nicosulfan (1 and 2x) did not affect plant fresh weight of two transgenic and control plants greatly. Data presented in Table 6 demonstrates that: on average, plants without herbicide treatments and plants treated

with two rice herbicides (AC322,140 cyclosufamuron and LONDAX bensulfuron-methyl) had 80-98% seed-set. Control (NT) plants treated with imidazolinone herbicides at most rates (except 1x PURSUIT® imazethapyr, 1 and 2x CADRE®), ACCENT® at all rates, and CLASSIC® clorimuron-ethyl at 4 and 6x died without producing seed. PURSUIT® imazethapyr, RAPTOR® imazamox, CADRE® imazameth, SCEPTER® imazaquin (at all rates tested) and low rates of ARSENAL® imazapyr (1 to 2x) had no influence on seed set (percentage of filled seeds over total number of kernels per panicle) and total number of kernels per panicle in two transonic lines. An average of about 85% seed set was obtained from these treatments. However, with applications of 4x (96 g ai/ha) and 6x (144 g ai/ha) ARSENAL® imazapyr, seed set dropped to 20% or lower in the two transgenic lines even though growth of these plants as reflected by fresh weight, and total number of kernels per panicle was not dramatically affected (Fig. 3 and Table 6). ACCENT® nicosulfan and CLASSIC® clorimuron-ethyl affected both seed set and total number of kernels per panicle in transgenic plants.

Example 9: Corn transformation and selection

Dissect immature embryos: Depending on the size of immature embryos, collect ears at 9-12 days after pollination. Seed (HiIIA and A188) was obtained from USDA/ARS and Crop Sciences, UIUC, Urbana, IL 61801-4798. Spray 70% ethanol and 10% Lysol several times from outer to inner husks to surface sterilize the cob. Remove all the husks, cut off silks with a scalpel, and shave the top of the kernel. Immature embryos ranging from 0.8 to 1.2 mm in size were isolated under a dissecting microscope and placed into a 2.5 ml tube containing the LS-inf medium (all media for corn and wheat transformation are adapted from Ishida et al., 1996). About 100 embryos for each tube are appropriate. Vortex the immature embryos in solution gently and remove the liquid solution. Wash the immature embryos one more time in the same manner with the LS-inf medium.

Co-cultivation: Add 1 ml *Agrobacterium* cells harboring the maize X112 mutant *ahas 2* gene to the tube containing the immature embryos and gently vortex the mixture for 30 sec followed by 5 minute incubation at room temperature. Transfer the immature embryos to a Petri-dish and gently plate the immature embryos with scutellum side up and away from the medium onto LSD1.5 medium supplemented with 100-200 μM AS (acetosyringeone). Up to two hundred embryos can be plated to one plate. Seal plates with Para-film film for three days followed by porous tape (3M, St Paul MN55144) until the end of the co-cultivation stage. Incubate the immature embryos at 26^0C in the dark for 7 days. Three to four days co-cultivation was recommended by Japan Tobacco, Inc. (see Ishida et al., 1996) but 7 days was needed in our hands.

1st selection: After 7 days of co-cultivation, carefully transfer the immature embryos to fresh LSD1.5 medium supplemented with 250 mg/l cefotaxime and 0.05 μM imazethapyr (AC 263,499) in a 100x25 mm Petri-dish. Usually 25 embryos are plated on one plate. Seal the plates with vegetable tape. Grow the immature embryos at 26^{0}C in the dark for 2 to 3 weeks.

2nd selection: Looking through a dissecting microscope and using a pair of forceps pick callus cells that are actively growing and transfer to fresh medium amended with 250 mg/l cefotaxime and 0.5 μM imazethapyr. Seal the plates with produce tape (purchased from Winans McShane, Benardsville, NJ 07924). Grow the material at 26^{0}C in the dark for 3 weeks.

3rd selection: Repeat the above selection processes and grow the material for another two to three weeks under the same conditions.

Plant regeneration: For shoot induction, select callus materials that are actively growing under a dissecting microscope and transfer to LSZT5 medium (the same as LSZ in Yoshida et al.,1996), supplemented with 0.25 μM imazethapyr. Grow the callus materials at 14/10 hr light/dark and 26/24^{0}C for two to three weeks or until visible shoot formation. Transfer the plantlets to a Magenta box containing MS4RG (MS medium supplemented with 3% sucrose, 0.8% agar and without plant regulator and imazethapyr) for root formation. Transplant the plantlets with roots to pot with MetroMix 360 (The Scotts Company, Marysville, OH 43040) in a 15-cell flat and grow the plants in the greenhouse.

Spray regenerated plants: Ten to 14 days after transplanting (when the plants reach 3-4 leaf stage), spray the regenerated plants with 2x or 4x PURSUIT® imazethapyr (1x =62.5 g/ha). Two to three weeks later, take score of the experiment and transfer the surviving plants to a one-gallon pot and grow plants to flowering. Self- or cross-pollinate transgenic plants as desired. About 45 days after pollination harvest the seed. Dry seed and store properly.

Evaluation of transgenic progeny: Grow one corn plant per pot (5x5 inch) in a 3x5 flat till three- to four-leaf stage (about 9 to 13 days depending on the weather). On the day of spraying, water the plants well and let excess water drip out. Spray plants on a belt-sprayer with the imidazolinone herbicides at desired rates. Do not top water plants for three days if weather permits. When temperature was too high, base water plants to prevent wilting. Closely observe the plants for the next two to three weeks. Symptoms of herbicide injury will be visible three days after spraying and susceptible plants will die within two to three weeks depending on temperature.

The plants were rated on a scale of 0 - 5 with 0 for dead plants and 5 for healthy plants with no or minimum injury.

The transformation efficiency of corn, calculated as percent of immature embryos

produced at least one imidazolinone-resistant transgenic plant averaging about 2% and reaching as high as 16%-20% in some experiments (Table 7).

Transgenic corn plants were evaluated in the greenhouse for resistant levels to various imidazolinone herbicides and inheritance of the transgene. For corn, our protocol involved self- or cross-pollinating the transgenic plants for 2 to 3 generations and spraying T1 progeny plants to identify the resistant vs. susceptible plants. Detailed analysis for levels of herbicide resistance and the inheritance pattern. Identification of lines homozygous for the transgene is carried out in T2 generation with self-pollination and cross-pollination derived progeny plants. For example, transgenic plant 1B1 (genotype A188) which survived initial spray treatment and showed positive in PCR reaction was self-pollinated and cross-pollinated with B73 to produce T1 seed which were again self- or cross pollinated to produce T2 seed (Fig. 5). B73 and Mo17 lines were obtained USDA/ARS and Crop Sciences, UIUC, Urbana, IL 61801-4798. T1 plants were treated with 4X PURSUIT® imazethapyr (250 ai g/ha) at 3-leaf stage and rated as either resistant or susceptible to the herbicide. A total of 6 self and 8 B73x1B1 T1 plants were sprayed and all survived the herbicide treatments.

Two transgenic hybrids BAa-4 and MAa-7 lines (see Fig. 5 for their pedigree), a non-transformed control line BxA (B73 x A188), 8962IT (heterozygous XI12 imi-tolerant corn line), 8962 (wild type control) and 3395IR (homozygous XA17 imi-resistant corn line) (8962IT and 8962 were from ICI, now Garst Seed Company, Slater, IA50244, and 3395IR was from Pioneer Hi-Bred International Inc, IA50306-3453). Plants were sprayed with 4, 8, 16 or 20X PURSUIT® imazethapyr, ARSENAL® imazapyr, CADRE® imazameth or RAPTOR® imazamox. Plants were scored 0 to 5 (with 0 being dead plants, 5 being healthy plants with no injury) individually 16 days after herbicide treatments. Table 8 lists the lines and herbicide rates used and summarizes the results of this experiment. Our observation indicated that both transgenic lines showed no injury up to 16x ARSENAL® imazapyr (384 g/ha), 8x PURSUIT® imazethapyr (500 g/ha), 4x CADRE® imazameth (800 g/ha) while all control plants were dead at 4x of any tested herbicides (Fig. 5). At 4x RAPTOR® imazamox (160 g/ha), one transgenic line (MAa-7) also performed well, while growth of the other transgenic line (BAa-4) was slightly affected. For the four tested imidazolinones, transgenic plants could tolerate higher rates of ARSENAL® imazapyr, PURSUIT® imazethapyr, than CADRE® imazameth and RAPTOR® imazamox.

Example 10: Wheat transformation and selection

Sterilization and dissecting immature embryos: Durum wheat, varieties Ciccio and Colosseo obtained from Eurogen S. r. l (C. da Grottacalda, Strada Turistica, Bivio Ramata, Grottacalda km 4,500, 94015 Piazza Amerina (EN), Italy) were used for transformation. Once wheat plants start

anthesis, mark each head with a small piece of tape with the date of first flowering on it. Depending on the size of immature embryos (range from 0.5 to 1.0mm), collect wheat spikes 12 to 16 days after anthesis. Separate kernels from branches and place 100-200 kernels in a 25x100 mm Petri-dish. The kernels were sterilized and immature embryos were dissected with a scalpel under a microscope. About 100-200 embryos were placed in a micro-centrifuge tube containing 2 ml LS-inf medium and vortexed gently. The immature embryos were washed twice with LS-inf medium.

Co-cultivation: Add 1ml bacteria cells to the tube containing the immature embryos and vortex gently for 30 sec. The immature embryos were incubated in the bacterial solution for 5 min at room temperature. After incubation, the immature embryos were plated to a Petri-dish containing LSD1.5 medium supplemented with 100 μM AS with scutellum side up and away from the medium. The plates were sealed with Para-film for three days followed by porous tape for 4 days. Total incubation time for co-cultivation was 7 days.

1st selection: After co-cultivation, carefully transfer the immature embryos to fresh LSD1.5 medium supplemented with 250 mg/l cefotaxime and 0.1 μM imazamox (AC299,263) in a 100x25 mm Petri-dish. If there is formation of embryonic shoots at this stage, cut off the shoots from the embryo before transferring. Plate 25 embryos per plate. Seal plates with vegetable tape. Grow the immature embryos at 14/10 day/night with 26^0C/24^0C temperature for 2 weeks.

