Attorney Docket No 035718/314461

COMPOSITIONS PROVIDING TOLERANCE TO MULTIPLE HERBICIDES AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

This invention is in the field of molecular biology. More specifically, this invention pertains to multiple herbicide tolerances conferred by expression of a sequence that confers tolerance to glyphosate in conjunction with the expression of at least one other herbicide tolerance gene.

BACKGROUND OF THE INVENTION

In the commercial production of crops, it is desirable to easily and quickly eliminate unwanted plants (*i.e.*, "weeds") from a field of crop plants. An ideal treatment would be one which could be applied to an entire field but which would eliminate only the unwanted plants while leaving the crop plants unharmed. One such treatment system would involve the use of crop plants which are tolerant to a herbicide so that when the herbicide was sprayed on a field of herbicide-tolerant crop plants, the crop plants would continue to thrive while non-herbicide-tolerant weeds were killed or severely damaged. Ideally, such treatment systems would take advantage of varying herbicide properties so that weed control could provide the best possible combination of flexibility and economy. For example, individual herbicides have different longevities in the field, and some herbicides persist and are effective for a relatively long time after they are applied to a field while other herbicides are quickly broken down into other and/or non-active compounds. An ideal treatment system would allow the use of different herbicides so that growers could tailor the choice of herbicides for a particular situation.

Crop tolerance to specific herbicides can be conferred by engineering genes into crops which encode appropriate herbicide metabolizing enzymes and/or insensitive herbicide targets. In some cases these enzymes, and the nucleic acids that encode them, originate in a plant. In other cases, they are derived from other organisms, such as microbes. *See, e.g.*, Padgette *et al.* (1996) "New weed control opportunities: Development of soybeans with a Roundup Ready® gene" and Vasil (1996) "Phosphinothricin-resistant crops," both in

Herbicide-Resistant Crops, ed. Duke (CRC Press, Boca Raton, Florida) pp. 54-84 and pp. 85-91. Indeed, transgenic plants have been engineered to express a variety of herbicide tolerance genes from a variety of organisms, including a gene encoding a chimeric protein of rat cytochrome P4507A1 and yeast NADPH-cytochrome P450 oxidoreductase (Shiota *et al.* (1994) *Plant Physiol.* 106: 17). Other genes that confer tolerance to herbicides include acetohydroxy acid synthase ("AHAS"), mutations in the native sequence have been found to confer resistance to multiple types of herbicides on plants expressing it and has been introduced into a variety of plants (*see*, *e.g.*, Hattori *et al.* (1995) *Mol. Gen. Genet.* 246: 419); glutathione reductase and superoxide dismutase (Aono *et al.* (1995) *Plant Cell Physiol.* 36: 1687); and genes for various phosphotransferases (Datta *et al.* (1992) *Plant Mol. Biol.* 20: 619).

One herbicide which has been studied extensively is N-phosphonomethylglycine, commonly referred to as glyphosate. Glyphosate is a broad spectrum herbicide that kills both broadleaf and grass-type plants due to inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (also referred to as "EPSP synthase" or "EPSPS"), an enzyme which is part of the biosynthetic pathway for the production of aromatic amino acids, hormones, and vitamins. Glyphosate-resistant transgenic plants have been produced which exhibit a commercially viable level of glyphosate resistance due to the introduction of a modified *Agrobacterium* CP4 EPSPS. This modified enzyme is targeted to the chloroplast where, even in the presence of glyphosate, it continues to synthesize EPSP from phosphoenolpyruvic acid ("PEP") and shikimate-3-phosphate. CP4 glyphosate-resistant soybean transgenic plants are presently in commercial use (*e.g.*, as sold by Monsanto under the name "Roundup Ready®").

Other herbicides of interest for commercial crop production include glufosinate (phosphinothricin) and acetolactate synthase (ALS) chemistry such as the sulfonylurea herbicides. Glufosinate is a broad spectrum herbicide which acts on the chloroplast glutamate synthase enzyme. Glufosinate-tolerant transgenic plants have been produced which carry the *bar* gene from *Streptomyces hygroscopicus*. The enzyme encoded by the *bar* gene has N-acetylation activity and modifies and detoxifies glufosinate. Glufosinate-tolerant plants are presently in commercial use (*e.g.*, as sold by Bayer under the name "Liberty Link®"). Sulfonylurea herbicides inhibit growth of higher plants by blocking acetolactate synthase (ALS). Plants containing particular mutations in ALS are tolerant to the ALS herbicides including sulfonylureas. Thus, for example, sulfonylurea herbicides such as Synchrony (a mixture of chlorimuron-ethyl plus thifensulfuron-methyl) can be used in conjunction with ALS herbicide-tolerant plants such as the STS® soybean (Synchrony

tolerant soybean) variety which contains a trait that enhances the soybean's natural tolerance to soybean sulfonylurea herbicides.

While a number of herbicide-tolerant crop plants are presently commercially available, one issue that has arisen for many commercial herbicides and herbicide/crop combinations is that individual herbicides typically have incomplete spectrum of activity against common weed species. For most individual herbicides which have been in use for some time, populations of herbicide resistant weed species and biotypes have become more prevalent (see, *e.g.*, Tranel and Wright (2002) *Weed Science* 50: 700-712; Owen and Zelaya (2005) *Pest Manag. Sci.* 61: 301-311). Transgenic plants which are resistant to more than one herbicide have been described (see, *e.g.*, WO2005/012515). However, improvements in every aspect of crop production, weed control options, extension of residual weed control, and improvement in crop yield are continuously in demand.

Particularly, due to local and regional variation in dominant weed species as well as preferred crop species, a continuing need exists for customized systems of crop protection and weed management which can be adapted to the needs of a particular region, geography, and/or locality. For example, a continuing need exists for methods of crop protection and weed management which can reduce: the number of herbicide applications necessary to control weeds in a field; the amount of herbicide necessary to control weeds in a field; the amount of tilling necessary to produce a crop; and/or programs which delay or prevent the development and/or appearance of herbicide-resistant weeds. A continuing need exists for methods of crop protection and weed management which allow the targeted use of particular herbicide combinations.

SUMMARY OF THE INVENTION

Methods and compositions relating to improved plants that are tolerant to more than one herbicide or class or subclass of herbicides are provided. Compositions include plants that are tolerant to glyphosate as well as at least one other herbicide or class or subclass of herbicide, as well as, methods of use thereof. Additional compositions comprise plants that comprise a polynucleotide encoding a polypeptide that can confer tolerance to glyphosate and a polynucleotide encoding an ALS inhibitor-tolerant polypeptide. In one non-limiting embodiment, compositions comprise a plant expressing a polynucleotide encoding a GAT (glyphosate-N-acetyltransferase) polypeptide and are tolerant to at least one additional herbicide. In some embodiments, a plant of the invention expresses a GAT polypeptide and an HRA polypeptide.

Methods for controlling weeds in an area of cultivation employing the plants of the invention are provided. Further provided are improved methods of transformation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides examples of constructs having 35S enhancer elements.

Figure 2 provides a schematic demonstrating the effect of 35S enhancers on TX efficiency.

Figure 3 provides a schematic demonstrating the effect of 35S enhancers on T0 efficiency.

Figure 4 provides a schematic demonstrating the effect of 35S enhancers on event copy number.

Figure 5 provides a table showing the effect of the 35S enhancers on T2 efficiency.

Figure 6 provides an insecticidal gene evaluation assay.

Figure 7 provides a schematic showing the development of a GAT selection scheme.

Figure 8 demonstrates that GAT can be used as a selectable marker.

Figure 9 provides a schematic demonstrating GAT transformation efficiencies.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for making and using a plant that is tolerant to more than one herbicide or class or subclass of herbicide. In some embodiments, a plant is provided that is tolerant to both glyphosate and at least one other herbicide (or class or subclass of herbicide) or another chemical (or class or subclass of another chemical). Such plants find use, for example, in methods of growing crop plants involving treatment with multiple herbicides. Thus, the invention provides improved plants which tolerate treatment with a herbicide or a combination of herbicides (including a combination of herbicides which act through different modes of action; i.e., application of mixtures having 2, 3, 4, or more modes of herbicide action) or a combination of at least one herbicide and at least one other chemical, including fungicides, insecticides, plant growth regulators and the like. In this manner, the invention provides improved methods of growing crop plants in which weeds are selectively controlled. In one embodiment, the plants of the invention comprise a polynucleotide which encodes a polypeptide that confers tolerance to glyphosate and a polynucleotide encoding an ALS inhibitor-tolerant polypeptide. As discussed in further detail below, such plants are referred to herein as "glyphosate/ALS inhibitor-tolerant plants."

The plants of the invention display a modified tolerance to herbicides and therefore allow for the application of herbicides at rates that would significantly damage plants and further allow for the application of mixtures of herbicides at lower concentrations than normally applied but which still continue to selectively control weeds. In addition, the glyphosate/ALS inhibitor-tolerant plants of the invention can be used in combination with herbicide blends technology and thereby make the application of chemical pesticides more convenient, economical, and effective for the producer. In the case of the glyphosate/ALS inhibitor-tolerant crops, the blends technology will provide easily formulated crop protection products, of ALS herbicides for example, in a dry granule form that enables delivery of customized mixtures designed to solve specific problems in conjunction with the glyphosate/ALS inhibitor-tolerant crop of the invention. With the addition of robust ALS tolerance afforded by the plants of the invention, the utility of ALS herbicides is further enabled whereby herbicidal crop response is eliminated. These uniquely selective herbicide offerings coupled with glyphosate/ALS inhibitor tolerate crops disclosed herein can now be designed and customized to meet ever-changing weed control needs. This breakthrough now enables a myriad of herbicide blends, including for example ALS inhibitor blends, that can be customized for improved weed management (since ALS inhibitor chemistries have different herbicidal attributes) including increased weed spectrum, the ability to provide specified residual activity, a second mode of action to combat or delay weed resistance (complementing glyphosate, glufosinate or the like), as well as, new offerings that can be designed either or both as pre-emergence or post-emergence. Blends also afford the ability to add or tank mix other agrochemicals at normal, labeled use rates such as additional herbicides with a 3rd or 4th mechanism of action, to fill spectrum holes or even the ability to include fungicides, insecticides, plant growth regulators and the like thereby saving costs associated with additional applications. As discussed in further detail below, the methods of the invention can be customized for a particular location or region. Improved methods of transformation are also provided.

I. Glyphosate/ALS Inhibitor-Tolerant Plants

a. Glyphosate Tolerance

Plants are provided which comprise a polynucleotide which encodes a polypeptide that confers tolerance to glyphosate and a polynucleotide encoding an ALS inhibitor-tolerant polypeptide. Various sequences which confer tolerance to glyphosate can be employed in the methods and compositions of the invention.

In one embodiment, the mechanism of glyphosate resistance is provided by the expression of a polynucleotide having transferase activity. As used herein, a "transferase" polypeptide has the ability to transfer the acetyl group from acetyl CoA to the N of glyphosate, transfer the propionyl group of propionyl CoA to the N of glyphosate, or to catalyze the acetylation of glyphosate analogs and/or glyphosate metabolites, *e.g.*, aminomethylphosphonic acid. Methods to assay for this activity are disclosed, for example, in U.S. Publication No. 2003/0083480, U.S. Publication No. 2004/0082770, and U.S. App. No. 10/835,615, filed April 29, 2004, WO2005/012515, WO2002/36782 and WO2003/092360. In one embodiment, the transferase polypeptide comprises a glyphosate-N-acetyltransferase "GAT" polypeptide.

As used herein, a GAT polypeptide or enzyme comprises a polypeptide which has glyphosate-N-acetyltransferase activity ("GAT" activity), *i.e.*, the ability to catalyze the acetylation of glyphosate. In specific embodiments, a polypeptide having glyphosate-N-acetyltransferase activity can transfer the acetyl group from acetyl CoA to the N of glyphosate. In addition, some GAT polypeptides transfer the propionyl group of propionyl CoA to the N of glyphosate. Some GAT polypeptides are also capable of catalyzing the acetylation of glyphosate analogs and/or glyphosate metabolites, *e.g.*, aminomethylphosphonic acid. GAT polypeptides are characterized by their structural similarity to one another, *e.g.*, in terms of sequence similarity when the GAT polypeptides are aligned with one another. Exemplary GAT polypeptides and the polynucleotides encoding them are known in the art and particularly disclosed, for example, in U.S. App. No. 10/004,357, filed October 29, 2001, U.S. App. No. 10/427,692, filed April 30, 2003, and U.S. App. No. 10/835,615, filed April 29, 2004, each of which is herein incorporated by reference in its entirety. In some embodiments, GAT polypeptides used in creating plants of the invention comprise the amino acid sequence set forth in: SEQ ID NO: 5, 14, 11, 8, 21, 27, 17, 24, 30, 35, 46, 47, 48, 49, 50, 51, 52, 53, 39, 42, 45, or 54. Each of these sequences is also disclosed in U.S. App. No. 10/835,615, filed April 29, 2004. In some embodiments, the corresponding GAT polynucleotides that encode these polypeptides are used; these polynucleotide sequences are set forth in SEQ ID NO: 3, 12, 9, 6, 19, 15, 25, 22, 28, 33, 4, 7, 10, 13, 16, 18, 20, 23, 26, 29, 32, 34, 36, 38, 41, 44, 43, 56, 31, 37, 40, 57, 58, 59, 60, 61, 62, 63, or 64. Each of these sequences is also disclosed in U.S. App. No. 10/835,615, filed April 29, 2004. As discussed in further detail elsewhere herein, the use of fragments and variants of GAT polynucleotides and other known herbicide-tolerance polynucleotides and polypeptides encoded thereby is also encompassed by the present invention.

In specific embodiments, the glyphosate/ALS inhibitor-tolerant plants of the invention express a GAT polypeptide, *i.e.*, a polypeptide having glyphosate-N-acetyltransferase activity wherein the acetyl group from acetyl CoA is transferred to the N of glyphosate. Thus, plants of the invention that have been treated with glyphosate contain the glyphosate metabolite N-acetylglyphosate ("NAG"). Thus, the invention also provides plants that contain NAG as well as a method for producing NAG by treating plants that contain a GAT gene (*i.e.*, that express a GAT polypeptide) with glyphosate. The presence of N-acetylglyphosate can serve as a diagnostic marker for the presence of an active GAT gene in a plant and can be evaluated by methods known in the art, for example, by mass spectrometry or by immunoassay. Generally, the level of NAG in a plant containing a GAT gene that has been treated with glyphosate is correlated with the activity of the GAT gene and the amount of glyphosate with which the plant has been treated.

The plants of the invention can comprise multiple GAT polynucleotides (i.e., at least 1, 2, 3, 4, 5, 6 or more). It is recognized that if multiple GAT polynucleotides are employed, the GAT polynucleotides may encode GAT polypeptides having different kinetic parameters, *i.e.*, a GAT variant having a lower K_m can be combined with one having a higher k_{cat}. In some embodiments, the different polynucleotides may be coupled to a chloroplast transit sequence or other signal sequence thereby providing polypeptide expression in different cellular compartments, organelles or secretion of one or more of the polypeptides.

The GAT polypeptide encoded by a GAT polynucleotide may have improved enzymatic activity in comparison to previously identified enzymes. Enzymatic activity can be characterized using the conventional kinetic parameters k_{cat}, K_M, and k_{cat}/K_M. k_{cat} can be thought of as a measure of the rate of acetylation, particularly at high substrate concentrations; K_M is a measure of the affinity of the GAT enzyme for its substrates (*e.g.*, acetyl CoA, propionyl CoA and glyphosate); and k_{cat}/K_M is a measure of catalytic efficiency that takes both substrate affinity and catalytic rate into account. k_{cat}/K_m is particularly important in the situation where the concentration of a substrate is at least partially rate-limiting. In general, a GAT with a higher k_{cat} or k_{cat}/K_M is a more efficient catalyst than another GAT with lower k_{cat} or k_{cat}/K_M. A GAT with a lower K_M is a more efficient catalyst than another GAT with a higher K_M. Thus, to determine whether one GAT is more effective than another, one can compare kinetic parameters for the two enzymes. The relative importance of k_{cat}, k_{cat}/K_M and K_M will vary depending upon the context in which the GAT will be expected to function, *e.g.*, the anticipated effective concentration of glyphosate relative to the K_M for glyphosate. GAT activity can also be characterized in terms of any of a

number of functional characteristics, including but not limited to stability, susceptibility to inhibition, or activation by other molecules.

Thus, for example, the GAT polypeptide may have a lower K_M for glyphosate than previously identified enzymes, for example, less than 1 mM, 0.9 mM, 0.8 mM, 0.7 mM, 0.6 mM, 0.5 mM, 0.4 mM, 0.3 mM, 0.2 mM, 0.1 mM, 0.05 mM, or less. The GAT polypeptide may have a higher k_{cat} for glyphosate than previously identified enzymes, for example, a k_{cat} of at least 500 min^{-1}, 1000 min^{-1}, 1100 min^{-1}, 1200 min^{-1}, 1250 min^{-1}, 1300 min^{-1}, 1400 min^{-1}, 1500 min^{-1}, 1600 min^{-1}, 1700 min^{-1}, 1800 min^{-1}, 1900 min^{-1}, or 2000 min^{-1} or higher. GAT polypeptides for use in the invention may have a higher k_{cat}/K_M for glyphosate than previously identified enzymes, for example, a k_{cat}/K_M of at least 1000 $mM^{-1}min^{-1}$, 2000 $mM^{-1}min^{-1}$, 3000 $mM^{-1}min^{-1}$, 4000 $mM^{-1}min^{-1}$, 5000 $mM^{-1}min^{-1}$, 6000 $mM^{-1}min^{-1}$, 7000 $mM^{-1}min^{-1}$, or 8000 $mM^{-1}min^{-1}$, or higher. The activity of GAT enzymes is affected by, for example, pH and salt concentration; appropriate assay methods and conditions are known in the art (see, *e.g.*, WO2005012515). Such improved enzymes may find particular use in methods of growing a crop in a field where the use of a particular herbicide or combination of herbicides and/or other agricultural chemicals would result in damage to the plant if the enzymatic activity (*i.e.*, k_{cat}, K_M, or k_{cat} / K_M) were lower.

Glyphosate-tolerant plants can also be produced by modifying the plant to increase the capacity to produce a higher level of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) as more fully described in U.S. Patent Nos. 6,248,876; 5,627,061; 5,804,425; 5,633,435; 5,145,783; 4,971,908; 5,312,910; 5,188,642; 4,940,835; 5,866,775; 6,225,114; 6,130,366; 5,310,667; 4,535,060; 4,769,061; 5,633,448; 5,510,471; Re. 36,449; RE 37,287 E; and 5,491,288; and international publications WO 97/04103; WO 00/66746; WO 01/66704; and WO 00/66747, which are incorporated herein by reference in their entireties for all purposes. Glyphosate resistance can also be imparted to plants that express a gene that encodes a glyphosate oxido-reductase enzyme as described more fully in U.S. Patent Nos. 5,776,760 and 5,463,175, which are incorporated herein by reference in their entireties for all purposes. Additionally, glyphosate tolerant plants can be generated through the selection of naturally occurring mutations that impart tolerance to glyphosate.

It is recognized that the methods and compositions of the invention can employ any combination of sequences (i.e., sequences that act via the same or different modes) that confer tolerance to glyphosate known in the art to produce plants and plant explants with superior glyphosate resistance.

b. Acetolactate Synthase (ALS) Inhibitor Tolerance

Glyphosate/ALS inhibitor-tolerant plants are provided which comprise a polynucleotide which encodes a polypeptide that confers tolerance to glyphosate and further comprise a polynucleotide encoding an acetolactate synthase (ALS) inhibitor-tolerant polypeptide. As used herein, an "ALS inhibitor-tolerant polypeptide" comprises any polypeptide which when expressed in a plant confers tolerance to at least one ALS inhibitor. A variety of ALS inhibitors are known and include, for example, sulfonylurea, imidazolinone, triazolopyrimidines, pryimidinyoxy(thio)benzoates, and/or sulfonylaminocarbonyltriazolinone herbicide. Additional ALS inhibitors are known and are disclosed elsewhere herein. It is known in the art that ALS mutations fall into different classes with regard to tolerance to sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidinyl(thio)benzoates, including mutations having the following characteristics: (1) broad tolerance to all four of these groups; (2) tolerance to imidazolinones and pyrimidinyl(thio)benzoates; (3) tolerance to sulfonylureas and triazolopyrimidines; and (4) tolerance to sulfonylureas and imidazolinones.

Various ALS inhibitor-tolerant polypeptides can be employed. In some embodiments, the ALS inhibitor-tolerant polynucleotides contain at least one nucleotide mutation resulting in one amino acid change in the ALS polypeptide. In specific embodiments, the change occurs in one of seven substantially conserved regions of acetolactate synthase. See, for example, Hattori *et al.* (1995) *Molecular Genetics and Genomes* 246:419-425; Lee *et al.* (1998) *EMBO Journal* 7:1241-1248; Mazur *et al.* (1989) *Ann. Rev. Plant Phys.* 40:441-470; and U.S. Patent No. 5,605,011, each of which is incorporated by reference in their entirety. The ALS inhibitor-tolerant polypeptide can be encoded by, for example, the SuRA or SuRB locus of ALS. In specific embodiments, the ALS inhibitor-tolerant polypeptide comprises the C3 ALS mutant, the HRA ALS mutant, the S4 mutant or the S4/HRA mutant or any combination thereof. Different mutations in ALS are known to confer tolerance to different herbicides and groups (and/or subgroups) of herbicides; see, e.g., Tranel and Wright (2002) *Weed Science* 50:700-712. See also, U.S. Patent No. 5,605,011, 5,378,824, 5,141,870, and 5,013,659, each of which is herein incorporated by reference in their entirety. See also, SEQ ID NO:65 comprising a soybean HRA sequence; SEQ ID NO:66 comprising a maize HRA sequence; SEQ ID NO:67 comprising an *Arabidopsis* HRA sequence; and SEQ ID NO:86 comprising an HRA sequence used in cotton. The HRA mutation in ALS finds particular use in one embodiment of the invention. The mutation results in the production of an acetolactate synthase polypeptide which is resistant to at least one ALS inhibitor chemistry in comparison

to the wild-type protein. For example, a plant expressing an ALS inhibitor-tolerant polypeptide may be tolerant of a dose of sulfonylurea, imidazolinone, triazolopyrimidines, pryimidinyloxy(thio)benzoates, and/or sulfonylaminocarbonyltriazolinone herbicide that is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 50, 70, 80, 100, 125, 150, 200, 500, or 1000 times higher than a dose of the herbicide that would cause damage to an appropriate control plant. In some embodiments, an ALS inhibitor-tolerant polypeptide comprises a number of mutations. Additionally, plants having an ALS inhibitor polypeptide can be generated through the selection of naturally occurring mutations that impart tolerance to glyphosate.

In some embodiments, the ALS inhibitor-tolerant polypeptide confers tolerance to sulfonylurea and imidazolinone herbicides. Sulfonylurea and imidazolinone herbicides inhibit growth of higher plants by blocking acetolactate synthase (ALS), also known as, acetohydroxy acid synthase (AHAS). For example, plants containing particular mutations in ALS (*e.g.*, the S4 and/or HRA mutations) are tolerant to sulfonylurea herbicides. The production of sulfonylurea-tolerant plants and imidazolinone-tolerant plants is described more fully in U.S. Patent Nos. 5,605,011; 5,013,659; 5,141,870; 5,767,361; 5,731,180; 5,304,732; 4,761,373; 5,331,107; 5,928,937; and 5,378,824; and international publication WO 96/33270, which are incorporated herein by reference in their entireties for all purposes. In specific embodiments, the ALS inhibitor-tolerant polypeptide comprises a sulfonamide-tolerant acetolactate synthase (otherwise known as a sulfonamide-tolerant acetohydroxy acid synthase) or an imidazolinone-tolerant acetolactate synthase (otherwise known as an imidazolinone-tolerant acetohydroxy acid synthase).

A plant of the invention that comprises at least one sequence which confers tolerance to glyphosate and at least one sequence which confers tolerance to an ALS inhibitor is referred to herein as a "glyphosate/ALS inhibitor-tolerant plant." A plant of the invention that contains at least one GAT polypeptide and at least one HRA polypeptide is referred to herein as a "GAT-HRA plant."

c. Additional Herbicide Tolerance

In some embodiments, plants are provided having enhanced tolerance to glyphosate and at least one ALS inhibitor herbicide, as well as, tolerance to at least one additional herbicide. In specific embodiments, tolerance to the additional herbicide is due to the expression of at least one polypeptide imparting tolerance to the additional herbicide. In some embodiments, a composition of the invention (*e.g.*, a plant) may comprise two, three,

four, five, six, seven, or more traits which confer tolerance to at least one herbicide, so that a plant of the invention may be tolerant to at least two, three, four, five, six, or seven or more different types of herbicides. Thus, a plant of the invention that is tolerant to more than two different herbicides may be tolerant to herbicides that have different modes of action and/or different sites of action. In some embodiments, all of these traits are transgenic traits, while in other embodiments, at least one of these traits is not transgenic.

In some of these embodiments, each herbicide tolerance gene confers tolerance to a different herbicide or class or subclass of herbicides. In some of these embodiments, at least two of the herbicide tolerance genes confer tolerance to the same herbicide or to members of the same class or subclass of herbicides. Accordingly, further provided are plants having a polynucleotide that encodes a polypeptide which can confer tolerance to glyphosate and a polynucleotide that encodes an ALS inhibitor-tolerant polypeptide can further comprise at least one additional herbicide-tolerance polynucleotide which when expressed imparts tolerance to an additional herbicide. Such additional herbicides, include but are not limited to, an acetyl Co-A carboxylase inhibitor such as quizalofop-P-ethyl, a synthetic auxin such as quinclorac, a protoporphyrinogen oxidase (PPO) inhibitor herbicide (such as sulfentrazone), a pigment synthesis inhibitor herbicide such as a hydroxyphenylpyruvate dioxygenase inhibitor (*e.g.*, mesotrione or sulcotrione), a phosphinothricin acetyltransferase or a phytoene desaturase inhibitor like diflufenican or pigment synthesis inhibitor. It is understood that the invention is not bound by the mechanism of action of a herbicide, so long as the goal of the invention (*i.e.*, herbicide tolerance to glyphosate and at least on ALS inhibitor) is achieved. Additional herbicides of interest are disclosed elsewhere herein.

In some embodiments, the compositions of the invention further comprise polypeptides conferring tolerance to herbicides which inhibit the enzyme glutamine synthase, such as phosphinothricin or glufosinate (*e.g.*, the *bar* gene or *pat* gene). Glutamine synthetase (GS) appears to be an essential enzyme necessary for the development and life of most plant cells, and inhibitors of GS are toxic to plant cells. Glufosinate herbicides have been developed based on the toxic effect due to the inhibition of GS in plants. These herbicides are non-selective; that is, they inhibit growth of all the different species of plants present. The development of plants containing an exogenous phosphinothricin acetyltransferase is described in U.S. Patent Nos. 5,969,213; 5,489,520; 5,550,318; 5,874,265; 5,919,675; 5,561,236; 5,648,477; 5,646,024; 6,177,616; and 5,879,903, which are incorporated herein by reference in their entireties for all purposes. Mutated phosphinothricin acetyltransferase having this activity are also disclosed.

In still other embodiments, the compositions of the invention further comprise polypeptides conferring tolerance to herbicides which inhibit protox (protoporphyrinogen oxidase). Protox is necessary for the production of chlorophyll, which is necessary for all plant survival. The protox enzyme serves as the target for a variety of herbicidal compounds. These herbicides also inhibit growth of all the different species of plants present. The development of plants containing altered protox activity which are resistant to these herbicides are described in U.S. Patent Nos. 6,288,306; 6,282,837; and 5,767,373; and international publication WO 01/12825, which are incorporated herein by reference in their entireties for all purposes.

In still other embodiments, compositions of the invention may comprise polypeptides involving other modes of herbicide resistance. For example, hydroxyphenylpyruvatedioxygenases are enzymes that catalyze the reaction in which para-hydroxyphenylpyruvate (HPP) is transformed into homogentisate. Molecules which inhibit this enzyme and which bind to the enzyme in order to inhibit transformation of the HPP into homogentisate are useful as herbicides. Plants more resistant to certain herbicides are described in U.S. Patent Nos. 6,245,968; 6,268,549; and 6,069,115; and international publication WO 99/23886, which are incorporated herein by reference in their entireties for all purposes. Mutated hydroxyphenylpyruvatedioxygenase having this activity are also disclosed.

d. Fragments and Variants of Sequences that Confer Herbicide Tolerance

Depending on the context, "fragment" refers to a portion of the polynucleotide or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a polynucleotide may encode protein fragments that retain the biological activity of the original protein and hence confer tolerance to a herbicide. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length polynucleotide encoding a herbicide-tolerance polypeptide.

A fragment of a herbicide-tolerance polynucleotide that encodes a biologically active portion of a herbicide-tolerance polypeptide will encode at least 15, 25, 30, 50, 100, 150, 200, or 250 contiguous amino acids, or up to the total number of amino acids present in a full-length herbicide-tolerance polypeptide. A biologically active portion of a herbicide-tolerance polypeptide can be prepared by isolating a portion of a herbicide-tolerance polynucleotide, expressing the encoded portion of the herbicide-tolerance polypeptide (*e.g.*, by recombinant expression *in vitro*), and assessing the activity of the encoded portion of the herbicide-

tolerance polypeptide. Polynucleotides that are fragments of a herbicide-tolerance polynucleotide comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, or 1,400 contiguous nucleotides, or up to the number of nucleotides present in a full-length herbicide-tolerance polynucleotide.

The term "variants" refers to substantially similar sequences. For polynucleotides, a variant comprises a polynucleotide having deletions (*i.e.*, truncations) at the 5′ and/or 3′ end; deletion and/or addition of one or more nucleotides at one or more internal sites in the native polynucleotide; and/or substitution of one or more nucleotides at one or more sites in the native polynucleotide. As used herein, a "native" polynucleotide or polypeptide comprises a naturally-occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of a herbicide-tolerance polypeptide. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques. Variant polynucleotides also include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis or "shuffling." Generally, variants of a particular polynucleotide have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters as described elsewhere herein.

Variants of a particular polynucleotide (*i.e.*, the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides of the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

"Variant" protein is intended to mean a protein derived from a native and/or original protein by deletion (so-called truncation) of one or more amino acids at the N-terminal and/or C-terminal end of the protein; deletion and/or addition of one or more amino acids at one or more internal sites in the protein; or substitution of one or more amino acids at one or more sites in the protein. Variant proteins encompassed by the present invention are biologically

active, that is they continue to possess the desired herbicide-tolerance activity as described herein. Biologically active variants of a herbicide-tolerance polypeptide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a herbicide-tolerance polypeptide may differ from that polypeptide by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue. Variant herbicide-tolerance polypeptides, as well as polynucleotides encoding these variants, are known in the art.

Herbicide-tolerance polypeptides may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants and fragments of herbicide-tolerance polypeptides can be prepared by mutations in the encoding polynucleotide. Methods for mutagenesis and polynucleotide alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82: 488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154: 367-382; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be made. One skilled in the art will appreciate that the activity of a herbicide-tolerance polypeptide can be evaluated by routine screening assays. That is, the activity can be evaluated by determining whether a transgenic plant has an increased tolerance to a herbicide, for example, as illustrated in working Example 1, or with an *in vitro* assay, such as the production of N-acetylglyphosphate from glyphosate by a GAT polypeptide (see, *e.g.*, WO 02/36782).

Variant polynucleotides and polypeptides also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different herbicide-tolerance polypeptide coding sequences can be manipulated to create a new herbicide-tolerance polypeptide possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have

substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between a herbicide-tolerance polypeptide and other known genes to obtain a new gene coding for a protein with an improved property of interest, such as an increased K_m in the case of an enzyme. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91: 10747-10751; Stemmer (1994) *Nature* 370: 389-391; Crameri *et al.* (1997) *Nature Biotech.* 15: 436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272: 336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA* 94: 4504-4509; Crameri *et al.* (1998) *Nature* 391: 288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The following terms are used to describe the sequence relationships between two or more polynucleotides or polypeptides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", and, (d) "percentage of sequence identity."