2nd selection: Pick up actively growing callus cells with a pair of forceps under a dissecting microscope and transfer to fresh medium amended with 250 mg/l cefotaxime and 0.3 μM imazamox (AC299,263). Grow the culture under the same conditions specified above for two weeks.

3rd selection: Repeat the above selection processes and grow the materials for two week.

Plant regeneration: Usually, there is small shoot formation already at this stage. Carefully transfer callus pieces with shoots on the surface to LSZT5 medium supplemented with 0.1 μM AC299,263. Grow the immature embryos at 14/10 day/night with 26^0C/24^0C temperature for 2 to 3 weeks. Transfer the shoots to Magenta box containing medium for root induction. Transplant the plantlets to pot mix in a 15-cell flat and grow the plants in the greenhouse.

Spray regenerated plants: About two weeks after transplanting when the plantlets reach 3- to 4-leaf stage, spray the regenerated plants with 25 mg/ha RAPTOR® imazamox. Three weeks later transplant survival plants to one-gallon pots and grow the plants to flowering. When the plants start to flowering, cover each head with a pollination-bag to ensure self-pollination. Allow plants to grow to maturity. Harvest, dry and store seed properly.

Evaluation of transgenic progeny: Sow seed to 3x5-cell flats at about 10 seed per cell. Grow the plants for about two weeks. On the day of spraying, water plants well and let excess water drip out. Spray plants on a belt-sprayer with the imidazolinone herbicides at desired rates. Do not water plants for three days. Resume regular water schedule three days after spraying. Closely observe the plants for the next two to four weeks. Symptoms of herbicide injury will be visible two weeks after spraying and susceptible plants will die four to six weeks later. We usually measure plant height and score the plant morphology with a 0 to 10 rating with 0 for dead plants and 10 for healthy plants with no or minimum injury.

Wheat transformation efficiency, calculated as percent of immature embryos produced at least one imidazolinone-resistant transgenic plant ranged from 0.4 to 3.1% (Table 7). A fertile regenerant 0407-1 generated from early transformation experiment with *Agrobacterium* (LBA4404) containing pAC1558 was evaluated for resistance to RAPTOR® imazamox post emergence. Untransformed Ciccio and Closseo plants were very sensitive to RAPTOR® imazamox and dead at very low rate. Transgenic wheat plants showed resistance to RAPTOR® imazamox over non-transformed control (Fig. 6B).

This rate (50g/ha) caused death in non-transformed Ciccio (Fig. 6). Out of the 288 T1 transgenic plants, 70 survived and 218 dead after the herbicide spray, making the ratio between resistant and sensitive plants close to 1:3. The resistant plants survived the herbicide treatment, but displayed increased tillers and 33-70% stunting in addition to delayed plant development. PCR analysis of the plant sample revealed a complete insert of the trasngene indicating that the 0407-10 plant and its progeny were transformed with the maize mutant XI12 ahas gene conferring resistant AHAS enzyme against the imidazolinone herbicides.

We also analyzed T2 progeny plants from one of the best lines of 0407-1-10. T2 seeds were planted at 10 seeds per pot and two pots per line were sprayed at 0, 10, 20, 30, 40 and 50 g/ha RAPTOR® imazamox. Most plants did show an increase in resistance over non-transformed control (Fig. 6B). For example, plant height of the resistant transgenic plants was reduced by about 25% at 20g/ha while non-transformed plants were completely killed at rate above 10 g/ha.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

Table 1. Transformation and plant regeneration efficiencies of rice protoplasts transformed with the maize mutant *ahas2* gene and selected on medium amended with 0.5 μM imazethapyr.

Expt. No	Transform No.	Protoplasts used x 10^{-6}	No. Res. calli recovered	No. transformants per 10^6 protoplasts	No. plants regenerated
1	1	25	26	1	0
1	2	25	110	4.4	3
2	1	7.5	31	4.1	1
2	2	7.5	96	12.8	11
2	3	7.5	71	9.5	8
2	4	7.5	101	13.5	10
3	1	20	85	2.1	9

Table 2. Results of PCR analysis of T1 plants and inheritance patterns of the imidazolinone resistant trait in transgenic rice plants.

T0 No.	T1 PCR+/- ratio	T2 progeny response to imazathepyr	χ^2 ratio*
26	12/3	5 lines all resistant 8 lines segregating (179 R : 49S) 3 lines all susceptible	1.31 n.s.*
29	13/2	5 lines all resistant 6 lines segregating (157R : 40S) 2 lines all susceptible	2.07 n.s.

* n.s.= the observed segregation ratio is not significantly different from expected 3:1 ratio at the 0.05 probability level when tested by χ^2 distribution.

Table 3. Summary on herbicides and amount applied for testing transgenic rice plants, and their commercial applications.

Commercial name or AC No.	Chemical name of active ingredient	Amount used as 1x rate (g ai/ha)	Field application major crop,method,rate (g ai/ha)
PURSUIT®	imazethapyr	**62.5**	Soybean, Post, 53-70; Imi-corn, Post, 70
CADRE®	imazameth	**32**	Peanut, Post, 70
ARSENAL®	imazapyr	**24**	Non-crop, Post, 560-1700
RAPTOR®	imazamox	**32**	Soybean, Post, 35-45
SCEPTER®	imazaquin	**125**	Soybean, Post, 70-140; Pre, 105-140
ACCENT®	nicosulfuron	**35**	Corn, Post, 70
CLASSIC®	chlorimuron-ethyl	**5**	Soybean, Post, 8.8-13
AC322, 140	cyclosulfamuron	**20**	Rice, Pre, 25-40
LONDAX®	bensulfuron-methyl	**68**	Rice, Pre/Post, 42-70

Table 4. Summary of morphological characteristics of rice measured for pre-emergence treatment

PURSUIT® imazethapyr		Plant height (cm)		No.tillers/plant		No. panicles/plant		Yield (g/5 plants)		
Line	g/ha	Mean	Sdv	Mean	Sdv	Mean	Sdv	Mean	Sdv	% 0x
29	0	93.5	4.2	18.1	1.8	17.9	1.3	168.3	5.5	100
	63	92.7	3.2	16.2	1.1	15.6	1.2	156.3	5.7	93
	125	95.1	5.1	14.3	2.6	16.3	1.5	166.7	12.4	99
	250	93.1	1.9	16.3	2.0	16.2	1.1	155.3	2.5	92
26	0	91.1	8.1	14.8	2.0	14.6	1.8	153.3	44.4	100
	63	92.2	4.4	14.3	1.7	13.2	0.5	155.3	2.1	101
	125	89.7	4.2	15.3	2.8	14.4	2.1	170.0	26.5	111
	250	88.5	3.3	13.3	0.9	15.7	0.4	167.0	6.9	109
Nt	0	111.8	3.4	11.3	1.0	10.1	0.3	197.3	10.8	100
	63	98.0	1.1	6.5	6.1	5.7	4.9	110.7	16.2	56
	125	0	0	0	0	0	0	0	0	0
	250	0	0	0	0	0	0	0	0	0

Table 5. Summary of morphological characteristics or rice measured for post-emergence treatments

PURSUIT® imazethapyr		Plant height (cm)		No.tillers/plant		No. panicles/plant		Yield (g/5 plants)		
Line	Rate	Mean	Sdv	Mean	Sdv	Mean	Sdv	Mean	Sdv	% 0x
29	0	113.5	1.1	30.7	8.4	27.5	8.3	213.3	33.5	100
	63	112.5	3.3	34.5	5.7	27.7	6.8	199.0	59.4	93
	125	114.1	3.0	28.3	1.0	24.5	4.8	240.7	45.2	113
	250	110.2	3.9	24.1	1.6	23.0	7.0	197.3	43.5	93
26	0	109.6	6.8	31.7	0.9	26.2	3.8	210.7	23.0	100
	63	110.1	6.0	27.1	4.6	25.3	6.0	206.7	38.2	98
	125	108.1	1.4	25.6	6.2	22.8	6.2	206.0	34.6	98
	250	110.3	2.2	25.4	4.7	21.7	4.5	202.7	50.6	96
Nt	0	127.8	4.9	20.0	4.3	20.5	3.8	229.0	33.5	100
	63	117.3	6.5	20.0	4.0	15.7	1.7	227.0	68.9	99
	125	94.5	10.3	13.5	1.6	13.9	3.6	120.0	58.5	52
	250	0	0	0	0	0	0	0	0	0