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two polynucleotides. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4: 11-17; the local alignment algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443-453; the search-for-local alignment method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85: 2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87: 2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90: 5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys Inc., 9685 Scranton Road, San Diego, California, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73: 237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5: 151-153; Corpet *et al.* (1988) *Nucleic Acids Res.* 16:10881-90; Huang *et al.* (1992) *CABIOS* 8: 155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24: 307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra.* A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra.* BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra.* When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (*e.g.*, BLASTN for nucleotide sequences, BLASTX for proteins) can be used. BLAST software is publicly available on the NCBI website. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical

nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the GCG Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the GCG Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89: 10915).

(c) As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (*e.g.*, charge or hydrophobicity) and therefore do not change

the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, *e.g.*, as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

The use of the term "polynucleotide" is not intended to be limited to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides can comprise ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. Thus, polynucleotides also encompass all forms of sequences including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

e. Herbicide Tolerance

A "herbicide" is a chemical that causes temporary or permanent injury to a plant. Non-limiting examples of herbicides that can be employed in the various methods and compositions of the invention are discussed in further detail elsewhere herein. A herbicide may be incorporated into the plant, or it may act on the plant without being incorporated into the plant or its cells. An "active ingredient" is the chemical in a herbicide formulation

primarily responsible for its phytotoxicity and which is identified as the active ingredient on the product label. Product label information is available from the U.S. Environmental Protection Agency and is updated online at the url oaspub.epa.gov/pestlabl/ppls.own; product label information is also available online at the url www.cdms.net. The term "acid equivalent" expresses the rate or quantity as the herbicidally active parent acid. For example, 2,4-D acid is often formulated in the form of a sodium or amine salt or an ester as the active ingredient in formulated products. The active acid equivalent per gallon of a widely used ester formulation is 3.8 lb a.e./gallon (about 0.454 kg a.e./L), while the active ingredient per gallon is 6.0 lb ai/gallon (about 0.717 kg ai/L). As used herein, an "agricultural chemical" is any chemical used in the context of agriculture.

"Herbicide-tolerant" or "tolerant" or "crop tolerance" in the context of herbicide or other chemical treatment as used herein means that a plant or other organism treated with a particular herbicide or class or subclass of herbicide or other chemical or class or subclass of other chemical will show no significant damage or less damage following that treatment in comparison to an appropriate control plant. A plant may be naturally tolerant to a particular herbicide or chemical, or a plant may be herbicide-tolerant as a result of human intervention such as, for example, breeding or genetic engineering. An "herbicide-tolerance polypeptide" is a polypeptide that confers herbicide tolerance on a plant or other organism expressing it (*i.e.*, that makes a plant or other organism herbicide-tolerant), and an "herbicide-tolerance polynucleotide" is a polynucleotide that encodes a herbicide-tolerance polypeptide. For example, a sulfonylurea tolerant polypeptide is one that confers tolerance to sulfonylurea herbicides on a plant or other organism that expresses it, an imidazolinone tolerant polypeptide is one that confers tolerance to imidazolinone herbicides on a plant or other organism that expresses it; and a glyphosate tolerant polypeptide is one that confers tolerance to glyphosate on a plant or other organism that expresses it.

Thus, a plant is tolerant to a herbicide or other chemical if it shows damage in comparison to an appropriate control plant that is less than the damage exhibited by the control plant by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or 1000% or more. In this manner, a plant that is tolerant to a herbicide or other chemical shows "improved tolerance" in comparison to an appropriate control plant. Damage resulting from herbicide or other chemical treatment is assessed by evaluating any parameter of plant growth or well-being deemed suitable by one of skill in the art. Damage can be assessed by visual inspection and/or by statistical analysis of suitable parameters of

individual plants or of a group of plants. Thus, damage may be assessed by evaluating, for example, parameters such as plant height, plant weight, leaf color, leaf length, flowering, fertility, silking, yield, seed production, and the like. Damage may also be assessed by evaluating the time elapsed to a particular stage of development (*e.g.*, silking, flowering, or pollen shed) or the time elapsed until a plant has recovered from treatment with a particular chemical and/or herbicide.

In making such assessments, particular values may be assigned to particular degrees of damage so that statistical analysis or quantitative comparisons may be made. The use of ranges of values to describe particular degrees of damage is known in the art, and any suitable range or scale may be used. For example, herbicide injury scores (also called tolerance scores) can be assigned as illustrated in Example 1 using the scale set forth in Table 7. In this scale, a rating of 9 indicates that a herbicide treatment had no effect on a crop, *i.e.*, that no crop reduction or injury was observed following the herbicide treatment. Thus, in this scale, a rating of 9 indicates that the crop exhibited no damage from the herbicide and therefore that the crop is tolerant to the herbicide. As indicated above, herbicide tolerance is also indicated by other ratings in this scale where an appropriate control plant exhibits a lower score on the scale, or where a group of appropriate control plants exhibits a statistically lower score in response to a herbicide treatment than a group of subject plants.

Damage caused by a herbicide or other chemical can be assessed at various times after a plant has been treated with a herbicide. Often, damage is assessed at about the time that the control plant exhibits maximum damage. Sometimes, damage is assessed after a period of time in which a control plant that was not treated with herbicide or other chemical has measurably grown and/or developed in comparison to the size or stage at which the treatment was administered. Damage can be assessed at various times, for example, at 12 hours or at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 days, or three weeks, four weeks, or longer after the test plant was treated with herbicide. Any time of assessment is suitable as long as it permits detection of a difference in response to a treatment of test and control plants.

A herbicide does not "significantly damage" a plant when it either has no effect on a plant or when it has some effect on a plant from which the plant later recovers, or when it has an effect which is detrimental but which is offset, for example, by the impact of the particular herbicide on weeds. Thus, for example, a crop plant is not "significantly damaged by" a herbicide or other treatment if it exhibits less than 50%, 40%, 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% decrease in at least one suitable parameter that is indicative of plant health and/or productivity in comparison to an appropriate control plant

(*e.g.*, an untreated crop plant). Suitable parameters that are indicative of plant health and/or productivity include, for example, plant height, plant weight, leaf length, time elapsed to a particular stage of development, flowering, yield, seed production, and the like. The evaluation of a parameter can be by visual inspection and/or by statistical analysis of any suitable parameter. Comparison may be made by visual inspection and/or by statistical analysis. Accordingly, a crop plant is not "significantly damaged by" a herbicide or other treatment if it exhibits a decrease in at least one parameter but that decrease is temporary in nature and the plant recovers fully within 1 week, 2 weeks, 3 weeks, 4 weeks, or 6 weeks.

Conversely, a plant is significantly damaged by a herbicide or other treatment if it exhibits more than a 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 150%, 170% decrease in at least one suitable parameter that is indicative of plant health and/or productivity in comparison to an appropriate control plant (*e.g.*, an untreated weed of the same species). Thus, a plant is significantly damaged if it exhibits a decrease in at least one parameter and the plant does not recover fully within 1 week, 2 weeks, 3 weeks, 4 weeks, or 6 weeks.

Damage resulting from a herbicide or other chemical treatment of a plant can be assessed by visual inspection by one of skill in the art and can be evaluated by statistical analysis of suitable parameters. The plant being evaluated is referred to as the "test plant." Typically, an appropriate control plant is one that expresses the same herbicide-tolerance polypeptide(s) as the plant being evaluated for herbicide tolerance (*i.e.*, the "test plant") but that has not been treated with herbicide. For example, in evaluating a herbicide-tolerant plant of the invention that confers tolerance to glyphosate and an ALS inhibitor, an appropriate control plant would be a plant that expresses each of these sequence but is not treated with the herbicide. In some circumstances, the control plant is one that that has been subjected to the same herbicide treatment as the plant being evaluated (*i.e.*, the test plant) but that does not express the enzyme intended to provide tolerance to the herbicide of interest in the test plant. One of skill in the art will be able to design, perform, and evaluate a suitable controlled experiment to assess the herbicide tolerance of a plant of interest, including the selection of appropriate test plants, control plants, and treatments.

Thus, as used herein, a "test plant or plant cell" is one in which genetic alteration has been effected as to at least one gene of interest, or is a plant or plant cell which is descended from a plant or cell so altered and which comprises the alteration. A genetic alteration may be introduced into the plant by breeding or by transformation. "Genetic alteration" is intended to mean a gene or mutation thereof which confers a phenotype on the plant that differs from the phenotype of a plant that does not contain the genetic alteration.

A "control" or "control plant" or "control plant cell" provides a reference point for measuring changes in phenotype of the subject plant or plant cell, and may be any suitable plant or plant cell. A control plant or plant cell may comprise, for example: (a) a wild-type plant or cell, *i.e.*, of the same genotype as the starting material for the genetic alteration which resulted in the subject plant or cell; (b) a plant or plant cell of the same genotype as the starting material but which has been transformed with a null construct (*i.e.*, with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant or plant cell which is a non-transformed segregant among progeny of a subject plant or plant cell; (d) a plant or plant cell which is genetically identical to the subject plant or plant cell but which is not exposed to the same treatment (*e.g.*, herbicide treatment) as the subject plant or plant cell; (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed; or (f) the subject plant or plant cell itself, under conditions in which it has not been exposed to a particular treatment such as, for example, a herbicide or combination of herbicides and/or other chemicals. In some instances, an appropriate control plant or control plant cell may have a different genotype from the subject plant or plant cell but may share the herbicide-sensitive characteristics of the starting material for the genetic alteration(s) which resulted in the subject plant or cell (see, *e.g.*, Green (1998) *Weed Technology* 12: 474-477; Green and Ulrich (1993) *Weed Science* 41: 508-516). In some instances, an appropriate control maize plant comprises a NK603 event (Nielson *et al.* (2004) *European Food Research and Technology* 219:421-427 and Ridley *et al.* (2002) *Journal of Agriculture and Food Chemistry* 50: 7235-7243), an elite stiff stalk inbred plant, a P3162 plant (Pioneer Hi-Bred International), a 39T66 plant (Pioneer Hi-Bred International), or a 34M91 plant (Pioneer Hi-Bred International),. In some instances, an appropriate control soybean plant is a "Jack" soybean plant (Illinois Foundation Seed, Champaign, Illinois).

Plants of the invention express a polypeptide that confers tolerance to glyphosate and at least one other polypeptide that confers tolerance to an ALS inhibitor. A plant of the invention shows at least one improved property relative to an appropriate control plant, such as, for example, improved herbicide tolerance, reduced lodging, increased height, reduced time to maturity, and improved yield. A plant has an improved property when it exhibits a statistically significant difference from an appropriate control plant wherein that difference is in a direction that represents an improvement over the control plant. For example, a plant has an improved property when it exhibits an increase in yield that is statistically significant in comparison to a control plant, and/or when it exhibits a decrease in damage resulting from treatment with a herbicide. Techniques for such assessments are known in the art. Any

suitable statistical analysis may be used, such as, for example, an ANOVA (available as a commercial package from SAS Institute, Inc., 100 SAS Campus Drive, Cary, NC).

f. Plants

As used herein, the term "plant" includes plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, explants, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, and the like. Grain is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced polynucleotides. Thus, the invention provides transgenic seeds produced by the plants of the invention.

The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plant species of interest include, but are not limited to, corn (*Zea mays*, also referred to herein as "maize"), *Brassica* spp. (*e.g.*, *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil (also referred to as "canola"), flax (*Linum* spp.), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (*e.g.*, pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, fruits, ornamentals (flowers), sugar cane, conifers, *Arabidopsis*.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (*e.g.*, *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea

(*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Any tree can also be employed. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Hardwood trees can also be employed including ash, aspen, beech, basswood, birch, black cherry, black walnut, buckeye, American chestnut, cottonwood, dogwood, elm, hackberry, hickory, holly, locust, magnolia, maple, oak, poplar, red alder, redbud, royal paulownia, sassafras, sweetgum, sycamore, tupelo, willow, yellow-poplar.

In specific embodiments, plants of the present invention are crop plants (for example, corn (also referred to as "maize"), alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, *etc.*).

Other plants of interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, *etc.* Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, *etc.* Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, *etc.*

Other plants of interest include Turfgrasses such as, for example, turfgrasses from the genus Poa, Agrostis, Festuca, Lolium, and Zoysia. Additional turfgrasses can come from the subfamily Panicoideae. Turfgrasses can further include, but are not limited to, Blue gramma (*Bouteloua gracilis* (H.B.K.) Lag. Ex Griffiths); Buffalograss (*Buchloe dactyloids* (Nutt.) Engelm.); Slender creeping red fescue (*Festuca rubra* ssp. *Litoralis);* Red fescue (*Festuca rubra)*; Colonial bentgrass (*Agrostis tenuis* Sibth.); Creeping bentgrass (*Agrostis palustris* Huds.); Fairway wheatgrass (*Agropyron cristatum* (L.) Gaertn.); Hard fescue (*Festuca longifolia* Thuill.); Kentucky bluegrass (*Poa pratensis* L.); Perennial ryegrass (*Lolium perenne* L.); Rough bluegrass (*Poa trivialis* L.); Sideoats grama (*Bouteloua curtipendula* Michx. Torr.); Smooth bromegrass (*Bromus inermis* Leyss.); Tall fescue (*Festuca arundinacea* Schreb.); Annual bluegrass (*Poa annua* L.); Annual ryegrass (*Lolium*

multiflorum Lam.); Redtop (*Agrostis alba* L.); Japanese lawn grass (*Zoysia japonica)*; bermudagrass (*Cynodon dactylon*; *Cynodon spp.* L.C. Rich; *Cynodon transvaalensis*); Seashore paspalum (*Paspalum vaginatum* Swartz); Zoysiagrass (*Zoysia spp.* Willd; *Zoysia japonica* and *Z. matrella* var. *matrella*); Bahiagrass (*Paspalum notatum* Flugge); Carpetgrass (*Axonopus affinis* Chase); Centipedegrass (*Eremochloa ophiuroides* Munro Hack.); Kikuyugrass (*Pennisetum clandesinum* Hochst Ex Chiov); Browntop bent (*Agrostis tenuis* also known as *A. capillaris*); Velvet bent (*Agrostis canina*); Perennial ryegrass (*Lolium perenne*); and, St. Augustinegrass (*Stenotaphrum secundatum* Walt. Kuntze). Additional grasses of interest include switchgrass (*Panicum virgatum*).

g. Stacking of Traits and Additional Traits of Interest

In some embodiments, the polynucleotide conferring the glyphosate tolerance and the ALS inhibitor tolerance in the plants of the invention are engineered into a molecular stack. In other embodiments, the molecular stack further comprises at least one additional polynucleotide that confers tolerance to a 3rd herbicide. Such sequences are disclosed elsewhere in herein. In one embodiment, the sequence confers tolerance to glufosinate, and 2in a specific embodiment, the sequence comprises pat.

In other embodiments, the glyphosate/ALS inhibitor-tolerant plants of the invention comprise one or more trait of interest, and in more specific embodiments, the plant is stacked with any combination of polynucleotide sequences of interest in order to create plants with a desired combination of traits. A trait, as used herein, refers to the phenotype derived from a particular sequence or groups of sequences. For example, herbicide-tolerance polynucleotides may be stacked with any other polynucleotides encoding polypeptides having pesticidal and/or insecticidal activity, such as *Bacillus thuringiensis* toxic proteins (described in U.S. Patent Nos. 5,366,892; 5,747,450; 5,737,514; 5,723,756; 5,593,881; Geiser *et al.* (1986) *Gene* 48: 109; Lee *et al.* (2003) *Appl. Environ. Microbiol.* 69: 4648-4657 (*Vip3A*); Galitzky *et al.* (2001) *Acta Crystallogr. D. Biol. Crystallogr.* 57: 1101-1109 (*Cry3Bb1*); and Herman *et al.* (2004) *J. Agric. Food Chem.* 52: 2726-2734 (*Cry1F*)), lectins (Van Damme *et al.* (1994) *Plant Mol. Biol.* 24: 825, pentin (described in U.S. Patent No. 5,981,722), and the like. The combinations generated can also include multiple copies of any one of the polynucleotides of interest.