Table 6. Summary of yield data for non-transgenic and transgenic rice lines

Line	Herbicide	No. filled seeds/3 panicles					Total No. of kernels/3 panicles					% seed-set				
		0x	1x	2x	4x	6x	0x	1x	2x	4x	6x	0x	1x	2x	4x	6x
	None	154	-	-	-	-	163	-	-	-	-	95	-	-	-	-
	PURSUIT®	-	38	0	19	0	-	47	0	21	0	-	35	0	10	0
	CADRE®	-	92	76	0	0	-	94	82	0	0	-	98	83	0	0
	ARSENAL®	-	0	0	0	0	-	0	0	0	0	-	0	0	0	0
Nt	RAPTOR®	-	0	0	0	0	-	0	0	0	0	-	0	0	0	0
	SCEPTER®	-	0	0	0	0	-	0	0	0	0	-	0	0	0	0
	ACCENT®	-	6	0	0	0	-	24	0	0	0	-	3	0	0	0
	CLASSIC®	-	87	90	0	0	-	99	97	0	0	-	87	92	0	0
	AC 322,140	-	175	158	83	118	-	187	171	86	121	-	93	93	95	98
	LONDAX®	-	147	142	113	109	-	172	154	127	114	-	85	93	91	96
	None	133	-	-	-	-	142	-	-	-	-	94	-	-	-	-
	PURSUIT®	-	143	133	152	107	-	157	155	172	138	-	91	86	89	77
	CADRE®	-	133	153	124	131	-	159	161	143	169	-	84	95	87	77
	ARSENAL®	-	156	132	10	2	-	183	170	170	178	-	85	77	6	1
26	RAPTOR®	-	156	172	166	136	-	180	193	183	167	-	87	89	91	77
	SCEPTER®	-	148	87	147	86	-	172	135	166	109	-	86	64	88	62
	ACCENT®	-	82	0	0	0	-	145	0	0	0	-	34	0	0	0
	CLASSIC®	-	95	100	26	10	-	114	108	65	18	-	83	93	28	20
	AC 322,140	-	144	158	166	134	-	160	168	182	158	-	90	94	91	85
	LONDAX®	-	140	140	102	127	-	173	156	136	143	-	80	90	65	89
	None	138	-	-	-	-	160	-	-	-	-	86	-	-	-	-
	PURSUIT®	-	125	136	138	141	-	146	155	156	160	-	85	88	88	87
	CADRE®	-	154	143	126	127	-	175	166	154	159	-	88	86	82	80
	ARSENAL®	-	122	116	41	27	-	137	144	179	140	-	90	81	26	19
29	RAPTOR®	-	161	137	152	125	-	180	171	167	149	-	89	80	91	80
	SCEPTER®	-	138	143	131	139	-	159	165	155	159	-	87	86	85	88
	ACCENT®	-	94	24	0	0	-	114	48	0	0	-	82	10	0	0
	CLASSIC®	-	115	112	35	5	-	130	132	45	14	-	88	85	37	4
	AC 322,140	-	150	172	129	134	-	166	187	160	154	-	91	92	80	87
	LONDAX®	-	125	133	155	150	-	156	148	177	175	-	80	90	87	86

Table 7. Transformation efficiency of corn and wheat

Expt. #	Species	Genotype	# IE	Constructs	# IE regenerated plants	# resistant plants	TE/Trt %
1	Maize	AxHiIIA F2	100	pAC1558/L	3	2	3.0
2	Maize	AxHiIIA F1	80	PAC1558/L	13	34	16.3
3	Maize	A188	100	PAC1558/L	2	2	2.0
4	Maize	AxHiIIA F2	48	PAC1558/L	2	19	4.2
5	Maize	AxHiIIA F2	102	PAC1558/L	3	9	2.9
6	Wheat	Ciccio	180	PAC1558/L	2	2	1.1
7	Wheat	Ciccio	200	PAC1558/L	1	1	0.5
8	Wheat	Ciccio	250	PAC1558/L	1	1	0.4
9	Wheat	Ciccio	220	PAC1558/L	2	2	0.9
10	Wheat	Ciccio	124	PAC1558/L	4	4	3.1

Table 8. Performance of transgenic corn lines treated with various herbicides

Treatment	Line	Score					M.S.
Control	BxA	5	5	5	5	5	5
	BAa-4	5	5	5	5	5	5
	MAa-7	5	5	5	5	5	5
	8692	5	5	5	5	5	5
	8692IT	5	5	5	5	5	5
	3395IR	5	5	5	5	5	5
4x PURSUIT® imazethapyr 250 g/ha	BxA	0	0	0	0	0	0
	BAa-4	5	5	5	5	5	5
	MAa-7	5	5	5	5	5	5
	8692	2	2	1	1	2	1.6
	8692IT	5	5	5	5	5	5
	3395IR	5	5	5	5	5	5
8x PURSUIT® imazethapyr 500 g/ha	BxA	0	0	0	0	0	0
	BAa-4	5	5	5	5	5	5
	MAa-7	5	5	5	5	5	5
	8692	0	0	0	0	0	0
	8692IT	5	5	5	5	4	4.8
	3395IR	5	5	5	5	5	5
20 x PURSUIT® imazethapyr 1.25 kg/ha	BxA	0	0	0	0	0	0
	BAa-4	4	3	2	4	4	3.4
	MAa-7	0	3	2	4	2	2.2
	8692	0	0	0	0	0	0
	8692IT	4	3	4	4	4	3.8
	3395IR	5	5	5	5	5	5

Treatment	Line	Score					M.S.
4x RAPTOR® imazamox 160 g/ha	BxA	0	0	0	0	0	0
	BAa-4	3	4	4	4	4	3.8
	MAa-7	4	5	5	5	5	4.8
	8692	0	0	0	0	0	0
	8692IT	4	3	2	3	3	3
	3395IR	5	5	5	5	5	5
8x RAPTOR® imazamox 320 g/ha	BxA	0	0	0	0	0	0
	BAa-4	4	4	0	4	4	3.2
	MAa-7	3	2	3	4	3	3
	8692	0	0	0	0	0	0
	8692IT	3	3	2	2	/	2.5
	3395IR	5	5	5	5	5	5
16x RAPTOR® imazamox 640 g/ha	BxA	0	0	0	0	0	0
	BAa-4	3	0	0	1	0	0.8
	MAa-7	1	1	1	1	1	1
	8692	0	0	0	0	0	0
	8692IT	2	1	0	2	0	1
	3395IR	5	5	5	5	5	5

Treatment	Line	Score					M.S.
4x ARSENAL® imazapyr 96 g/ha	BxA	0	0	0	0	0	0
	BAa-4	5	5	5	5	5	5
	MAa-7	5	5	5	5	5	5
	8692	0	0	0	0	0	0
	8692IT	4	4	4	4	4	4
	3395IR	5	5	5	5	5	5
8x ARSENAL® imazapyr 192 g/ha	BxA	0	0	0	0	0	0
	BAa-4	5	5	5	5	5	5
	MAa-7	5	5	5	5	5	5
	8692	0	0	0	0	0	0
	8692IT	4	4	3	3	3	3.4
	3395IR	5	5	5	5	5	5
16x ARSENAL® imazapyr 384 g/ha	BxA	0	0	0	0	0	0
	BAa-4	5	4	5	4	4	4.4
	MAa-7	5	5	4	5	5	4.8
	8692	0	0	0	0	0	0
	8692IT	4	3	3	3	3	3.2
	3395IR	4	5	5	5	5	4.8
4x CADRE® imazameth 400 g/ha	BxA	0	0	0	0	0	0
	BAa-4	5	5	5	5	5	5
	MAa-7	5	5	4	5	5	4.8
	8692	0	0	0	0	0	0
	8692IT	4	4	5	5	4	4.4
	3395IR	5	5	5	5	5	5

Treatment	Line	Score					M.S.
8x CADRE® imazameth 800 g/ha	BxA	0	0	0	0	0	0
	BAa-4	3	3	2	2	2	2.4
	MAa-7	2	2	4	4	3	3
	8692	0	0	0	0	0	0
	8692IT	3	2	3	2	3	2.6
	3395IR	5	5	5	5	5	5
16 x CADRE® imazameth 1.6 kg/ha	BxA	0	0	0	0	0	0
	BAa-4	3	1	0	0	0	0.8
	MAa-7	0	0	1	1	1	0.6
	8692	0	0	0	0	0	0
	8692IT	1	2	1	2	2	1.6
	3395IR	4	4	5	5	5	4.6

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WHAT IS CLAIMED IS:

1. A method of using a maize mutant *ahas* 2 gene as a selectable marker, the method comprising the steps of:

(a) recombinantly transforming a plant material with a nucleic acid construct comprising the maize mutant *ahas* 2 gene;

(b) placing the transformed plant material of step (a) on a growth medium comprising at least one imidazolinone; and

(c) identifying transformed plant material capable of growth in the presence of an imidazolinone.

2. The method according to claim 1 wherein the plant material is monocot plant cells.

3. The method according to claim 2, wherein the monocot plant cells are rice protoplasts.

4. The method according to claim 2, wherein the monocot plant cells are from maize embryos.

5. The method according to claim 2, wherein the monocot plant cells are from wheat embryos.

6. The method according to claim 1, wherein the nucleic acid construct comprises the maize mutant *ahas* 2 gene linked to a suitable promoter.

7. The method according to claim 1, wherein the nucleic acid construct comprises at least one additional gene.

8. The use of the method according to claim 7 to select for a transformed plant material harboring the additional gene.

9. The use of the method according to claim 7 to measure the transformation efficiency of the additional gene.

10. The method according to claim 1, wherein the maize mutant *ahas* 2 gene is a mutant X112 *ahas* 2 gene.

11. The method according claim 1, wherein the imidazolinone is an imidazolinone herbicide selected from the group consisting of imazethapyr, imazameth, imazapyr, imazamox, and imazaquin.

12. A process of selection which comprises the use of the maize *mutant* X112 *ahas* 2 gene as a selectable marker coupled with selection on imidazolinones for new traits such as herbicide resistance.

13. A transformed plant cell produced according to the method of claim 2 which is capable of growth in the presence of an imidazolinone.

14. A transgenic plant produced from the transformed plant cell according to claim 13, wherein the transgenic plant is resistant to imidazolinones.

15. The transgenic plant according to claim 14 which exhibits resistance to at least one imidazolinone herbicide.

16. The transgenic plant according to claim 15, wherein the imidazolinone herbicide is selected from the group consisting of imazethapyr, imazameth, imazapyr, imazamox, and imazaquin.

17. A transformed rice plant protoplast produced according to the method of claim 3 which is capable of growth in the presence of an imidazolinone.

18. A transgenic rice plant produced from the transformed rice protoplast according to claim 17, wherein the transgenic rice plant is resistant to imidazolinones.

19. The transgenic rice plant according to claim 18 which exhibits resistance to at least one imidazolinone herbicide.

20. The transgenic rice plant according to claim 19, wherein the imidazolinone herbicide is selected from the group consisting of imazethapyr, imazameth, imazapyr, imazamox, and imazaquin.

21. A transformed maize embryo produced according to the method of claim 4 which is capable of growth in the presence of an imidazolinone.

22. A transgenic maize plant produced from the transformed maize embryo according to claim 21, wherein the transgenic maize plant is resistant to imidazolinones.

23. The transgenic maize plant according to claim 22 which exhibits resistance to at least one imidazolinone herbicide.

24. The transgenic maize plant according to claim 23, wherein the imidazolinone herbicide is selected from the group consisting of imazethapyr, imazameth, imazapyr, imazamox, and imazaquin.

25. A transformed wheat embryo produced according to the method of claim 5 which is capable of growth in the presence of an imidazolinone.

26. A transgenic wheat plant produced from the transformed wheat cell according to claim 25, wherein the transgenic wheat plant is resistant to imidazolinones.

27. The transgenic wheat plant according to claim 26 which exhibits resistance to at least one imidazolinone herbicide.

28. The transgenic wheat plant according to claim 27, wherein the imidazolinone herbicide is selected from the group consisting of imazethapyr, imazameth, imazapyr, imazamox, and imazaquin.

29.Viable plant materials derived from the transgenic monocot plants of claim 2.

30. Viable plant materials of claim 29 selected from the group consisting of a cell tissue, plant organ or seed.

31. A tissue culture wherein the cells or protoplasts are derived from the viable plant materials of claim 30.

32. Progeny derived from the transgenic plant of claim 14, wherein the progeny are hybrid plants which are resistant to at least one imidazolinone herbicides.

33. Progeny derived from transgenic plant of claim 14, wherein the progeny are fertile plants which are resistant to at least one imidazolinone herbicides.

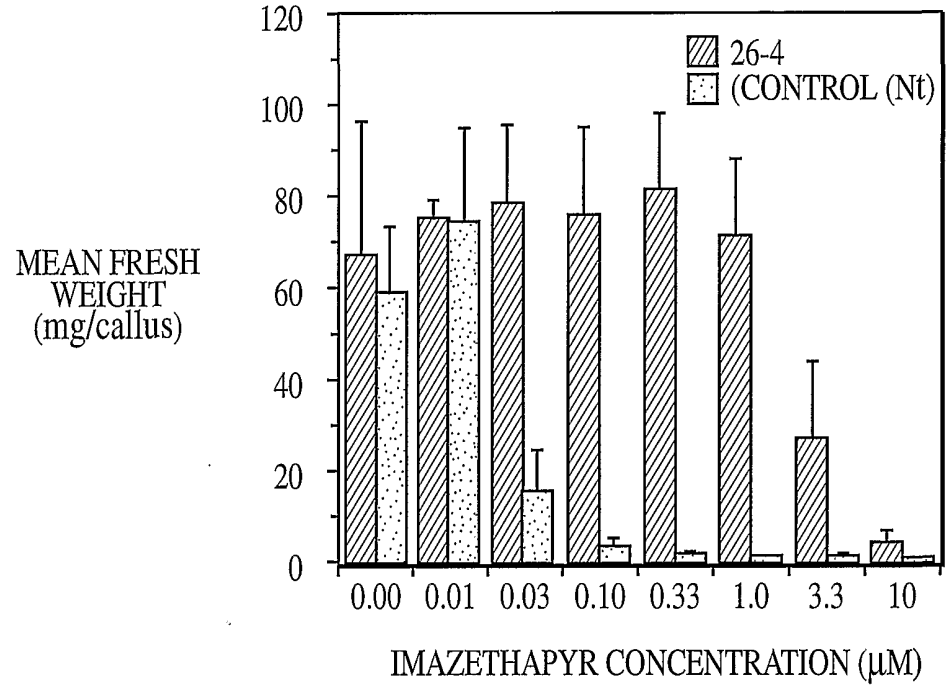
MEAN FRESH WEIGHT (mg/callus)
120
100
80
60
40
20
0
26-4
(CONTROL (Nt)
0.00 0.01 0.03 0.10 0.33 1.0 3.3 10
IMAZETHAPYR CONCENTRATION (µM)

FIG. 1A

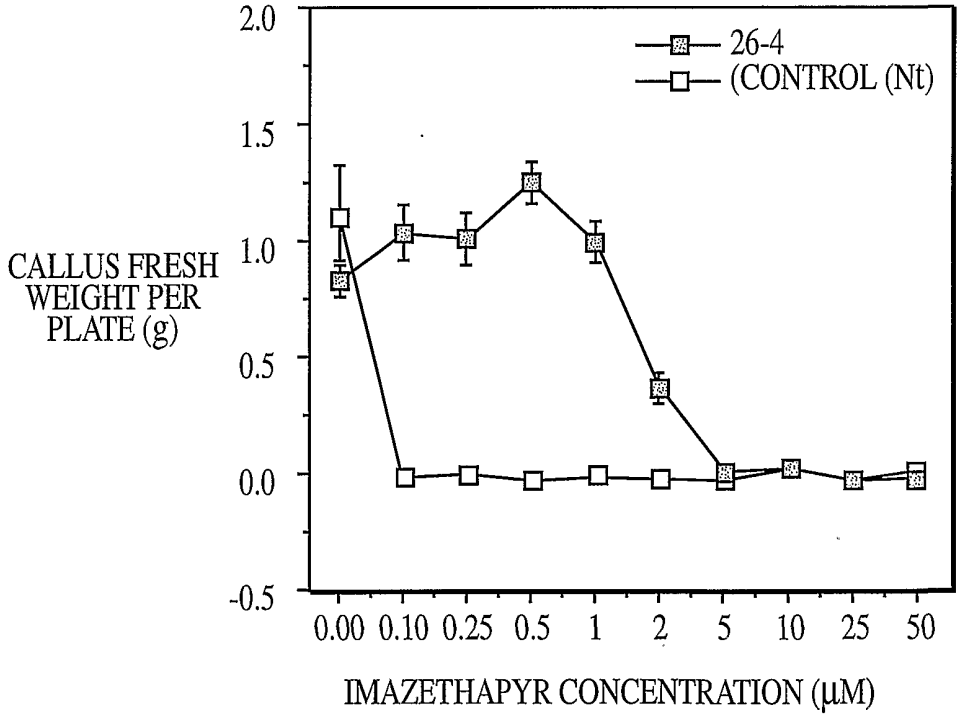
CALLUS FRESH WEIGHT PER PLATE (g)
2.0
1.5
1.0
0.5
0.0
-0.5
26-4
(CONTROL (Nt)
0.00 0.10 0.25 0.5 1 2 5 10 25 50
IMAZETHAPYR CONCENTRATION (µM)