In some embodiments, herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., such as plant comprising GAT and HRA) may be stacked with other herbicide-tolerance traits to create a transgenic plant of the invention with further

improved properties. Other herbicide-tolerance polynucleotides that could be used in such embodiments include those conferring tolerance to glyphosate or to ALS inhibitors by other modes of action, such as, for example, a gene that encodes a glyphosate oxido-reductase enzyme as described more fully in U.S. Patent Nos. 5,776,760 and 5,463,175. Other traits that could be combined with herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., such as GAT and HRA sequence) include those derived from polynucleotides that confer on the plant the capacity to produce a higher level of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), for example, as more fully described in U.S. Patent Nos. 6,248,876 B1; 5,627,061; 5,804,425; 5,633,435; 5,145,783; 4,971,908; 5,312,910; 5,188,642; 4,940,835; 5,866,775; 6,225,114 B1; 6,130,366; 5,310,667; 4,535,060; 4,769,061; 5,633,448; 5,510,471; Re. 36,449; RE 37,287 E; and 5,491,288; and international publications WO 97/04103; WO 00/66746; WO 01/66704; and WO 00/66747. Other traits that could be combined with herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., such as GAT and HRA sequences) include those conferring tolerance to sulfonylurea and/or imidazolinone, for example, as described more fully in U.S. Patent Nos. 5,605,011; 5,013,659; 5,141,870; 5,767,361; 5,731,180; 5,304,732; 4,761,373; 5,331,107; 5,928,937; and 5,378,824; and international publication WO 96/33270.

In some embodiments, herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., such as GAT and HRA sequence) may be stacked with, for example, hydroxyphenylpyruvatedioxygenases which are enzymes that catalyze the reaction in which para-hydroxyphenylpyruvate (HPP) is transformed into homogentisate. Molecules which inhibit this enzyme and which bind to the enzyme in order to inhibit transformation of the HPP into homogentisate are useful as herbicides. Traits conferring tolerance to such herbicides in plants are described in U.S. Patent Nos. 6,245,968 B1; 6,268,549; and 6,069,115; and international publication WO 99/23886. Other examples of suitable herbicide-tolerance traits that could be stacked with herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., such GAT and HRA sequences) include aryloxyalkanoate dioxygenase polynucleotides (which reportedly confer tolerance to 2,4-D and other phenoxy auxin herbicides as well as to aryloxyphenoxypropionate herbicides as described, for example, in WO2005/107437) and dicamba-tolerance polynucleotides as described, for example, in Herman *et al.* (2005) *J. Biol. Chem.* 280: 24759-24767.

Other examples of herbicide-tolerance traits that could be combined with herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., GAT and HRA plants) include those conferred by polynucleotides encoding an exogenous

phosphinothricin acetyltransferase, as described in U.S. Patent Nos. 5,969,213; 5,489,520; 5,550,318; 5,874,265; 5,919,675; 5,561,236; 5,648,477; 5,646,024; 6,177,616; and 5,879,903. Plants containing an exogenous phosphinothricin acetyltransferase can exhibit improved tolerance to glufosinate herbicides, which inhibit the enzyme glutamine synthase. Other examples of herbicide-tolerance traits that could be combined with the herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., GAT and HRA plants) include those conferred by polynucleotides conferring altered protoporphyrinogen oxidase (protox) activity, as described in U.S. Patent Nos. 6,288,306 B1; 6,282,837 B1; and 5,767,373; and international publication WO 01/12825. Plants containing such polynucleotides can exhibit improved tolerance to any of a variety of herbicides which target the protox enzyme (also referred to as "protox inhibitors").

Other examples of herbicide-tolerance traits that could be combined with herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., GAT and HRA plants) include those conferring tolerance to at least one herbicide in a plant such as, for example, a maize plant or horseweed. Herbicide-tolerant weeds are known in the art, as are plants that vary in their tolerance to particular herbicides. See, *e.g.*, Green and Williams (2004) "Correlation of Corn (*Zea mays*) Inbred Response to Nicosulfuron and Mesotrione," poster presented at the WSSA Annual Meeting in Kansas City, Missouri, February 9-12, 2004; Green (1998) *Weed Technology* 12: 474-477; Green and Ulrich (1993) *Weed Science* 41: 508-516. The trait(s) responsible for these tolerances can be combined by breeding or via other methods with herbicide-tolerance polynucleotides of the glyphosate/ALS inhibitor-tolerant plants (i.e., GAT and HRA plants) to provide a plant of the invention as well as methods of use thereof.

In this manner, the invention provides plants that are more tolerant to glyphosate and other ALS inhibitor chemistries and also provides plants that are more tolerant to the herbicide for which each of the traits discussed above confers tolerance. Accordingly, the invention provides methods for growing a crop (*i.e.*, for selectively controlling weeds in an area of cultivation) that comprise treating an area of interest (*e.g.*, a field or area of cultivation) with at least one herbicide to which the plant of the invention is tolerant, such as for example, glyphosate, an ALS inhibitor chemistry, a mixture of ALS inhibitor chemistries, or a mixture of glyphosate and ALS inhibitor chemistry. In some embodiments, methods of the invention further comprise treatment with additional herbicides to which the plant of the invention is tolerant. In such embodiments, generally the methods of the invention permit selective control of weeds without significantly damaging the crop. As used herein, an "area

of cultivation" comprises any region in which one desires to grow a plant. Such areas of cultivations include, but are not limited to, a field in which a plant is cultivated (such as a crop field, a sod field, a tree field, a managed forest, a field for culturing fruits and vegetables, etc), a greenhouse, a growth chamber, etc.

Herbicide-tolerant traits can also be combined with at least one other trait to produce plants of the present invention that further comprise a variety of desired trait combinations including, but not limited to, traits desirable for animal feed such as high oil content (*e.g.*, U.S. Patent No. 6,232,529); balanced amino acid content (*e.g.*, hordothionins (U.S. Patent Nos. 5,990,389; 5,885,801; 5,885,802; and 5,703,409; U.S. Patent No. 5,850,016); barley high lysine (Williamson *et al.* (1987) *Eur. J. Biochem.* 165: 99-106; and WO 98/20122) and high methionine proteins (Pedersen *et al.* (1986) *J. Biol. Chem.* 261: 6279; Kirihara *et al.* (1988) *Gene* 71: 359; and Musumura *et al.* (1989) *Plant Mol. Biol.* 12:123)); increased digestibility (*e.g.*, modified storage proteins (U.S. Application Serial No. 10/053,410, filed November 7, 2001); and thioredoxins (U.S. Application Serial No. 10/005,429, filed December 3, 2001)); the disclosures of which are herein incorporated by reference. Desired trait combinations also include LLNC (low linolenic acid content; see, *e.g.*, Dyer *et al.* (2002) *Appl. Microbiol. Biotechnol.* 59: 224-230) and OLCH (high oleic acid content; see, *e.g.*, Fernandez-Moya *et al.* (2005) *J. Agric. Food Chem.* 53: 5326-5330).

Herbicide-tolerant traits of interest can also be combined with other desirable traits such as, for example, fumonisim detoxification genes (U.S. Patent No. 5,792,931), avirulence and disease resistance genes (Jones *et al.* (1994) *Science* 266: 789; Martin *et al.* (1993) *Science* 262: 1432; Mindrinos *et al.* (1994) *Cell* 78: 1089), and traits desirable for processing or process products such as modified oils (*e.g.*, fatty acid desaturase genes (U.S. Patent No. 5,952,544; WO 94/11516)); modified starches (*e.g.*, ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE), and starch debranching enzymes (SDBE)); and polymers or bioplastics (*e.g.*, U.S. Patent No. 5,602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert *et al.* (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs)); the disclosures of which are herein incorporated by reference. One could also combine herbicide-tolerant polynucleotides with polynucleotides providing agronomic traits such as male sterility (*e.g.*, see U.S. Patent No. 5.583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regulation or gene targeting (*e.g.*, WO 99/61619, WO 00/17364, and WO 99/25821); the disclosures of which are herein incorporated by reference.

In another embodiment, the herbicide-tolerant traits of interest can also be combined with the Rcg1 sequence or biologically active variant or fragment thereof. The Rcg1 sequence is an anthracnose stalk rot resistance gene in corn. See, for example, U.S. Patent Application No. 11/397,153, 11/397,275, and 11/397,247, each of which is herein incorporated by reference.

These stacked combinations can be created by any method including, but not limited to, breeding plants by any conventional or TopCross methodology, or genetic transformation. If the sequences are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. For example, a transgenic plant comprising one or more desired traits can be used as the target to introduce further traits by subsequent transformation. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transformation cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucleotide of interest. This may be combined with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference.

Various changes in phenotype are of interest including modifying the fatty acid composition in a plant, altering the amino acid content of a plant, altering a plant's pathogen defense mechanism, and the like. These results can be achieved by providing expression of heterologous products or increased expression of endogenous products in plants. Alternatively, the results can be achieved by providing for a reduction of expression of one or more endogenous products, particularly enzymes or cofactors in the plant. These changes result in a change in phenotype of the transformed plant.

Genes of interest are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as developing nations open up world markets, new crops and technologies will emerge also. In addition, as our understanding of agronomic traits and characteristics such as yield and

heterosis increase, the choice of genes for transformation will change accordingly. General categories of genes of interest include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics, and commercial products. Genes of interest include, generally, those involved in oil, starch, carbohydrate, or nutrient metabolism as well as those affecting kernel size, sucrose loading, and the like. Agronomically important traits such as oil, starch, and protein content can be genetically altered in addition to using traditional breeding methods. Modifications include increasing content of oleic acid, saturated and unsaturated oils, increasing levels of lysine and sulfur, providing essential amino acids, and also modification of starch.

Derivatives of the coding sequences can be made by site-directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example, the gene encoding the barley high lysine polypeptide (BHL) is derived from barley chymotrypsin inhibitor, U.S. Application Serial No. 08/740,682, filed November 1, 1996, and WO 98/20133, the disclosures of which are herein incorporated by reference. Other proteins include methionine-rich plant proteins such as from sunflower seed (Lilley *et al.* (1989) *Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs*, ed. Applewhite (American Oil Chemists Society, Champaign, Illinois), pp. 497-502; herein incorporated by reference); corn (Pedersen *et al.* (1986) *J. Biol. Chem.* 261:6279; Kirihara *et al.* (1988) *Gene* 71:359; both of which are herein incorporated by reference); and rice (Musumura *et al.* (1989) *Plant Mol. Biol.* 12:123, herein incorporated by reference). Other agronomically important genes encode latex, Floury 2, growth factors, seed storage factors, and transcription factors.

Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for example, *Bacillus thuringiensis* toxic protein genes (U.S. Patent Nos. 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881; and Geiser *et al.* (1986) *Gene* 48: 109); and the like.

Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (U.S. Patent No. 5,792,931); avirulence (avr) and disease resistance (R) genes (Jones *et al.* (1994) *Science* 266: 789; Martin *et al.* (1993) *Science* 262: 1432; and Mindrinos *et al.* (1994) *Cell* 78: 1089); and the like.

Sterility genes can also be encoded in an expression cassette and provide an alternative to physical detasseling. Examples of genes used in such ways include male tissue-preferred genes and genes with male sterility phenotypes such as QM, described in U.S. Patent No. 5,583,210. Other genes include kinases and those encoding compounds toxic to either male or female gametophytic development.

The quality of grain is reflected in traits such as levels and types of oils, saturated and unsaturated, quality and quantity of essential amino acids, and levels of cellulose. In corn, modified hordothionin proteins are described in U.S. Patent Nos. 5,703,049, 5,885,801, 5,885,802, and 5,990,389.

Commercial traits can also be encoded on a gene or genes that could increase for example, starch for ethanol production, or provide expression of proteins. Another important commercial use of transformed plants is the production of polymers and bioplastics such as described in U.S. Patent No. 5,602,321. Genes such as β-Ketothiolase, PHBase (polyhydroxybutryrate synthase), and acetoacetyl-CoA reductase (see Schubert *et al.* (1988) *J. Bacteriol.* 170: 5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs).

Exogenous products include plant enzymes and products as well as those from other sources including procaryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like. The level of proteins, particularly modified proteins having improved amino acid distribution to improve the nutrient value of the plant, can be increased. This is achieved by the expression of such proteins having enhanced amino acid content.

II. Polynucleotide Constructs

In specific embodiments, one or more of the herbicide-tolerant polynucleotides employed in the methods and compositions can be provided in an expression cassette for expression in the plant or other organism of interest. The cassette will include 5′ and 3′ regulatory sequences operably linked to a herbicide-tolerance polynucleotide. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a polynucleotide of interest and a regulatory sequence (*e.g.*, a promoter) is functional link that allows for expression of the polynucleotide of interest. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, by "operably linked" is intended that the coding regions are in the same reading frame. When used to refer to the effect of an enhancer, "operably linked" indicates that the enhancer increases the expression of a

particular polynucleotide or polynucleotides of interest. Where the polynucleotide or polynucleotides of interest encode a polypeptide, the encoded polypeptide is produced at a higher level.

The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes. Such an expression cassette is provided with a plurality of restriction sites and/or recombination sites for insertion of the herbicide-tolerance polynucleotide to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain other genes, including other selectable marker genes. Where a cassette contains more than one polynucleotide, the polynucleotides in the cassette may be transcribed in the same direction or in different directions (also called "divergent" transcription).

An expression cassette comprising a herbicide-tolerance polynucleotide will include in the 5′-3′ direction of transcription a transcriptional and translational initiation region (*i.e.*, a promoter), a herbicide-tolerance polynucleotide (*e.g.*, a GAT polynucleotide, a ALS inhibitor-tolerant polynucleotide, an HRA polynucleotide, or any combination thereof, etc.), and a transcriptional and translational termination region (*i.e.*, termination region) functional in plants or the other organism of interest. Accordingly, plants having such expression cassettes are also provided. The regulatory regions (*i.e.*, promoters, transcriptional regulatory regions, and translational termination regions) and/or the herbicide-tolerance polynucleotide may be native (*i.e.*, analogous) to the host cell or to each other. Alternatively, the regulatory regions and/or the herbicide-tolerance polynucleotide of the invention may be heterologous to the host cell or to each other. As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same (*i.e.*, analogous) species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide.

While it may be optimal to express polynucleotides using heterologous promoters, native promoter sequences may be used. Such constructs can change expression levels and/or expression patterns of the encoded polypeptide in the plant or plant cell. Expression levels and/or expression patterns of the encoded polypeptide may also be changed as a result of an

additional regulatory element that is part of the construct, such as, for example, an enhancer. Thus, the phenotype of the plant or cell can be altered even though a native promoter is used.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked herbicide-tolerance polynucleotide of interest, may be native with the plant host, or may be derived from another source (*i.e.*, foreign or heterologous) to the promoter, the herbicide-tolerance polynucleotide of interest, the plant host, or any combination thereof. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions, or can be obtained from plant genes such as the *Solanum tuberosum* proteinase inhibitor II gene. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262: 141-144; Proudfoot (1991) *Cell* 64: 671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5: 141-149; Mogen *et al.* (1990) *Plant Cell* 2: 1261-1272; Munroe *et al.* (1990) *Gene* 91: 151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17: 7891-7903; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15: 9627-9639.

A number of promoters can be used in the practice of the invention, including the native promoter of the polynucleotide sequence of interest. The promoters can be selected based on the desired outcome. The polynucleotides of interest can be combined with constitutive, tissue-preferred, or other promoters for expression in plants.

Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313: 810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2: 163-171); the maize actin promoter; the ubiquitin promoter (see, *e.g.*, Christensen *et al.* (1989) *Plant Mol. Biol.* 12: 619-632; Christensen *et al.* (1992) *Plant Mol. Biol.* 18: 675-689; Callis *et al.* (1995) *Genetics* 139: 921-39); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81: 581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3: 2723-2730); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, those described in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611. Some promoters show improved expression when they are used in conjunction with a native 5′ untranslated region and/or other elements such as, for example, an intron. For example, the maize ubiquitin promoter is often placed upstream of a polynucleotide of interest along with at least a portion of the 5′ untranslated region of the ubiquitin gene, including the first intron of the maize ubiquitin gene.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the

objective, the promoter may be a chemical-inducible promoter for which application of the chemical induces gene expression or the promoter may be a chemical-repressible promoter for which application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88: 10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2): 247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227: 229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced herbicide-tolerance polypeptide expression within a particular plant tissue. Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J.* 12(2): 255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7): 792-803; Hansen *et al.* (1997) *Mol. Gen Genet.* 254(3): 337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2): 157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2): 525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2): 513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5): 773-778; Lam (1994) *Results Probl. Cell Differ.* 20: 181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6): 1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20): 9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3): 495-505. Such promoters can be modified, if necessary, for weak expression.

Leaf-preferred promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2): 255-265; Kwon *et al.* (1994) *Plant Physiol.* 105: 357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5): 773-778; Gotor *et al.* (1993) *Plant J.* 3: 509-18; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6): 1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20): 9586-9590.

Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2): 207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10): 1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.*

14(3): 433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1): 11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7): 633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1): 69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J.* 8(2): 343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4): 759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4): 681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays* 10: 108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); milps (myo-inositol-1-phosphate synthase) (see WO 00/11177 and U.S. Patent No. 6,225,529; herein incorporated by reference). Gamma-zein is an endosperm-specific promoter. Globulin 1 (Glb-1) is a representative embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, gamma-zein, waxy,

shrunken 1, shrunken 2, Globulin 1, *etc.* See also WO 00/12733, where seed-preferred promoters from *end1* and *end2* genes are disclosed; herein incorporated by reference.

Additional promoters of interest include the SCP1 promoter (US 6,072,050), the HB2 promoter (US 6,177,611) and the SAMS promoter (US20030226166 and SEQ ID NO: 87 and biologically active variants and fragments thereof); each of which is herein incorporated by reference. In addition, as discussed elsewhere herein, various enhancers can be used with these promoters including, for example, the ubiquitin intron (i.e, the maize ubiquitin intron 1 (see, for example, NCBI sequence S94464), the omega enhancer or the omega prime enhancer (Gallie *et al.* (1989) *Molecular Biology of RNA* ed. Cech (Liss, New York) 237-256 and Gallie *et al. Gene* (1987) 60:217-25), or the 35S enhancer; each of which is incorporated by reference.

The expression cassette can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). Additional selectable markers include phenotypic markers such as β-galactosidase and fluorescent proteins such as green fluorescent protein (GFP) (Su et *al.* (2004) *Biotechnol Bioeng* 85: 610-9 and Fetter *et al.* (2004) *Plant Cell* 16: 215-28), cyan florescent protein (CYP) (Bolte *et al.* (2004) *J. Cell Science* 117: 943-54 and Kato *et al.* (2002) *Plant Physiol* 129: 913-42), and yellow fluorescent protein (PhiYFP from Evrogen, see, Bolte *et al.* (2004) *J. Cell Science* 117: 943-54). For additional selectable markers, see generally, Yarranton (1992) *Curr. Opin. Biotech.* 3: 506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89: 6314-6318; Yao *et al.* (1992) *Cell* 71: 63-72; Reznikoff (1992) *Mol. Microbiol.* 6: 2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48: 555-566; Brown *et al.* (1987) *Cell* 49: 603-612; Figge *et al.* (1988) *Cell* 52: 713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA* 86: 5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 2549-2553; Deuschle *et al.* (1990) *Science* 248: 480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90: 1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10: 3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89: 3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88: 5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19: 4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10: 143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:

1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry* 27: 1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89: 5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36: 913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature* 334: 721-724. Such disclosures are herein incorporated by reference. The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention, including the GAT gene and/or HRA gene.

Methods are known in the art of increasing the expression level of a polypeptide of the invention in a plant or plant cell, for example, by inserting into the polypeptide coding sequence one or two G/C-rich codons (such as GCG or GCT) immediately adjacent to and downstream of the initiating methionine ATG codon. Where appropriate, the polynucleotides may be optimized for increased expression in the transformed plant. That is, the polynucleotides can be synthesized substituting in the polypeptide coding sequence one or more codons which are less frequently utilized in plants for codons encoding the same amino acid(s) which are more frequently utilized in plants, and introducing the modified coding sequence into a plant or plant cell and expressing the modified coding sequence. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92: 1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17: 477-498, herein incorporated by reference. Embodiments comprising such modifications are also a feature of the invention.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures. "Enhancers" such as the CaMV 35S enhancer may also be used (see, *e.g.*, Benfey *et al.* (1990) *EMBO J.* 9: 1685-96), or other enhancers may be used. For example, the sequence set forth in SEQ ID NO: 1, 72, 79, 84, 85, 88, or 89 or a biologically active variant or fragment thereof can be used. See, also, U.S. Utility Application No. ____, entitled "*Methods and Compositions for the Expression of a Polynucleotide of Interest*", filed concurrently herewith, and herein incorporated by reference in its entirety. The term "promoter" is intended to mean a regulatory region of DNA

comprising a transcriptional initiation region, which in some embodiments, comprises a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular coding sequence. The promoter can further be operably linked to additional regulatory elements that influence transcription, including, but not limited to, introns, 5' untranslated regions, and enhancer elements. As used herein, an "enhancer sequence," "enhancer domain," "enhancer element," or "enhancer," when operably linked to an appropriate promoter, will modulate the level of transcription of an operably linked polynucleotide of interest. Biologically active fragments and variants of the enhancer domain may retain the biological activity of modulating (increase or decrease) the level of transcription when operably linked to an appropriate promoter.

Fragments of a polynucleotide for the enhancer domain or a promoter may range from at least about 50 nucleotides, about 100 nucleotides, about 150 nucleotides, about 200 nucleotides, about 250 nucleotides, about 300 nucleotides, about 350 nucleotides, about 400 nucleotides, about 450 nucleotides, about 500 nucleotides, and up to the full-length nucleotide sequence of the invention for the enhancer domain of the invention. In other embodiments, a fragment of the enhancer domain comprises a length of about 50 to about 100, 100 to about 150, 150 to about 200, 200 to about 250, about 250 to about 300, about 300 to about 350, about 350 to about 400, about 400 to about 450, about 450 to about 500, about 500 to about 535 nucleotides. Generally, variants of a particular polynucleotides of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotides as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of an enhancer or a promoter may differ from that sequence by as few as 1-15 nucleic acid residues, as few as 1-10, such as 6-10, as few as 10, 9, 8, 7, 6, 5, 4, 3, 2, or even 1 nucleic acid residue. Such active variants and fragments will continue to modulate transcription.

Multiple copies of the enhancer domain or active variants and fragments thereof can be operably linked to a promoter. In specific embodiment, the chimeric transcriptional regulatory control region comprises at least 1, 2, 3, 4, 5, 6, 7 or more copies of the enhancer domain. In further embodiments, the enhancer domain employed does not comprise the sequence set forth in SEQ ID NO:5. In addition, the enhancer can be orientated in either orientation (i.e., sense or reverse).

The distance between the promoter and the enhancer domain can vary, so long as the chimeric transcriptional regulatory region continues to direct transcription of the operably linked polynucleotide of interest in the desired manner. For example, an enhancer domain can be positioned at least about 10000 to about 15000, about 10000 to about a 9000, about 9000 to about 8000, about 8000 to about 7000, about 7000 to about 6000, about 6000 to about 5000, about 5000 to about 4000, about 4000 to about 3000, about 3000 to about 2000, about 2000 to about 1000, about 1000 to about 500, about 500 to about 250, about 250 to immediately adjacent to the promoter. It is further recognized that one or more copies of the enhancer can be placed upstream (5') of the promoter or alternatively, one or more copies of the enhancer can be located 3' to the promoter. In specific embodiments, when located 3' of the promoter, the enhancer is downstream of the terminator region. In still further embodiments, one or more of the enhancers can be arranged either in the 5' or 3' orientation (as shown in SEQ ID NO:1 or 72) or in the 3' to 5' orientation.

If multiple enhancers are employed, the enhancers can be positioned in the construct with respect to the promoter such that the desired affect on expression is achieved. For example, the enhances can be immediately adjacent to each other or at least between 1 to 100, 100 to 300, 300 to 500, 500 to 1000 nucleotides apart.

It is further recognized that the enhancer employed in the invention can be positioned in a DNA construct between and operably linked to a first and a second promoter. In such embodiments, the enhancer allows for a modulation in expression of both the first and the second promoters from a divergent direction. Exemplary, but non-limiting, examples of such DNA constructs comprise in the 5' to 3' or 3' to 5' orientation: a first polynucleotide of interest operably linked to a first promoter, operably linked to at least one copy of an enhancer of the invention, operably linked to a second promoter, operably linked to a second polynucleotide of interest. In specific embodiments, the enhancer sequence is heterologous to the first and the second enhancer sequence. In other embodiments, the first promoter is operably linked to a polynucleotide encoding an ALS inhibitor and the second promoter is operably linked to a polynucleotide encoding a polypeptide that confers tolerance to glyphosate. Such polynucleotides are disclosed elsewhere herein.

The expression cassette may additionally contain 5′ leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5′ noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie *et al.* (1995) *Gene*

165(2): 233-238), MDMV leader (Maize Dwarf Mosaic Virus) (*Virology* 154: 9-20), and human immunoglobulin heavy-chain binding protein (BiP) (Macejak *et al.* (1991) *Nature* 353: 90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325: 622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81: 382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol.* 84: 965-968.

In preparing the expression cassette, the various polynucleotide fragments may be manipulated, so as to provide for sequences to be in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous material such as the removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.*, transitions and transversions, may be involved. Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully, for example, in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor) (also known as "Maniatis").

In some embodiments, the polynucleotide of interest is targeted to the chloroplast for expression. In this manner, where the polynucleotide of interest is not directly inserted into the chloroplast, the expression cassette will additionally contain a nucleic acid encoding a transit peptide to direct the gene product of interest to the chloroplasts. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9: 104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264: 17544-17550; Della-Cioppa *et al.* (1987) *Plant Physiol.* 84: 965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196: 1414-1421; and Shah *et al.* (1986) *Science* 233: 478-481.

Chloroplast targeting sequences are known in the art and include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) (de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol.* 30: 769-780; Schnell *et al.* (1991) *J. Biol. Chem.* 266(5): 3335-3342); 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer *et al.* (1990) *J. Bioenerg. Biomemb.* 22(6): 789-810); tryptophan synthase (Zhao *et al.* (1995) *J. Biol. Chem.* 270(11): 6081-6087); plastocyanin (Lawrence *et al.* (1997) *J. Biol. Chem.* 272(33): 20357-20363); chorismate synthase (Schmidt *et al.* (1993) *J. Biol. Chem.* 268(36): 27447-27457); and the light harvesting chlorophyll a/b binding protein (LHBP) (Lamppa *et al.* (1988) *J. Biol. Chem.*

263: 14996-14999). See also Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9: 104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264: 17544-17550; Della-Cioppa *et al.* (1987) *Plant Physiol.* 84: 965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196: 1414-1421; and Shah *et al.* (1986) *Science* 233: 478-481.

Methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87: 8526-8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90: 913-917; Svab and Maliga (1993) *EMBO J.* 12: 601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-preferred expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91: 7301-7305.

The polynucleotides of interest to be targeted to the chloroplast may be optimized for expression in the chloroplast to account for differences in codon usage between the plant nucleus and this organelle. In this manner, the polynucleotide of interest may be synthesized using chloroplast-preferred codons. See, for example, U.S. Patent No. 5,380,831, herein incorporated by reference.

"Gene" refers to a polynucleotide that expresses a specific protein, generally including regulatory sequences preceding (5′ non-coding sequences) and following (3′ non-coding sequences) the coding sequence (*i.e.*, the portion of the sequence that encodes the specific protein). "Native gene" refers to a gene as found in nature, generally with its own regulatory sequences. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. Accordingly, a "transgenic plant" is a plant that contains a transgene, whether the transgene was introduced into that particular plant by transformation or by breeding; thus, descendants of an originally-transformed plant are encompassed by the definition.

III. Methods of Introducing

The plants of the invention are generated by introducing a polypeptide or polynucleotide into a plant. "Introducing" is intended to mean presenting to the plant the polynucleotide or polypeptide in such a manner that the sequence gains access to the interior of a cell of the plant. The methods of the invention do not depend on a particular method for introducing a sequence into a plant, only that the polynucleotide or polypeptides gains access

to the interior of at least one cell of the plant. Methods for introducing polynucleotide or polypeptides into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, virus-mediated methods, and breeding.

"Stable transformation" is intended to mean that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by the progeny thereof. "Transient transformation" is intended to mean that a polynucleotide is introduced into the plant and does not integrate into the genome of the plant or a polypeptide is introduced into a plant.

Transformation protocols as well as protocols for introducing polypeptides or polynucleotide sequences into plants may vary depending on the type of plant or plant cell (*i.e.*, monocot or dicot) targeted for transformation. Suitable methods of introducing polypeptides and polynucleotides into plant cells include microinjection (Crossway *et al.* (1986) *Biotechniques* 4: 320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83: 5602-5606, *Agrobacterium*-mediated transformation (U.S. Patent No. 5,563,055 and U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3: 2717-2722), and ballistic particle acceleration (see, for example, U.S. Patent Nos. 4,945,050; U.S. Patent No. 5,879,918; U.S. Patent No. 5,886,244; and, 5,932,782; Tomes *et al.* (1995) in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe *et al.* (1988) *Biotechnology* 6: 923-926); and Lec1 transformation (WO 00/28058). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22: 421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5: 27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87: 671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6: 923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P: 175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8: 736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); U.S. Patent Nos. 5,240,855; 5,322,783; and, 5,324,646; Klein *et al.* (1988) *Plant Physiol.* 91: 440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8: 833-839 (maize); protocols published electronically by "IP.com" under the permanent publication identifiers IPCOM000033402D, IPCOM000033402D, and IPCOM000033402D and available at the "IP.com" website (cotton); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311: 763-764; U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84: 5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman *et al.* (Longman,

New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9: 415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84: 560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4: 1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12: 250-255 and Christou and Ford (1995) *Annals of Botany* 75: 407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14: 745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

In specific embodiments, herbicide-tolerance or other desirable sequences can be provided to a plant using a variety of transient transformation methods. Such transient transformation methods include, but are not limited to, the introduction of the polypeptide or variants and fragments thereof directly into the plant or the introduction of a transcript into the plant. Such methods include, for example, microinjection or particle bombardment. See, for example, Crossway *et al.* (1986) *Mol Gen. Genet.* 202: 179-185; Nomura *et al.* (1986) *Plant Sci.* 44: 53-58; Hepler *et al.* (1994) *Proc. Natl. Acad. Sci.* 91: 2176-2180 and Hush *et al.* (1994) *The Journal of Cell Science* 107: 775-784, all of which are herein incorporated by reference. Alternatively, a herbicide-tolerance polynucleotide can be transiently transformed into the plant using techniques known in the art. Such techniques include viral vector system and the precipitation of the polynucleotide in a manner that precludes subsequent release of the DNA. Thus, the transcription from the particle-bound DNA can occur, but the frequency with which it is released to become integrated into the genome is greatly reduced. Such methods include the use particles coated with polyethylimine (PEI; Sigma #P3143).

In other embodiments, polynucleotides may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct within a viral DNA or RNA molecule. It is recognized that a polypeptide of interest may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant protein. Further, it is recognized that useful promoters may include promoters utilized for transcription by viral RNA polymerases. Methods for introducing polynucleotides into plants and expressing a polypeptide encoded thereby, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367, 5,316,931, and Porta *et al.* (1996) *Molecular Biotechnology* 5: 209-221; herein incorporated by reference.

Methods are known in the art for the targeted insertion of a polynucleotide at a specific location in the plant genome. In one embodiment, the insertion of the polynucleotide at a desired genomic location is achieved using a site-specific recombination system. See, for

example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference. Briefly, a polynucleotide can be contained in transfer cassette flanked by two non-recombinogenic recombination sites. The transfer cassette is introduced into a plant having stably incorporated into its genome a target site which is flanked by two non-recombinogenic recombination sites that correspond to the sites of the transfer cassette. An appropriate recombinase is provided and the transfer cassette is integrated at the target site. The polynucleotide of interest is thereby integrated at a specific chromosomal position in the plant genome.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5: 81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting progeny having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as "transgenic seed") having a polynucleotide of the invention, for example, an expression cassette of the invention, stably incorporated into their genome.

In specific embodiments, a polypeptide or the polynucleotide of interest is introduced into the plant cell. Subsequently, a plant cell having the introduced sequence of the invention is selected using methods known to those of skill in the art such as, but not limited to, Southern blot analysis, DNA sequencing, PCR analysis, or phenotypic analysis. A plant or plant part altered or modified by the foregoing embodiments is grown under plant forming conditions for a time sufficient to modulate the concentration and/or activity of polypeptides in the plant. Plant forming conditions are well known in the art and discussed briefly elsewhere herein.