FIG. 1B

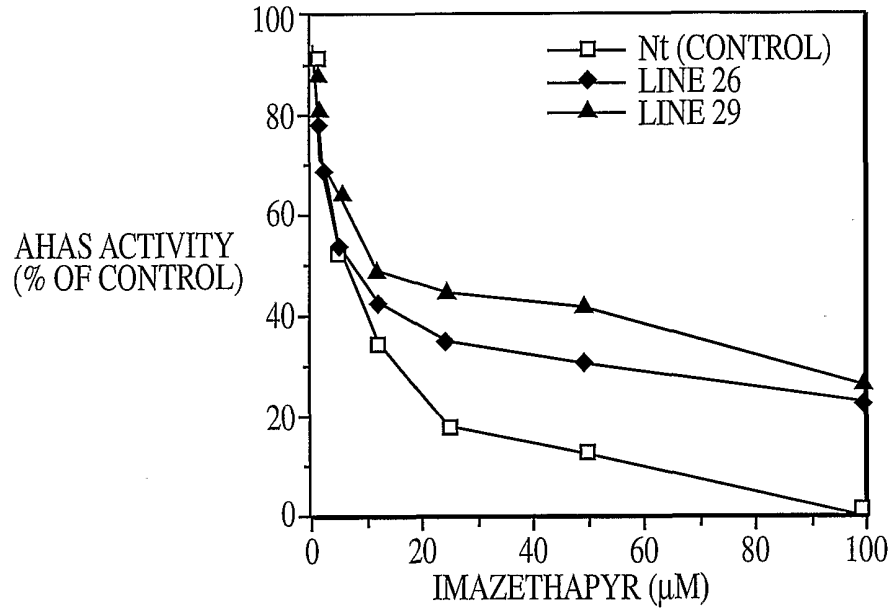

FIG. 2A

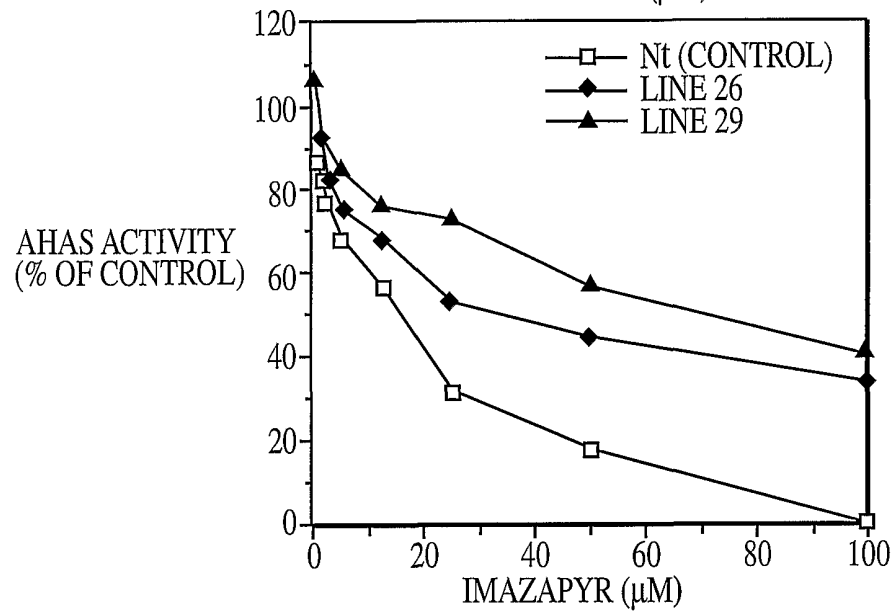

FIG. 2B

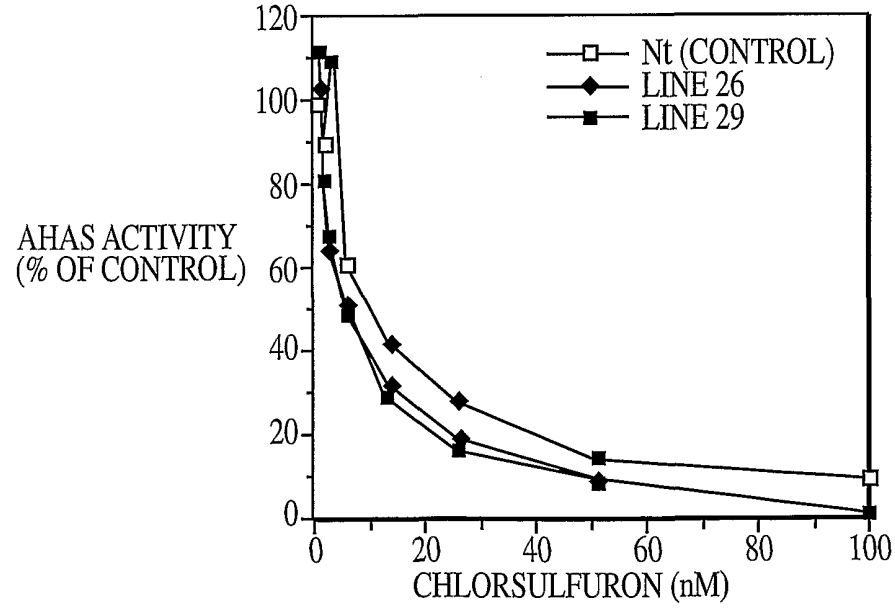

FIG. 2C

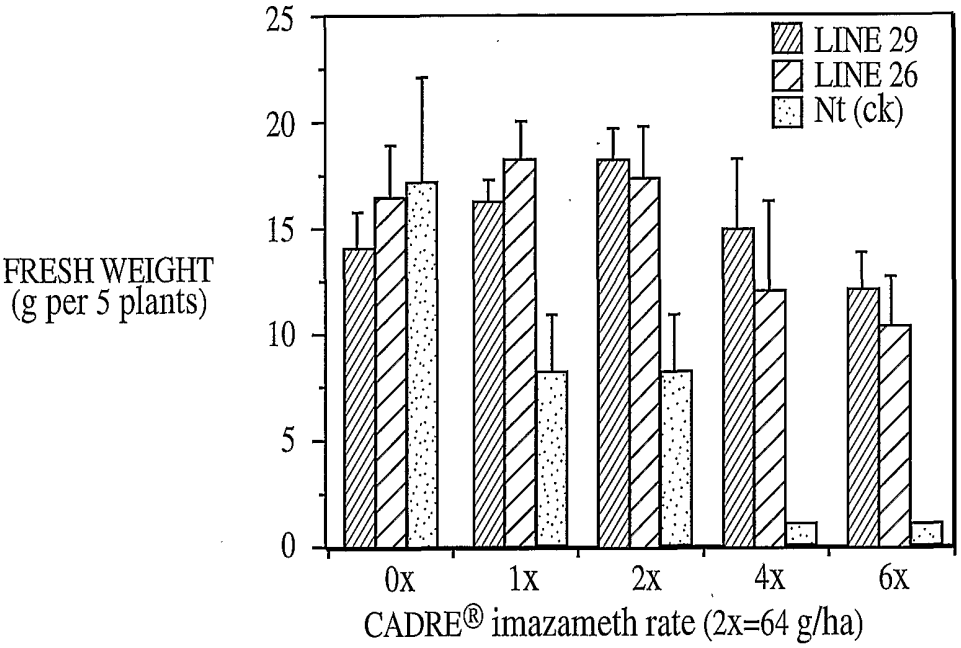
FRESH WEIGHT
(g per 5 plants)
25
20
15
10
5
0
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
CADRE® imazameth rate (2x=64 g/ha)

FIG. 3A

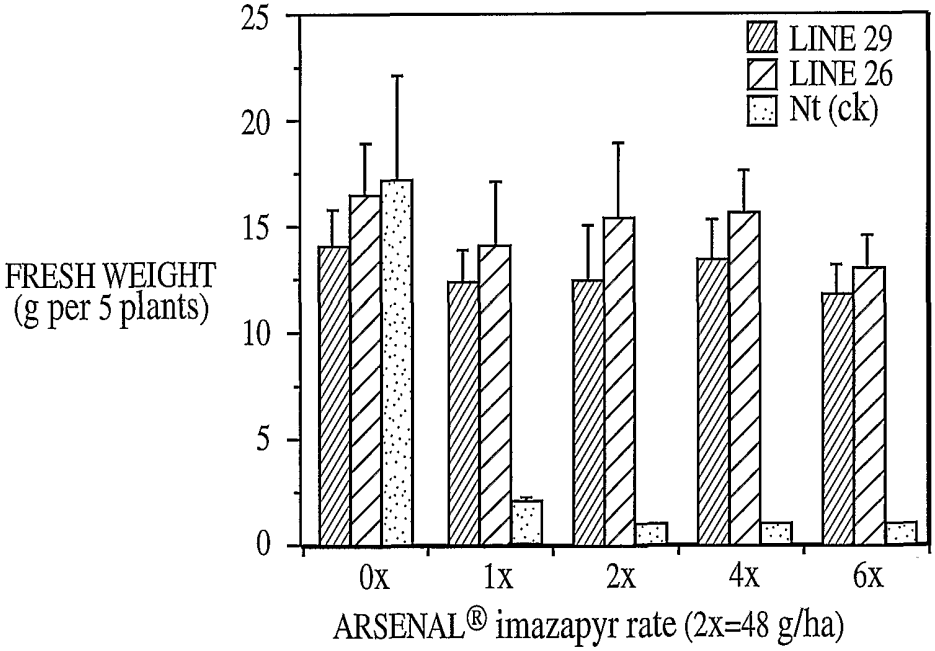
FRESH WEIGHT
(g per 5 plants)
25
20
15
10
5
0
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
ARSENAL® imazapyr rate (2x=48 g/ha)

FIG. 3B

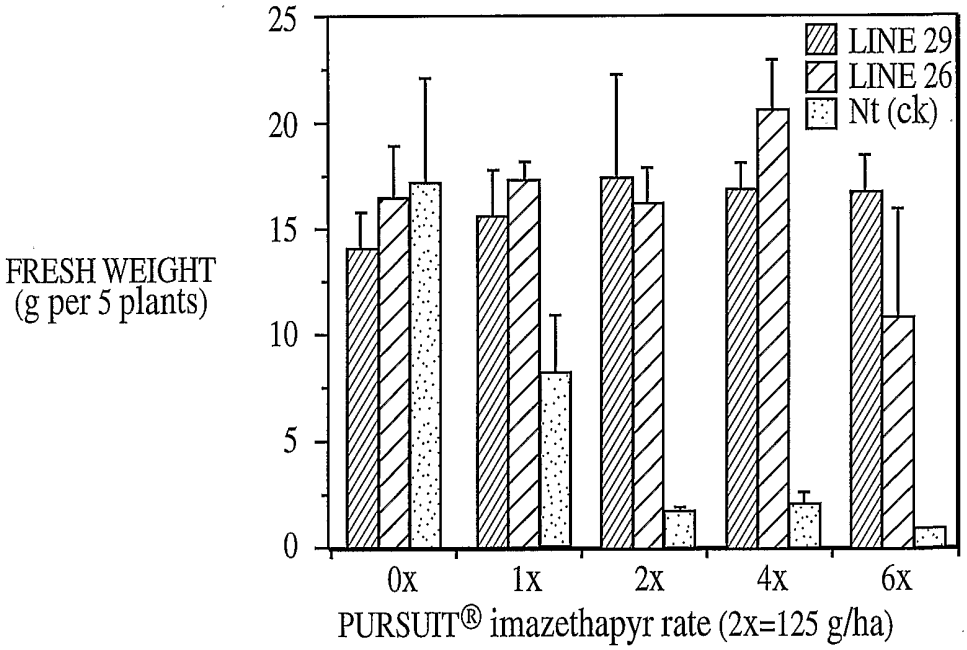
FRESH WEIGHT
(g per 5 plants)
25
20
15
10
5
0
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
PURSUIT® imazethapyr rate (2x=125 g/ha)

FIG. 3C

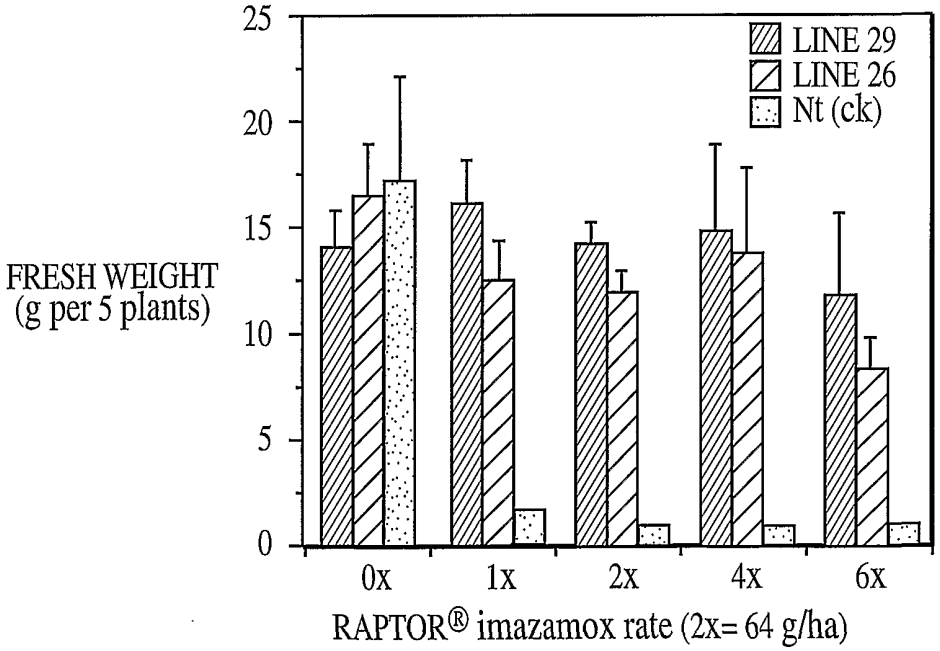
FRESH WEIGHT
(g per 5 plants)
25
20
15
10
5
0
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
RAPTOR® imazamox rate (2x= 64 g/ha)

FIG. 3D

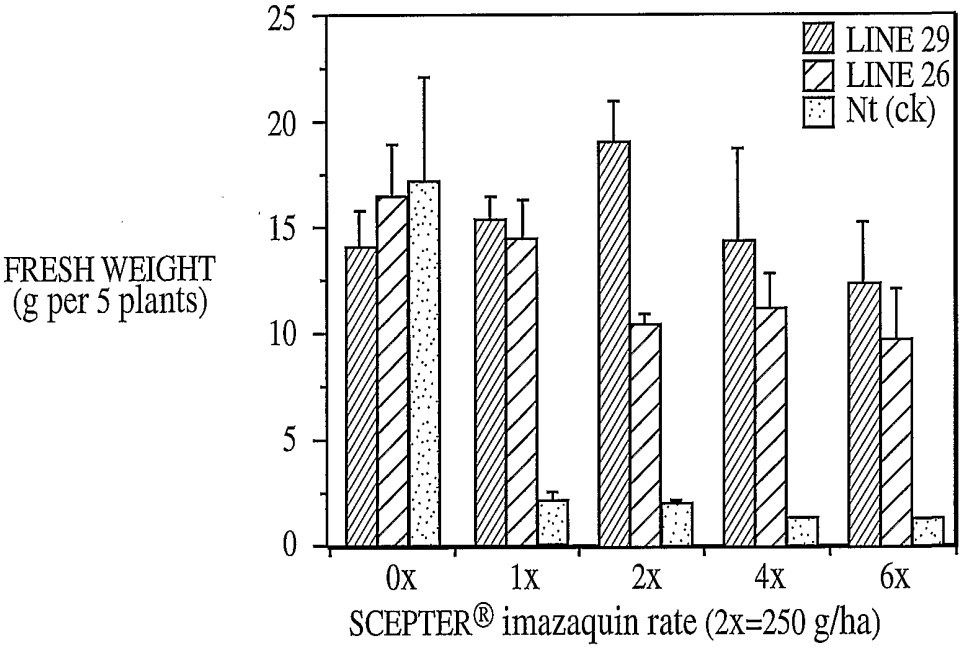
FRESH WEIGHT
(g per 5 plants)
25
20
15
10
5
0
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
SCEPTER® imazaquin rate (2x=250 g/ha)

FIG. 3E

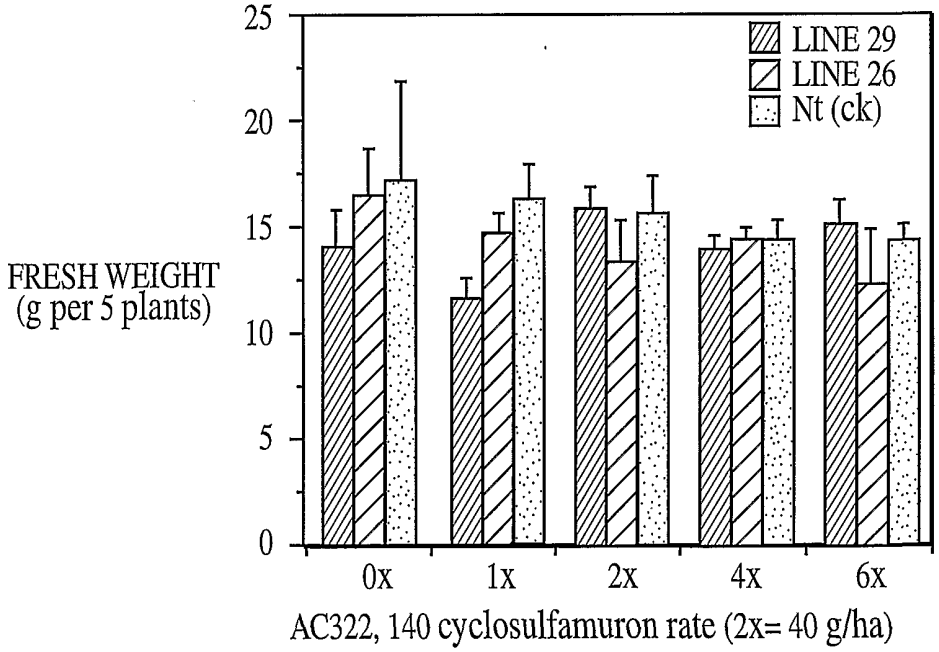
FRESH WEIGHT
(g per 5 plants)
25
20
15
10
5
0
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
AC322, 140 cyclosulfamuron rate (2x= 40 g/ha)

FIG. 3F

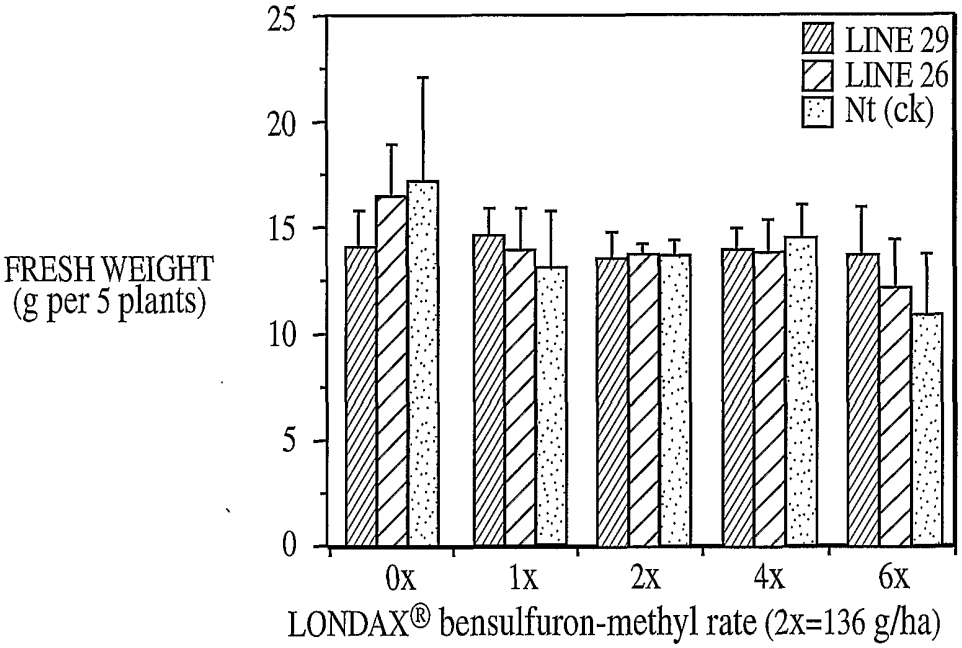
FRESH WEIGHT
(g per 5 plants)
25
20
15
10
5
0
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
LONDAX® bensulfuron-methyl rate (2x=136 g/ha)

FIG. 3G

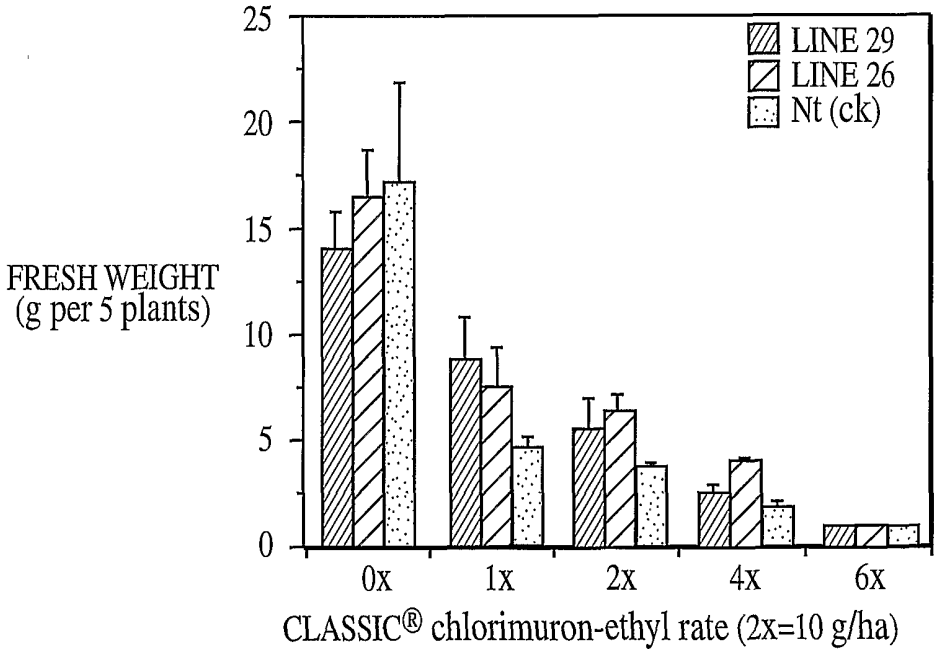
FRESH WEIGHT
(g per 5 plants)
25
20
15
10
5
0
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
CLASSIC® chlorimuron-ethyl rate (2x=10 g/ha)