It is also recognized that the level and/or activity of a polypeptide of interest may be modulated by employing a polynucleotide that is not capable of directing, in a transformed plant, the expression of a protein or an RNA. For example, the polynucleotides of the invention may be used to design polynucleotide constructs that can be employed in methods for altering or mutating a genomic nucleotide sequence in an organism. Such polynucleotide constructs include, but are not limited to, RNA:DNA vectors, RNA:DNA mutational vectors, RNA:DNA repair vectors, mixed-duplex oligonucleotides, self-complementary RNA:DNA oligonucleotides, and recombinogenic oligonucleobases. Such nucleotide constructs and

methods of use are known in the art. See, U.S. Patent Nos. 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871,984; all of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96: 8774-8778; herein incorporated by reference.

It is therefore recognized that methods of the present invention do not depend on the incorporation of the entire polynucleotide into the genome, only that the plant or cell thereof is altered as a result of the introduction of the polynucleotide into a cell. In one embodiment of the invention, the genome may be altered following the introduction of the polynucleotide into a cell. For example, the polynucleotide, or any part thereof, may incorporate into the genome of the plant. Alterations to the genome of the present invention include, but are not limited to, additions, deletions, and substitutions of nucleotides into the genome. While the methods of the present invention do not depend on additions, deletions, and substitutions of any particular number of nucleotides, it is recognized that such additions, deletions, or substitutions comprises at least one nucleotide.

Plants of the invention may be produced by any suitable method, including breeding. Plant breeding can be used to introduce desired characteristics (*e.g.*, a stably incorporated transgene or a genetic variant or genetic alteration of interest) into a particular plant line of interest, and can be performed in any of several different ways. Pedigree breeding starts with the crossing of two genotypes, such as an elite line of interest and one other elite inbred line having one or more desirable characteristics (*i.e.*, having stably incorporated a polynucleotide of interest, having a modulated activity and/or level of the polypeptide of interest, *etc.*) which complements the elite plant line of interest. If the two original parents do not provide all the desired characteristics, other sources can be included in the breeding population. In the pedigree method, superior plants are selfed and selected in successive filial generations. In the succeeding filial generations the heterozygous condition gives way to homogeneous lines as a result of self-pollination and selection. Typically in the pedigree method of breeding, five or more successive filial generations of selfing and selection is practiced: F1 $\rightarrow$ F2; F2$\rightarrow$ F3; F3 $\rightarrow$ F4; F4 $\rightarrow$ F_5, *etc.* After a sufficient amount of inbreeding, successive filial generations will serve to increase seed of the developed inbred. In specific embodiments, the inbred line comprises homozygous alleles at about 95% or more of its loci. Various techniques known in the art can be used to facilitate and accelerate the breeding (*e.g.*, backcrossing) process, including, for example, the use of a greenhouse or growth chamber

with accelerated day/night cycles, the analysis of molecular markers to identify desirable progeny, and the like.

In addition to being used to create a backcross conversion, backcrossing can also be used in combination with pedigree breeding to modify an elite line of interest and a hybrid that is made using the modified elite line. As discussed previously, backcrossing can be used to transfer one or more specifically desirable traits from one line, the donor parent, to an inbred called the recurrent parent, which has overall good agronomic characteristics yet lacks that desirable trait or traits. However, the same procedure can be used to move the progeny toward the genotype of the recurrent parent but at the same time retain many components of the non-recurrent parent by stopping the backcrossing at an early stage and proceeding with selfing and selection. For example, an F1, such as a commercial hybrid, is created. This commercial hybrid may be backcrossed to one of its parent lines to create a BC1 or BC2. Progeny are selfed and selected so that the newly developed inbred has many of the attributes of the recurrent parent and yet several of the desired attributes of the non-recurrent parent. This approach leverages the value and strengths of the recurrent parent for use in new hybrids and breeding.

Therefore, an embodiment of this invention is a method of making a backcross conversion of an inbred line of interest comprising the steps of crossing a plant from the inbred line of interest with a donor plant comprising at least one mutant gene or transgene conferring a desired trait (*e.g.*, herbicide tolerance), selecting an F1 progeny plant comprising the mutant gene or transgene conferring the desired trait, and backcrossing the selected F1 progeny plant to a plant of the inbred line of interest. This method may further comprise the step of obtaining a molecular marker profile of the inbred line of interest and using the molecular marker profile to select for a progeny plant with the desired trait and the molecular marker profile of the inbred line of interest. In the same manner, this method may be used to produce an F1 hybrid seed by adding a final step of crossing the desired trait conversion of the inbred line of interest with a different plant to make F1 hybrid seed comprising a mutant gene or transgene conferring the desired trait.

Recurrent selection is a method used in a plant breeding program to improve a population of plants. The method entails individual plants cross pollinating with each other to form progeny. The progeny are grown and the superior progeny selected by any number of selection methods, which include individual plant, half-sib progeny, full-sib progeny, selfed progeny and topcrossing. The selected progeny are cross-pollinated with each other to form progeny for another population. This population is planted and again superior plants are

selected to cross pollinate with each other. Recurrent selection is a cyclical process and therefore can be repeated as many times as desired. The objective of recurrent selection is to improve the traits of a population. The improved population can then be used as a source of breeding material to obtain inbred lines to be used in hybrids or used as parents for a synthetic cultivar. A synthetic cultivar is the resultant progeny formed by the intercrossing of several selected inbreds.

Mass selection is a useful technique when used in conjunction with molecular marker enhanced selection. In mass selection seeds from individuals are selected based on phenotype and/or genotype. These selected seeds are then bulked and used to grow the next generation. Bulk selection requires growing a population of plants in a bulk plot, allowing the plants to self-pollinate, harvesting the seed in bulk and then using a sample of the seed harvested in bulk to plant the next generation. Instead of self pollination, directed pollination could be used as part of the breeding program.

Mutation breeding is one of many methods that could be used to introduce new traits into an elite line. Mutations that occur spontaneously or are artificially induced can be useful sources of variability for a plant breeder. The goal of artificial mutagenesis is to increase the rate of mutation for a desired characteristic. Mutation rates can be increased by many different means including temperature, long-term seed storage, tissue culture conditions, radiation such as X-rays, Gamma rays (*e.g.*, cobalt 60 or cesium 137), neutrons, (product of nuclear fission of uranium 235 in an atomic reactor), Beta radiation (emitted from radioisotopes such as phosphorus 32 or carbon 14), or ultraviolet radiation (preferably from 2500 to 2900nm), or chemical mutagens (such as base analogues (5-bromo-uracil), related compounds (8-ethoxy caffeine), antibiotics (streptonigrin), alkylating agents (sulfur mustards, nitrogen mustards, epoxides, ethylenamines, sulfates, sulfonates, sulfones, lactones), azide, hydroxylamine, nitrous acid, or acridines. Once a desired trait is observed through mutagenesis the trait may then be incorporated into existing germplasm by traditional breeding techniques, such as backcrossing. Details of mutation breeding can be found in "Principals of Cultivar Development" Fehr, 1993 Macmillan Publishing Company the disclosure of which is incorporated herein by reference. In addition, mutations created in other lines may be used to produce a backcross conversion of elite lines that comprises such mutations.

IV. Methods of Modulating Expression

In some embodiments, the activity and/or level of the polypeptide is modulated (i.e., increased or decreased). An increase in the level and/or activity of the polypeptide can be achieved by providing the polypeptide to the plant. As discussed elsewhere herein, many methods are known the art for providing a polypeptide to a plant including, but not limited to, direct introduction of the polypeptide into the plant, introducing into the plant (transiently or stably) a polynucleotide construct encoding a polypeptide having the desired activity. It is also recognized that the methods of the invention may employ a polynucleotide that is not capable of directing, in the transformed plant, the expression of a protein or an RNA. Thus, the level and/or activity of a polypeptide may be modulated by altering the gene encoding the polypeptide or its promoter. See, *e.g.*, Kmiec, U.S. Patent 5,565,350; Zarling *et al.*, PCT/US93/03868. Therefore mutagenized plants that carry mutations in genes, where the mutations increase expression of the gene or increase the activity of the encoded polypeptide are provided.

In other embodiments, the activity and/or level of a polypeptide is reduced or eliminated by introducing into a plant a polynucleotide that inhibits the level or activity of the polypeptide. The polynucleotide may inhibit the expression of the polypeptide directly, by preventing translation of the corresponding messenger RNA, or indirectly, by encoding a polypeptide that inhibits the transcription or translation of a gene encoding the protein. Methods for inhibiting or eliminating the expression of a gene in a plant are well known in the art, and any such method may be used in the present invention to inhibit the expression of a gene in a plant. In other embodiments of the invention, the activity of a polypeptide is reduced or eliminated by transforming a plant cell with a sequence encoding a polypeptide that inhibits the activity of the polypeptide. In other embodiments, the activity of a polypeptide may be reduced or eliminated by disrupting the gene encoding the polypeptide. The invention encompasses mutagenized plants that carry mutations in genes of interest, where the mutations reduce expression of the gene or inhibit the activity of the encoded polypeptide.

Reduction of the activity of specific genes (also known as gene silencing or gene suppression) is desirable for several aspects of genetic engineering in plants. Many techniques for gene silencing are well known to one of skill in the art, including, but not limited to, antisense technology (see, *e.g.*, Sheehy *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85: 8805-8809; and U.S. Patent Nos. 5,107,065; 5,453,566; and 5,759,829); cosuppression (*e.g.*, Taylor (1997) *Plant Cell* 9: 1245; Jorgensen (1990) *Trends Biotech.* 8(12): 340-344;

Flavell (1994) *Proc. Natl. Acad. Sci. USA* 91: 3490-3496; Finnegan *et al.* (1994) *Bio/Technology* 12: 883-888; and Neuhuber *et al.* (1994) *Mol. Gen. Genet.* 244: 230-241); RNA interference (Napoli *et al.* (1990) *Plant Cell* 2: 279-289; U.S. Patent No. 5,034,323; Sharp (1999) *Genes Dev.* 13: 139-141; Zamore *et al.* (2000) *Cell* 101: 25-33; and Montgomery *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95: 15502-15507), virus-induced gene silencing (Burton *et al.* (2000) *Plant Cell* 12: 691-705; and Baulcombe (1999) *Curr. Op. Plant Bio.* 2: 109-113); target-RNA-specific ribozymes (Haseloff *et al.* (1988) *Nature* 334: 585-591); hairpin structures (Smith *et al.* (2000) *Nature* 407: 319-320; WO 99/53050; WO 02/00904; WO 98/53083; Chuang and Meyerowitz (2000) *Proc. Natl. Acad. Sci. USA* 97: 4985-4990; Stoutjesdijk *et al.* (2002) *Plant Physiol.* 129: 1723-1731; Waterhouse and Helliwell (2003) *Nat. Rev. Genet.* 4: 29-38; Pandolfini *et al. BMC Biotechnology* 3: 7, U.S. Patent Publication No. 20030175965; Panstruga *et al.* (2003) *Mol. Biol. Rep.* 30: 135-140; Wesley *et al.* (2001) *Plant J.* 27: 581-590; Wang and Waterhouse (2001) *Curr. Opin. Plant Biol.* 5: 146-150; U.S. Patent Publication No. 20030180945; and WO 02/00904, all of which are herein incorporated by reference); ribozymes (Steinecke *et al.* (1992) *EMBO J.* 11: 1525; and Perriman *et al.* (1993) *Antisense Res. Dev.* 3: 253); oligonucleotide-mediated targeted modification (*e.g.*, WO 03/076574 and WO 99/25853); Zn-finger targeted molecules (*e.g.*, WO 01/52620; WO 03/048345; and WO 00/42219); transposon tagging (Maes *et al.* (1999) *Trends Plant Sci.* 4: 90-96; Dharmapuri and Sonti (1999) *FEMS Microbiol. Lett.* 179: 53-59; Meissner *et al.* (2000) *Plant J.* 22: 265-274; Phogat *et al.* (2000) *J. Biosci.* 25: 57-63; Walbot (2000) *Curr. Opin. Plant Biol.* 2: 103-107; Gai *et al.* (2000) *Nucleic Acids Res.* 28: 94-96; Fitzmaurice *et al.* (1999) *Genetics* 153: 1919-1928; Bensen *et al.* (1995) *Plant Cell* 7: 75-84; Mena *et al.* (1996) *Science* 274: 1537-1540; and U.S. Patent No. 5,962,764); each of which is herein incorporated by reference; and other methods or combinations of the above methods known to those of skill in the art.

It is recognized that antisense constructions complementary to at least a portion of the messenger RNA (mRNA) for a polynucleotide of interest can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having at least 70%, optimally 80%, more optimally 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50

nucleotides, 100 nucleotides, 200 nucleotides, 300, 400, 450, 500, 550, or greater may be used.

Polynucleotides may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using polynucleotides in the sense orientation are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a polynucleotide that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, generally greater than about 65%, 85%, or 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference. Thus, many methods may be used to reduce or eliminate the activity of a polypeptide. More than one method may be used to reduce the activity of a single polypeptide. In addition, combinations of methods may be employed to reduce or eliminate the activity of a polypeptide.

In one embodiment, the expression level of a polypeptide may be measured directly, for example, by assaying for the level of the polynucleotide or polypeptide or a known metabolite in the plant (*e.g.*, by assaying for the level of N-acetylglyphosate ("NAG") in a plant containing a GAT gene), or indirectly, for example, by evaluating the plant containing it for the trait to be conferred by the polypeptide, *e.g.*, herbicide resistance.

V. Methods of Controlling Weeds

Methods are provided for controlling weeds in an area of cultivation, preventing the development or the appearance of herbicide resistant weeds in an area of cultivation, producing a crop, and increasing crop safety. The term "controlling," and derivations thereof, for example, as in "controlling weeds" refers to one or more of inhibiting the growth, germination, reproduction, and/or proliferation of; and/or killing, removing, destroying, or otherwise diminishing the occurrence and/or activity of a weed.

The glyphosate/ALS inhibitor plants of the invention display a modified tolerance to herbicides and therefore allow for the application of one or more herbicides at rates that would significantly damage control plants and further allow for the application of combinations of herbicides at lower concentrations than normally applied which still continue to selectively control weeds. In addition, the glyphosate/ALS inhibitor-tolerant plants of the invention can be used in combination with herbicide blends technology and thereby make the

application of chemical pesticides more convenient, economical, and effective for the producer.

The methods of the invention comprise planting the area of cultivation with glyphosate/ALS inhibitor-tolerant crop seeds or plants of the invention, and in specific embodiments, applying to any crop, crop part, weed or area of cultivation thereof an effective amount of a herbicide of interest. It is recognized that the herbicide can be applied before or after the crop is planted in the area of cultivation. Such herbicide applications can include an application of glyphosate, an ALS inhibitor chemistry, or any combination thereof. In specific embodiments, a mixture of ALS inhibitor chemistry in combination with glyphosate is applied to the glyphosate/ALS inhibitor-tolerant plant, wherein the effective concentration of at least two of the ALS inhibitor chemistries would significantly damage an appropriate control plant. In one non-limiting embodiment, the herbicide comprises at least one of a sulfonylaminocarbonyltriazolinone; a triazolopyrimidine; a pyrimidinyl(thio)benzoate; an imidazolinone; a triazine; and/or a phosphinic acid.

In another non-limiting embodiment, the combination of herbicides comprises glyphosate, imazapyr, chlorimuron-ethyl, quizalofop, and fomesafen, wherein said effective amount is tolerated by the crop and controls weeds. As disclosed elsewhere herein, any effective amount of these herbicides can be applied. In specific embodiments, this combination of herbicides comprises an effective amount of glyphosate comprising about 1110 to about 1130 g ai/hectare; an effective amount of imazapyr comprising about 7.5 to about 27.5 g ai/hectare; an effective amount of chlorimuron-ethyl comprising about 7.5 to about 27.5 g ai/hectare; an effective amount of quizalofop comprising about 50 to about 70 g ai/hectare; and, an effective amount of fomesafen comprising about 240 to about 260 g ai/hectare.

In other embodiments, at least a combination of two herbicides are applied, wherein the combination does not include glyphosate. In other embodiments, at least one ALS inhibitor and glyphosate is applied to the plant. More details regarding the various herbicide combinations that can be employed in the methods of the invention are discussed elsewhere herein.