FIG. 3H

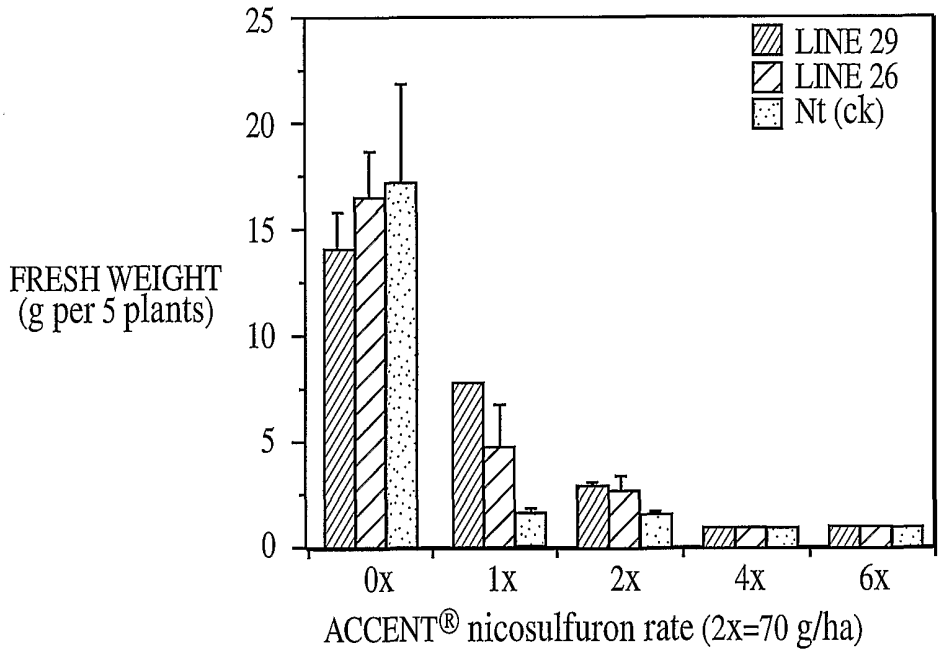
25
20
15
10
5
0
FRESH WEIGHT
(g per 5 plants)
LINE 29
LINE 26
Nt (ck)
0x
1x
2x
4x
6x
ACCENT® nicosulfuron rate (2x=70 g/ha)

FIG. 3I

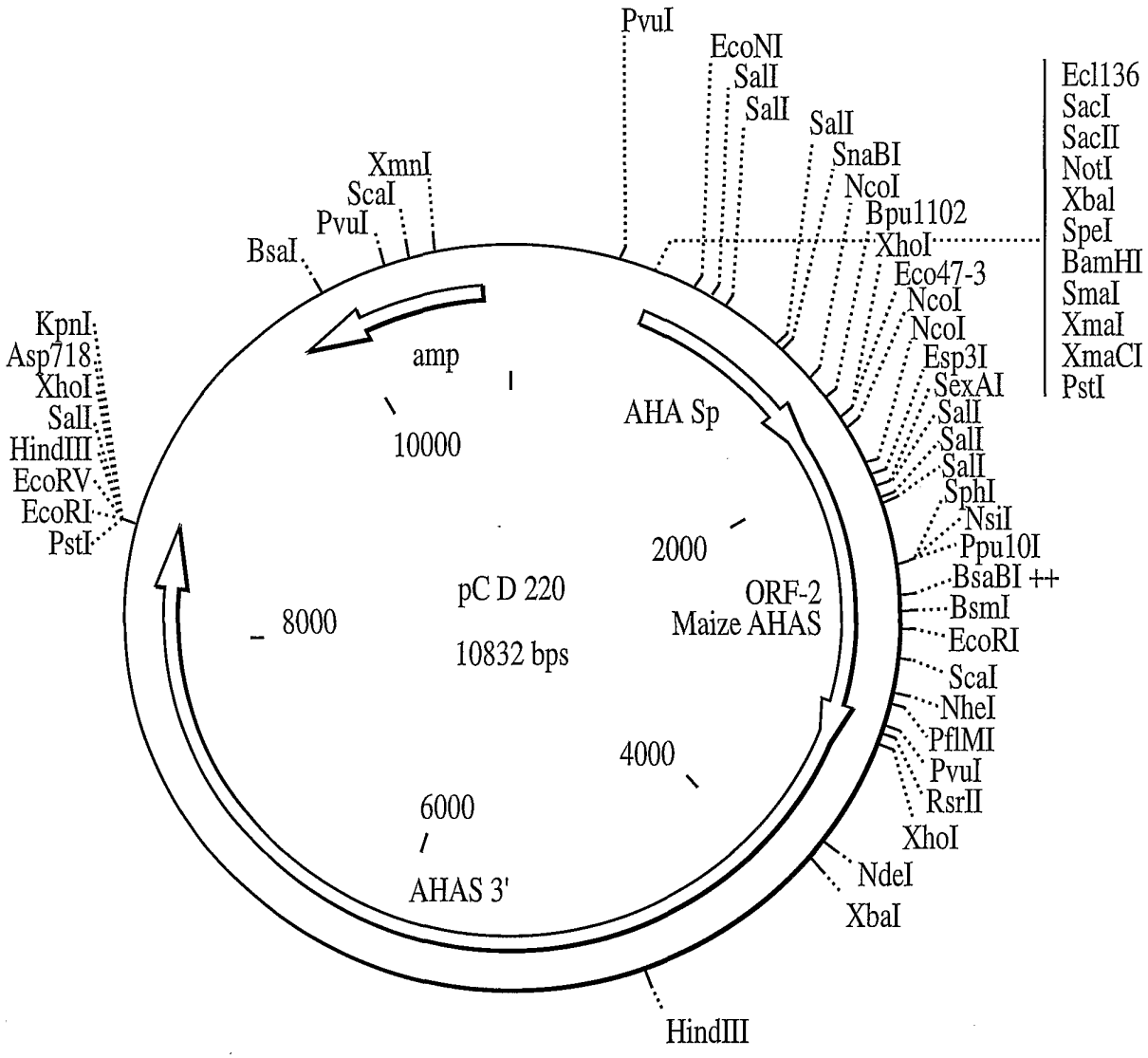
PvuI
EcoNI
SalI
SalI
SalI
SnaBI
NcoI
Bpu1102
XhoI
Eco47-3
NcoI
NcoI
Esp3I
SexAI
SalI
SalI
SalI
SphI
NsiI
Ppu10I
BsaBI ++
BsmI
EcoRI
ScaI
NheI
PflMI
PvuI
RsrII
XhoI
NdeI
XbaI
HindIII
Ecl136
SacI
SacII
NotI
XbaI
SpeI
BamHI
SmaI
XmaI
XmaCI
PstI
XmnI
ScaI
PvuI
BsaI
KpnI
Asp718
XhoI
SalI
HindIII
EcoRV
EcoRI
PstI
amp
10000
AHA Sp
2000
pC D 220
10832 bps
ORF-2
Maize AHAS
8000
4000
6000
AHAS 3'

FIG. 4A

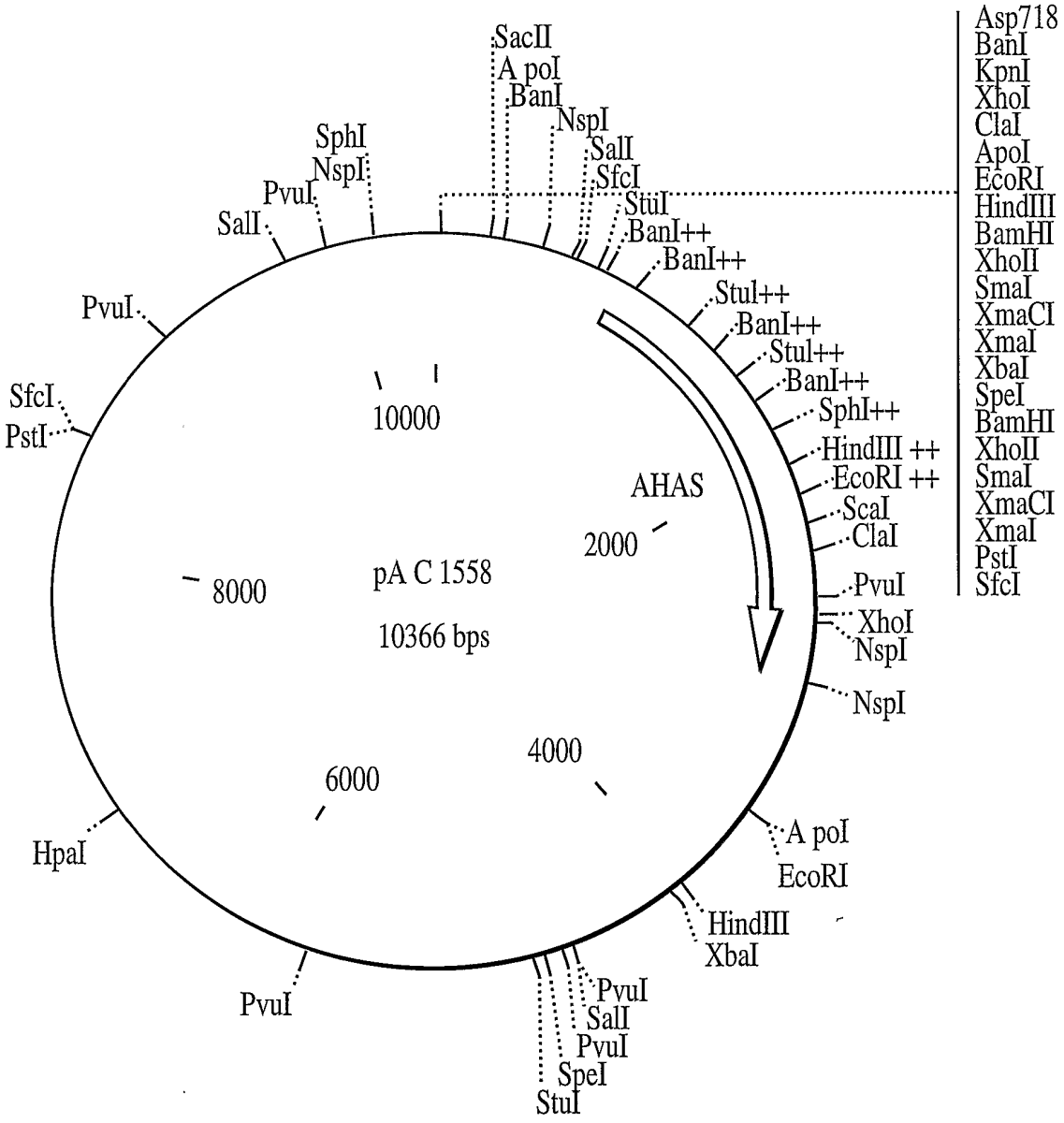
Asp718
BanI
KpnI
XhoI
ClaI
ApoI
EcoRI
HindIII
BamHI
XhoII
SmaI
XmaCI
XmaI
XbaI
SpeI
BamHI
XhoII
SmaI
XmaCI
XmaI
PstI
SfcI
SacII
A poI
BanI
NspI
SalI
SfcI
StuI
BanI++
BanI++
StuI++
BanI++
StuI++
BanI++
SphI++
HindIII ++
EcoRI ++
ScaI
ClaI
PvuI
XhoI
NspI
NspI
A poI
EcoRI
HindIII
XbaI
PvuI
SalI
PvuI
SpeI
StuI
PvuI
HpaI
PvuI
SfcI
PstI
SalI
PvuI
SphI
NspI
10000
AHAS
2000
4000
6000
8000
pA C 1558
10366 bps