In one embodiment, the method of controlling weeds comprises planting the area with a glyphosate/ALS inhibitor-tolerant crop seeds or plant and applying to the crop, crop part, seed of said crop or the area under cultivation, an effective amount of a herbicide, wherein said effective amount comprises

i) an amount that is not tolerated by a first control crop when applied to the first control crop, crop part, seed or the area of cultivation, wherein said first control crop expresses a first polynucleotide that confers tolerance to glyphosate and does not express a second polynucleotide that encodes an ALS inhibitor-tolerant polypeptide;

ii) an amount that is not tolerated by a second control crop when applied to the second crop, crop part, seed or the area of cultivation, wherein said second control crop expresses the second polynucleotide and does not express the first polynucleotide; and,

iii) an amount that is tolerated when applied to the glyphosate/ALS inhibitor-tolerant crop, crop part, seed, or the area of cultivation thereof. The herbicide can comprise a combination of herbicides that either includes or does not include glyphosate. In specific embodiments, the combination of herbicides comprises ALS inhibitor chemistries as discussed in further detail below.

In another embodiment, the method of controlling weeds comprises planting the area with a glyphosate/ALS inhibitor-tolerant crop seeds or plant and applying to the crop, crop part, seed of said crop or the area under cultivation, an effective amount of a herbicide, wherein said effective amount comprises a level that is above the recommended label use rate for the crop, wherein said effective amount is tolerated when applied to the glyphosate/ALS inhibitor-tolerant crop, crop part, seed, or the area of cultivation thereof. The herbicide applied can comprise a combination of herbicides that either includes or does not include glyphosate. In specific embodiments, the combination of herbicides comprises at least one ALS inhibitor chemistries as discussed in further detail below. Further herbicides and combinations thereof that can be employed in the various methods of the invention are discussed in further detail below.

In another non-limiting embodiment, the herbicide applied in any method disclosed herein does not comprise glyphosate, chlorimuron-methyl, rimsulfuron, tribenuron-methyl or thifensufuron-methyl.

a. Types of Herbicides

Any herbicide can be applied to the glyphosate/ALS inhibitor-tolerant crop, crop part, or the area of cultivation containing said crop plant. Classifications of herbicides (*i.e.*, the grouping of herbicides into classes and subclasses) is well-known in the art and includes classifications by HRAC (Herbicide Resistance Action Committee) and WSSA (the Weed Science Society of America) (see also, Retzinger and Mallory-Smith (1997) *Weed*

Technology 11: 384-393). An abbreviated version of the HRAC classification (with notes regarding the corresponding WSSA group) is set forth below in Table 1.

Herbicides can be classified by their mode of action and/or site of action and can also be classified by the time at which they are applied (*e.g.*, preemergent or postemergent), by the method of application (*e.g.*, foliar application or soil application), or by how they are taken up by or affect the plant. For example, thifensulfuron-methyl and tribenuron-methyl are applied to the foliage of a crop (*e.g.*, maize) and are generally metabolized there, while rimsulfuron and chlorimuron-ethyl are generally taken up through both the roots and foliage of a plant. "Mode of action" generally refers to the metabolic or physiological process within the plant that the herbicide inhibits or otherwise impairs, whereas "site of action" generally refers to the physical location or biochemical site within the plant where the herbicide acts or directly interacts. Herbicides can be classified in various ways, including by mode of action and/or site of action (see, *e.g.*, Table 1).

Often, a herbicide-tolerance gene that confers tolerance to a particular herbicide or other chemical on a plant expressing it will also confer tolerance to other herbicides or chemicals in the same class or subclass, for example, a class or subclass set forth in Table 1. Thus, in some embodiments of the invention, a transgenic plant of the invention is tolerant to more than one herbicide or chemical in the same class or subclass, such as, for example, an inhibitor of PPO, a sulfonylurea, or a synthetic auxin.

Typically, the plants of the present invention can tolerate treatment with different types of herbicides (*i.e.*, herbicides having different modes of action and/or different sites of action) as well as with higher amounts of herbicides than previously known plants, thereby permitting improved weed management strategies that are recommended in order to reduce the incidence and prevalence of herbicide-tolerant weeds. Specific herbicide combinations can be employed to effectively control weeds.

The invention thereby provides a transgenic crop plant which can be selected for use in crop production based on the prevalence of herbicide-tolerant weed species in the area where the transgenic crop is to be grown. Methods are known in the art for assessing the herbicide tolerance of various weed species. Weed management techniques are also known in the art, such as for example, crop rotation using a crop that is tolerant to a herbicide to which the local weed species are not tolerant. A number of entities monitor and publicly report the incidence and characteristics of herbicide-tolerant weeds, including the Herbicide Resistance Action Committee (HRAC), the Weed Science Society of America, and various state agencies (see, *e.g.*, see, for example, herbicide tolerance scores for various broadleaf

weeds from the 2004 Illinois Agricultural Pest Management Handbook), and one of skill in the art would be able to use this information to determine which crop and herbicide combinations should be used in a particular location.

These entities also publish advice and guidelines for preventing the development and/or appearance of and controlling the spread of herbicide tolerant weeds (see, *e.g.*, Owen and Hartzler (2004), *2005 Herbicide Manual for Agricultural Professionals*, Pub. WC 92 Revised (Iowa State University Extension, Iowa State University of Science and Technology, Ames, Iowa); *Weed Control for Corn, Soybeans, and Sorghum*, Chapter 2 of "2004 Illinois Agricultural Pest Management Handbook" (University of Illinois Extension, University of Illinois at Urbana-Champaign, Illinois)); *Weed Control Guide for Field Crops*, MSU Extension Bulletin E434 (Michigan State University, East Lansing, Michigan)).

Table 1: Abbreviated version of HRAC Herbicide Classification

I. ALS Inhibitors (WSSA Group 2)
  A. Sulfonylureas
    1. Azimsulfuron
    2. Chlorimuron-ethyl
    3. Metsulfuron-methyl
    4. Nicosulfuron
    5. Rimsulfuron
    6. Sulfometuron-methyl
    7. Thifensulfuron-methyl
    8. Tribenuron-methyl
    9. Amidosulfuron
    10. Bensulfuron-methyl
    11. Chlorsulfuron
    12. Cinosulfuron
    13. Cyclosulfamuron
    14. Ethametsulfuron-methyl
    15. Ethoxysulfuron
    16. Flazasulfuron
    17. Flupyrsulfuron- methyl
    18. Foramsulfuron
    19. Imazosulfuron
    20. Iodosulfuron-methyl
    21. Mesosulfuron-methyl
    22. Oxasulfuron
    23. Primisulfuron-methyl
    24. Prosulfuron
    25. Pyrazosulfuron-ethyl
    26. Sulfosulfuron
    27. Triasulfuron
    28. Trifloxysulfuron

29. Triflusulfuron-methyl
30. Tritosulfuron
31. Halosulfuron-methyl
32. Flucetosulfuron

B. Sulfonylaminocarbonyltriazolinones
1. Flucarbazone
2. Procarbazone

C. Triazolopyrimidines
1. Cloransulam-methyl
2. Flumetsulam
3. Diclosulam
4. Florasulam
5. Metosulam
6. Penoxsulam
7. Pyroxsulam

D. Pyrimidinyloxy(thio)benzoates
1. Bispyribac
2. Pyriftalid
3. Pyribenzoxim
4. Pyrithiobac
5. Pyriminobac-methyl

E. Imidazolinones
1. Imazapyr
2. Imazethapyr
3. Imazaquin
4. Imazapic
5. Imazamethabenz-methyl
6. Imazamox

II. Other Herbicides--Active Ingredients/ Additional Modes of Action

A. Inhibitors of Acetyl CoA carboxylase (ACCase) (WSSA Group 1)

1. Aryloxyphenoxypropionates ('FOPs')
a. Quizalofop-P-ethyl
b. Diclofop-methyl
c. Clodinafop-propargyl
d. Fenoxaprop-P-ethyl
e. Fluazifop-P-butyl
f. Propaquizafop
g. Haloxyfop-P-methyl
h. Cyhalofop-butyl
i. Quizalofop-P-ethyl

2. Cyclohexanediones ('DIMs')
   a. Alloxydim
   b. Butroxydim
   c. Clethodim
   d. Cycloxydim
   e. Sethoxydim
   f. Tepraloxydim
   g. Tralkoxydim

B. Inhibitors of Photosystem II—HRAC Group C1/ WSSA Group 5

1. Triazines
   a. Ametryne
   b. Atrazine
   c. Cyanazine
   d. Desmetryne
   e. Dimethametryne
   f. Prometon
   g. Prometryne
   h. Propazine
   i. Simazine
   j. Simetryne
   k. Terbumeton
   l. Terbuthylazine
   m. Terbutryne
   n. Trietazine

2. Triazinones
   a. Hexazinone
   b. Metribuzin
   c. Metamitron

3. Triazolinone
   a. Amicarbazone

4. Uracils
   a. Bromacil
   b. Lenacil
   c. Terbacil

5. Pyridazinones
   a. Pyrazon

6. Phenyl carbamates
   a. Desmedipham
   b. Phenmedipham

C. Inhibitors of Photosystem II--HRAC Group C2/WSSA Group 7

1. Ureas
   a. Fluometuron
   b. Linuron

c. Chlorobromuron
d. Chlorotoluron
e. Chloroxuron
f. Dimefuron
g. Diuron
h. Ethidimuron
i. Fenuron
j. Isoproturon
k. Isouron
l. Methabenzthiazuron
m. Metobromuron
n. Metoxuron
o. Monolinuron
p. Neburon
q. Siduron
r. Tebuthiuron

2. Amides
a. Propanil
b. Pentanochlor

D. Inhibitors of Photosystem II--HRAC Group C3/ WSSA Group 6
1. Nitriles
a. Bromofenoxim
b. Bromoxynil
c. Ioxynil

2. Benzothiadiazinone (Bentazon)
a. Bentazon

3. Phenylpyridazines
a. Pyridate
b. Pyridafol

E. Photosystem-I-electron diversion (Bipyridyliums) (WSSA Group 22)
1. Diquat
2. Paraquat

F. Inhibitors of PPO (protoporphyrinogen oxidase) (WSSA Group 14)
1. Diphenylethers
a. Acifluorfen-Na
b. Bifenox
c. Chlomethoxyfen
d. Fluoroglycofen-ethyl
e. Fomesafen
f. Halosafen
g. Lactofen
h. Oxyfluorfen

2. Phenylpyrazoles
   a. Fluazolate
   b. Pyraflufen-ethyl

3. N-phenylphthalimides
   a. Cinidon-ethyl
   b. Flumioxazin
   c. Flumiclorac-pentyl

4. Thiadiazoles
   a. Fluthiacet-methyl
   b. Thidiazimin

5. Oxadiazoles
   a. Oxadiazon
   b. Oxadiargyl

6. Triazolinones
   a. Carfentrazone-ethyl
   b. Sulfentrazone

7. Oxazolidinediones
   a. Pentoxazone

8. Pyrimidindiones
   a. Benzfendizone
   b. Butafenicil

9. Others
   a. Pyrazogyl
   b. Profluazol

G. Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS) (WSSA Group 12)

1. Pyridazinones
   a. Norflurazon

2. Pyridinecarboxamides
   a. Diflufenican
   b. Picolinafen

3. Others
   a. Beflubutamid
   b. Fluridone
   c. Flurochloridone
   d. Flurtamone

H. Bleaching: Inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD) (WSSA Group 28)

1. Triketones
   a. Mesotrione
   b. Sulcotrione

2. Isoxazoles
   a. Isoxachlortole
   b. Isoxaflutole

3. Pyrazoles
   a. Benzofenap
   b. Pyrazoxyfen
   c. Pyrazolynate

4. Others
   a. Benzobicyclon

I. Bleaching: Inhibition of carotenoid biosynthesis (unknown target) (WSSA Group 11 and 13)

1. Triazoles (WSSA Group 11)
   a. Amitrole

2. Isoxazolidinones (WSSA Group 13)
   a. Clomazone

3. Ureas
   a. Fluometuron

3. Diphenylether
   a. Aclonifen

J. Inhibition of EPSP Synthase

1. Glycines (WSSA Group 9)
   a. Glyphosate
   b. Sulfosate

K. Inhibition of glutamine synthetase

1. Phosphinic Acids
   a. Glufosinate-ammonium
   b. Bialaphos

L. Inhibition of DHP (dihydropteroate) synthase (WSSA Group 18)

1 Carbamates
   a. Asulam

M. Microtubule Assembly Inhibition (WSSA Group 3)

1. Dinitroanilines
   a. Benfluralin

b. Butralin
c. Dinitramine
d. Ethalfluralin
e. Oryzalin
f. Pendimethalin
g. Trifluralin

2. Phosphoroamidates
a. Amiprophos-methyl
b. Butamiphos

3. Pyridines
a. Dithiopyr
b. Thiazopyr

4. Benzamides
a. Pronamide
b. Tebutam

5. Benzenedicarboxylic acids
a. Chlorthal-dimethyl

N. Inhibition of mitosis/microtubule organization WSSA Group 23)

1. Carbamates
a. Chlorpropham
b. Propham
c. Carbetamide

O. Inhibition of cell division (Inhibition of very long chain fatty acids as proposed mechanism; WSSA Group 15)

1. Chloroacetamides
a. Acetochlor
b. Alachlor
c. Butachlor
d. Dimethachlor
e. Dimethanamid
f. Metazachlor
g. Metolachlor
h. Pethoxamid
i. Pretilachlor
j. Propachlor
k. Propisochlor
l. Thenylchlor

2. Acetamides
a. Diphenamid
b. Napropamide
c. Naproanilide

3. Oxyacetamides
a. Flufenacet

b. Mefenacet

4. Tetrazolinones
   a. Fentrazamide

5. Others
   a. Anilofos
   b. Cafenstrole
   c. Indanofan
   d. Piperophos

P. Inhibition of cell wall (cellulose) synthesis

1. Nitriles (WSSA Group 20)
   a. Dichlobenil
   b. Chlorthiamid

2. Benzamides (isoxaben (WSSA Group 21))
   a. Isoxaben

3. Triazolocarboxamides (flupoxam)
   a. Flupoxam

Q. Uncoupling (membrane disruption): (WSSA Group 24)

1. Dinitrophenols
   a. DNOC
   b. Dinoseb
   c. Dinoterb

R. Inhibition of Lipid Synthesis by other than ACC inhibition

1. Thiocarbamates (WSSA Group 8)
   a. Butylate
   b. Cycloate
   c. Dimepiperate
   d. EPTC
   e. Esprocarb
   f. Molinate
   g. Orbencarb
   h. Pebulate
   i. Prosulfocarb
   j. Benthiocarb
   k. Tiocarbazil
   l. Triallate
   m. Vernolate

2. Phosphorodithioates
   a. Bensulide

3. Benzofurans
   a. Benfuresate
   b. Ethofumesate

4. Halogenated alkanoic acids (WSSA Group 26)
    a. TCA
    b. Dalapon
    c. Flupropanate

S. Synthetic auxins (IAA-like) (WSSA Group 4)
1. Phenoxycarboxylic acids
    a. Clomeprop
    b. 2,4-D
    c. Mecoprop

2. Benzoic acids
    a. Dicamba
    b. Chloramben
    c. TBA

3. Pyridine carboxylic acids
    a. Clopyralid
    b. Fluroxypyr
    c. Picloram
    d. Tricyclopyr
4. Quinoline carboxylic acids
    a. Quinclorac
    b. Quinmerac

5. Others (benazolin-ethyl)
    a. Benazolin-ethyl

T. Inhibition of Auxin Transport
1. Phthalamates; semicarbazones (WSSA Group 19)
    a. Naptalam
    b. Diflufenzopyr-Na

U. Other Mechanism of Action
1. Arylaminopropionic acids
    a. Flamprop-M-methyl /-isopropyl

2. Pyrazolium
    a. Difenzoquat

3. Organoarsenicals
    a. DSMA
    b. MSMA

4. Others
    a. Bromobutide
    b. Cinmethylin
    c. Cumyluron
    d. Dazomet

e. Daimuron-methyl
f. Dimuron
g. Etobenzanid
h. Fosamine
i. Metam
j. Oxaziclomefone
k. Oleic acid
l. Pelargonic acid
m. Pyributicarb

In one embodiment, one ALS inhibitor or at least two ALS inhibitors are applied to the glyphosate/ALS inhibitor-tolerant crop or area of cultivation. In one non-limiting embodiment, the combination of ALS herbicides does not include glyphosate. The ALS inhibitor can be applied at any effective rate that selectively controls weeds and does not significantly damage the crop. In specific embodiments, at least one ALS inhibitor is applied at a level that would significantly damage an appropriate control plant. In other embodiments, at least one ALS inhibitor is applied above the recommended label use rate for the crop. In still other embodiments, a mixture of ALS inhibitors is applied at a lower rate than the recommended use rate and weeds continue to be selectively controlled. Herbicides that inhibit acetolactate synthase (also known as acetohydroxy acid synthase) and are therefore useful in the methods of the invention include sulfonylureas as listed in Table 1, including agriculturally suitable salts (*e.g.*, sodium salts) thereof; sulfonylaminocarbonyltriazolinones as listed in Table 1, including agriculturally suitable salts (*e.g.*, sodium salts) thereof; triazolopyrimidines as listed in Table 1, including agriculturally suitable salts (*e.g.*, sodium salts) thereof; pyrimidinyloxy(thio)benzoates as listed in Table 1, including agriculturally suitable salts (*e.g.*, sodium salts) thereof; and imidazolinones as listed in Table 1, including agriculturally suitable salts (*e.g.*, sodium salts) there. In some embodiments, methods of the invention comprise the use of a sulfonylurea which is not chlorimuron-ethyl, chlorsulfuron, rimsulfuron, thifensulfuron-methyl, or tribenuron-methyl.