FIG. 4B

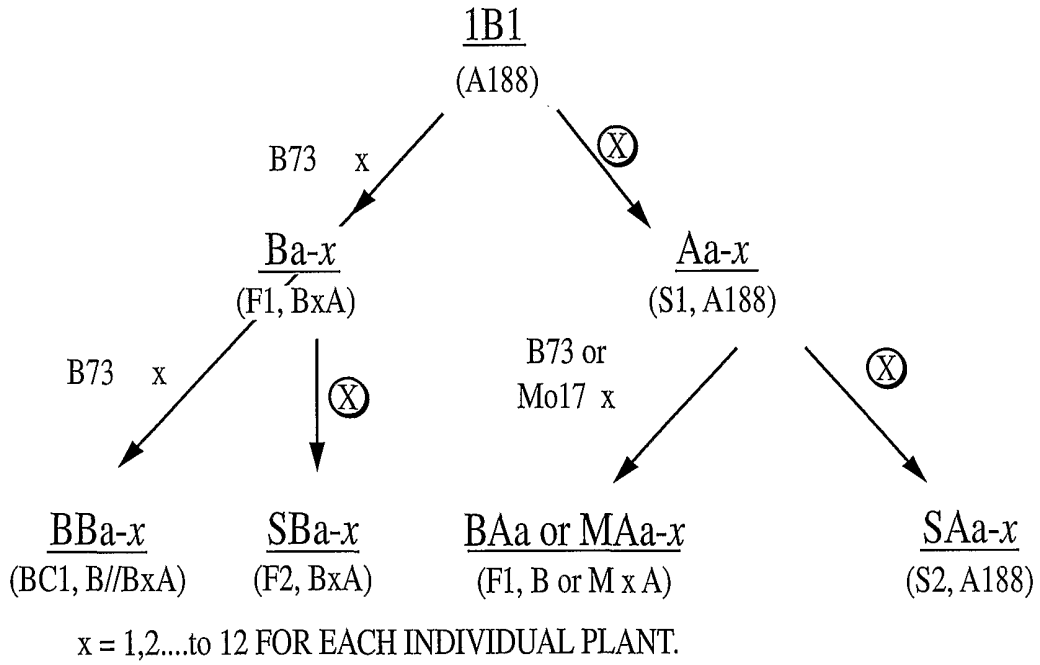
1B1
(A188)
B73 x
⊗
Ba-x
(F1, BxA)
Aa-x
(S1, A188)
B73 x
⊗
B73 or
Mo17 x
⊗
BBa-x
(BC1, B//BxA)
SBa-x
(F2, BxA)
BAa or MAa-x
(F1, B or M x A)
SAa-x
(S2, A188)
x = 1,2....to 12 FOR EACH INDIVIDUAL PLANT.

FIG. 5

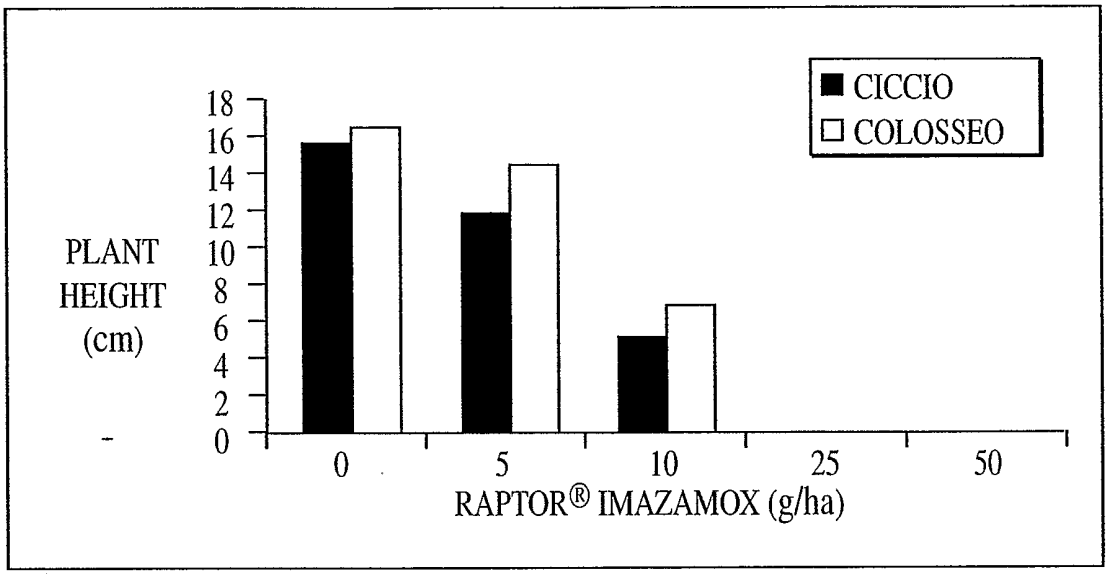
CICCIO
COLOSSEO
PLANT HEIGHT (cm)
18
16
14
12
10
8
6
4
2
0
0
5
10
25
50
RAPTOR® IMAZAMOX (g/ha)

FIG. 6A

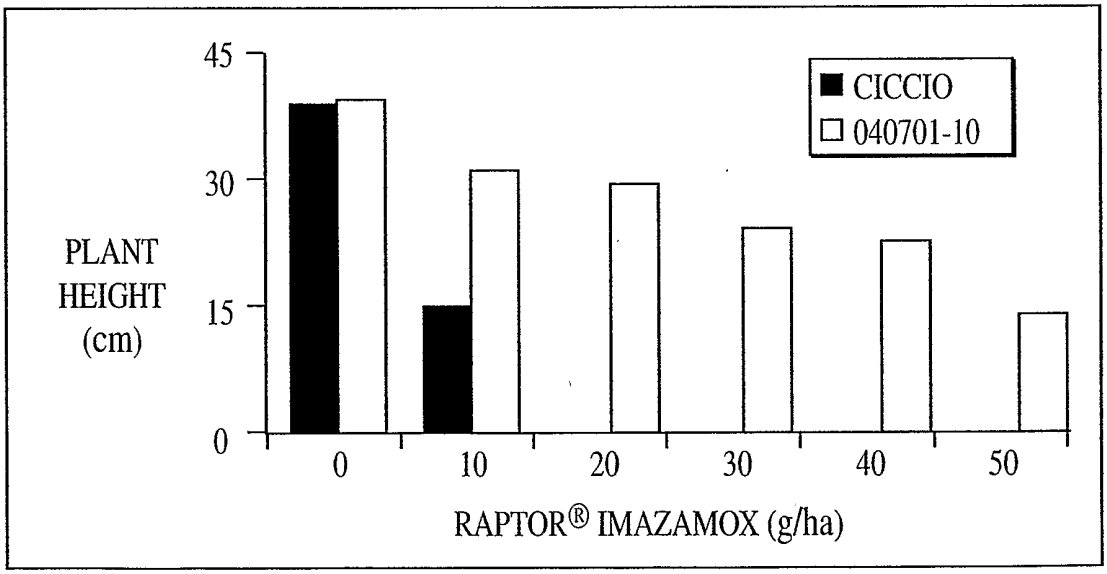
CICCIO
040701-10
PLANT HEIGHT (cm)
45
30
15
0
0
10
20
30
40
50
RAPTOR® IMAZAMOX (g/ha)

FIG. 6B

SEQUENCE LISTING

<110> AMERICAN CYANAMID COMPANY

<120> USE OF THE MAIZE X112 MUTANT AHAS GENE AS A SELECTABLE MARKER AND THE IMIDAZOLINONE HERBICIDES FOR SELECTION OF TRANSGENIC MONOCOTS, MAIZE, RICE AND WHEAT PLANTS RESISTANT TO THE IMIDAZOLINONE HERBICIDES

<130> BASF 15020

<140>
<141>

<150> 60/200,658
<151> 2000-04-24

<160> 2

<170> PatentIn Ver. 2.1

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<211> 20
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 1
agcagggagg cggtgcttgc 20

<210> 2
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 2
aagggtcaac attccagcgg t 21

WO 01/82685 INTERNATIONAL SEARCH REPORT

International PCT/US01/13650

PCT/US01/13650

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01H 5/00; C12N 5/14, 15/82

US CL : 435/418, 419; 800/278,300,300.1,320.2,320.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/418, 419; 800/278,300,300.1,320.2,320.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST (USPAT); STN (Agricola, Biosis, CaPlus, Embase)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,731,180 A (DIETRICH) 24 March 1998 (24.03.1998), see entire document.	1-16,21-24,29-33
---		---
Y		17-20,25-28
X	US 5,767,361 A (DIETRICH) 16 June 1998 (16.06.1998), see entire document.	1-16,21-24,29-33
---		---
Y		17-20,25-28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 2001 (16.06.2001)	02 AUG 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer David Kruse Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)