In still further methods, glyphosate, in combination with another herbicide of interest, can be applied to the glyphosate/ALS inhibitor-tolerant plants or their area of cultivation. Non-limiting examples of glyphosate formations are set forth in Table 2. In specific embodiments, the glyphosate is in the form of a salt, such as, ammonium, isopropylammonium, potassium, sodium (including sesquisodium) or trimesium (alternatively named sulfosate). In still further embodiments, a mixture of a synergistically effective amount of a combination of glyphosate and an ALS inhibitor (such as a

sulfonylurea) is applied to the glyphosate/ALS inhibitor-tolerant plants or their area of cultivation.

Table 2. Glyphosate formulations comparisons.

Herbicide by Registered Trademark	*Manufacturer*	*Salt*	*Active ingredient per gallon*	*Acid equivalent per gallon*	*Apply: fl oz/ acre*	*Acid equivalent per acre*
Roundup Original	Monsanto	Isopropylamine	4	3	32	0.750
Roundup Original II	Monsanto	Isopropylamine	4	3	32	0.750
Roundup Original MAX	Monsanto	Potassium	5.5	4.5	22	0.773
Roundup UltraMax	Monsanto	Isopropylamine	5	3.68	26	0.748
Roundup UltraMax II	Monsanto	Potassium	5.5	4.5	22	0.773
Roundup Weathermax	Monsanto	Potassium	5.5	4.5	22	0.773
Touchdown	Syngenta	Diammonium	3.7	3	32	0.750
Touchdown HiTech	Syngenta	Potassium	6.16	5	20	0.781
Touchdown Total	Syngenta	Potassium	5.14	4.17	24	0.782
Durango	Dow AgroSciences	Isopropylamine	5.4	4	24	0.750
Glyphomax	Dow AgroSciences	Isopropylamine	4	3	32	0.750
Glyphomax Plus	Dow AgroSciences	Isopropylamine	4	3	32	0.750
Glyphomax XRT	Dow AgroSciences	Isopropylamine	4	3	32	0.750
Gly Star Plus	Albaugh/Agri Star	Isopropylamine	4	3	32	0.750
Gly Star 5	Albaugh/Agri Star	Isopropylamine	5.4	4	24	0.750
Gly Star Original	Albaugh/Agri Star	Isopropylamine	4	3	32	0.750
Gly-Flo	Micro Flo	Isopropylamine	4	3	32	0.750
Credit	Nufarm	Isopropylamine	4	3	32	0.750
Credit Extra	Nufarm	Isopropylamine	4	3	32	0.750
Credit Duo	Nufarm	Isopro.+ monoamm.	4	3	32	0.750
Credit Duo Extra	Nufarm	Isopro.+ monoamm.	4	3	32	0.750
Extra Credit 5	Nufarm	Isopropylamine	5	3.68	26	0.748
Cornerstone	Agriliance	Isopropylamine	4	3	32	0.750
Cornerstone Plus	Agriliance	Isopropylamine	4	3	32	0.750
Glyfos	Cheminova	Isopropylamine	4	3	32	0.750
Glyfos X-TRA	Cheminova	Isopropylamine	4	3	32	0.750
Rattler	Helena	Isopropylamine	4	3	32	0.750
Rattler Plus	Helena	Isopropylamine	4	3	32	0.750
Mirage	UAP	Isopropylamine	4	3	32	0.750
Mirage Plus	UAP	Isopropylamine	4	3	32	0.750
Glyphosate 41%	Helm Agro USA	Isopropylamine	4	3	32	0.750
Buccaneer	Tenkoz	Isopropylamine	4	3	32	0.750
Buccaneer Plus	Tenkoz	Isopropylamine	4	3	32	0.750
Honcho	Monsanto	Isopropylamine	4	3	32	0.750
Honcho Plus	Monsanto	Isopropylamine	4	3	32	0.750
Gly-4	Univ. Crop Prot. Alli.	Isopropylamine	4	3	32	0.750
Gly-4 Plus	Univ. Crop Prot. Alli.	Isopropylamine	4	3	32	0.750
ClearOut 41	Chemical Products Tech.	Isopropylamine	4	3	32	0.750
ClearOut 41 Plus	Chemical Products Tech.	Isopropylamine	4	3	32	0.750
Spitfire	Control Soultions	Isopropylamine	4	3	32	0.750
Spitfire Plus	Control Soultions	Isopropylamine	4	3	32	0.750
Glyphosate 4	FarmerSavor.com	Isopropylamine	4	3	32	0.750
FS Glyphosate Plus	Growmark	Isopropylamine	4	3	32	0.750
Glyphosate Original	Griffin, LLC.	Isopropylamine	4	3	32	0.750

Thus, in some embodiments, a transgenic plant of the invention is used in a method of growing a glyphosate/ALS inhibitor-tolerant crop by the application of herbicides to which the plant is tolerant. In this manner, treatment with a combination of one of more herbicides which include, but are not limited to: acetochlor, acifluorfen and its sodium salt, aclonifen,

acrolein (2-propenal), alachlor, alloxydim, ametryn, amicarbazone, amidosulfuron, aminopyralid, amitrole, ammonium sulfamate, anilofos, asulam, atrazine, azimsulfuron, beflubutamid, benazolin, benazolin-ethyl, bencarbazone, benfluralin, benfuresate, bensulfuron-methyl, bensulide, bentazone, benzobicyclon, benzofenap, bifenox, bilanafos, bispyribac and its sodium salt, bromacil, bromobutide, bromofenoxim, bromoxynil, bromoxynil octanoate, butachlor, butafenacil, butamifos, butralin, butroxydim, butylate, cafenstrole, carbetamide, carfentrazone-ethyl, catechin, chlomethoxyfen, chloramben, chlorbromuron, chlorflurenol-methyl, chloridazon, chlorimuron-ethyl, chlorotoluron, chlorpropham, chlorsulfuron, chlorthal-dimethyl, chlorthiamid, cinidon-ethyl, cinmethylin, cinosulfuron, clethodim, clodinafop-propargyl, clomazone, clomeprop, clopyralid, clopyralid-olamine, cloransulam-methyl, CUH-35 (2-methoxyethyl 2-[[[4-chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl](3-fluorobenzoyl)amino]carbonyl]-1-cyclohexene-1-carboxylate), cumyluron, cyanazine, cycloate, cyclosulfamuron, cycloxydim, cyhalofop-butyl, 2,4-D and its butotyl, butyl, isoctyl and isopropyl esters and its dimethylammonium, diolamine and trolamine salts, daimuron, dalapon, dalapon-sodium, dazomet, 2,4-DB and its dimethylammonium, potassium and sodium salts, desmedipham, desmetryn, dicamba and its diglycolammonium, dimethylammonium, potassium and sodium salts, dichlobenil, dichlorprop, diclofop-methyl, diclosulam, difenzoquat metilsulfate, diflufenican, diflufenzopyr, dimefuron, dimepiperate, dimethachlor, dimethametryn, dimethenamid, dimethenamid-P, dimethipin, dimethylarsinic acid and its sodium salt, dinitramine, dinoterb, diphenamid, diquat dibromide, dithiopyr, diuron, DNOC, endothal, EPTC, esprocarb, ethalfluralin, ethametsulfuron-methyl, ethofumesate, ethoxyfen, ethoxysulfuron, etobenzanid, fenoxaprop-ethyl, fenoxaprop-P-ethyl, fentrazamide, fenuron, fenuron-TCA, flamprop-methyl, flamprop-M-isopropyl, flamprop-M-methyl, flazasulfuron, florasulam, fluazifop-butyl, fluazifop-P-butyl, flucarbazone, flucetosulfuron, fluchloralin, flufenacet, flufenpyr, flufenpyr-ethyl, flumetsulam, flumiclorac-pentyl, flumioxazin, fluometuron, fluoroglycofen-ethyl, flupyrsulfuron-methyl and its sodium salt, flurenol, flurenol-butyl, fluridone, flurochloridone, fluroxypyr, flurtamone, fluthiacet-methyl, fomesafen, foramsulfuron, fosamine-ammonium, glufosinate, glufosinate-ammonium, glyphosate and its salts such as ammonium, isopropylammonium, potassium, sodium (including sesquisodium) and trimesium (alternatively named sulfosate), halosulfuron-methyl, haloxyfop-etotyl, haloxyfop-methyl, hexazinone, HOK-201 (*N*-(2,4-difluorophenyl)-1,5-dihydro-*N*-(1-methylethyl)-5-oxo-1-[(tetrahydro-2*H*-pyran-2-yl)methyl]-4*H*-1,2,4-triazole-4-carboxamide), imazamethabenz-methyl, imazamox, imazapic, imazapyr, imazaquin,

imazaquin-ammonium, imazethapyr, imazethapyr-ammonium, imazosulfuron, indanofan, iodosulfuron-methyl, ioxynil, ioxynil octanoate, ioxynil-sodium, isoproturon, isouron, isoxaben, isoxaflutole, isoxachlortole, lactofen, lenacil, linuron, maleic hydrazide, MCPA and its salts (e.g., MCPA-dimethylammonium, MCPA-potassium and MCPA-sodium, esters (e.g., MCPA-2-ethylhexyl, MCPA-butotyl) and thioesters (e.g., MCPA-thioethyl), MCPB and its salts (e.g., MCPB-sodium) and esters (e.g., MCPB-ethyl), mecoprop, mecoprop-P, mefenacet, mefluidide, mesosulfuron-methyl, mesotrione, metam-sodium, metamifop, metamitron, metazachlor, methabenzthiazuron, methylarsonic acid and its calcium, monoammonium, monosodium and disodium salts, methyldymron, metobenzuron, metobromuron, metolachlor, S-metholachlor, metosulam, metoxuron, metribuzin, metsulfuron-methyl, molinate, monolinuron, naproanilide, napropamide, naptalam, neburon, nicosulfuron, norflurazon, orbencarb, oryzalin, oxadiargyl, oxadiazon, oxasulfuron, oxaziclomefone, oxyfluorfen, paraquat dichloride, pebulate, pelargonic acid, pendimethalin, penoxsulam, pentanochlor, pentoxazone, perfluidone, pethoxyamid, phenmedipham, picloram, picloram-potassium, picolinafen, pinoxaden, piperofos, pretilachlor, primisulfuron-methyl, prodiamine, profoxydim, prometon, prometryn, propachlor, propanil, propaquizafop, propazine, propham, propisochlor, propoxycarbazone, propyzamide, prosulfocarb, prosulfuron, pyraclonil, pyraflufen-ethyl, pyrasulfotole, pyrazogyl, pyrazolynate, pyrazoxyfen, pyrazosulfuron-ethyl, pyribenzoxim, pyributicarb, pyridate, pyriftalid, pyriminobac-methyl, pyrimisulfan, pyrithiobac, pyrithiobac-sodium, pyroxsulam, quinclorac, quinmerac, quinoclamine, quizalofop-ethyl, quizalofop-P-ethyl, quizalofop-P-tefuryl, rimsulfuron, sethoxydim, siduron, simazine, simetryn, sulcotrione, sulfentrazone, sulfometuron-methyl, sulfosulfuron, 2,3,6-TBA, TCA, TCA-sodium, tebutam, tebuthiuron, tefuryltrione, tembotrione, tepraloxydim, terbacil, terbumeton, terbuthylazine, terbutryn, thenylchlor, thiazopyr, thiencarbazone, thifensulfuron-methyl, thiobencarb, tiocarbazil, topramezone, tralkoxydim, tri-allate, triasulfuron, triaziflam, tribenuron-methyl, triclopyr, triclopyr-butotyl, triclopyr-triethylammonium, tridiphane, trietazine, trifloxysulfuron, trifluralin, triflusulfuron-methyl, tritosulfuron and vernolate.

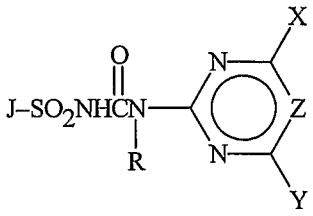
Other suitable herbicides and agricultural chemicals are known in the art, such as, for example, those described in WO 2005/041654. Other herbicides also include bioherbicides such as *Alternaria destruens* Simmons, *Colletotrichum gloeosporiodes* (Penz.) Penz. & Sacc., *Drechsiera monoceras* (MTB-951), *Myrothecium verrucaria* (Albertini & Schweinitz) Ditmar: Fries, *Phytophthora palmivora* (Butl.) Butl. and *Puccinia thlaspeos* Schub. Combinations of various herbicides can result in a greater-than-additive (*i.e.*, synergistic)

effect on weeds and/or a less-than-additive effect (*i.e.* safening) on crops or other desirable plants. In certain instances, combinations of glyphosate with other herbicides having a similar spectrum of control but a different mode of action will be particularly advantageous for preventing the development of resistant weeds. Herbicidally effective amounts of any particular herbicide can be easily determined by one skilled in the art through simple experimentation.

Herbicides may be classified into groups and/or subgroups as described herein above with reference to their mode of action, or they may be classified into groups and/or subgroups in accordance with their chemical structure.

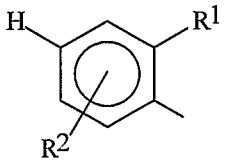
Sulfonamide herbicides have as an essential molecular structure feature a sulfonamide moiety (–$S(O)_2NH$–). As referred to herein, sulfonamide herbicides particularly comprise sulfonylurea herbicides, sulfonylaminocarbonyltriazolinone herbicides and triazolo-pyrimidine herbicides. In sulfonylurea herbicides the sulfonamide moiety is a component in a sulfonylurea bridge (–$S(O)_2NHC(O)NH(R)$–). In sulfonylurea herbicides the sulfonyl end of the sulfonylurea bridge is connected either directly or by way of an oxygen atom or an optionally substituted amino or methylene group to a typically substituted cyclic or acyclic group. At the opposite end of the sulfonylurea bridge, the amino group, which may have a substituent such as methyl (R being CH_3) instead of hydrogen, is connected to a heterocyclic group, typically a symmetric pyrimidine or triazine ring, having one or two substituents such as methyl, ethyl, trifluoromethyl, methoxy, ethoxy, methylamino, dimethylamino, ethylamino and the halogens. In sulfonylaminocarbonyltriazolinone herbicides, the sulfonamide moiety is a component is a sulfonylaminocarbonyl bridge (–$S(O)_2NHC(O)$–). In sulfonylamino-carbonyltriazolinone herbicides the sulfonyl end of the sulfonylaminocarbonyl bridge is typically connected to substituted phenyl ring. At the opposite end of the sulfonylaminocarbonyl bridge, the carbonyl is connected to the 1-position of a triazolinone ring, which is typically substituted with groups such as alkyl and alkoxy. In triazolo-pyrimidine herbicides the sulfonyl end of the sulfonamide moiety is connected to the 2-position of a substituted [1,2,4]triazolopyrimidine ring system and the amino end of the sulfonamide moiety is connected to a substituted aryl, typically phenyl, group or alternatively the amino end of the sulfonamide moiety is connected to the 2-position of a substituted [1,2,4]triazolopyrimidine ring system and the sulfonyl end of the sulfonamide moiety is connected to a substituted aryl, typically pyridinyl, group.

Representative of the sulfonylurea herbicides useful in the present invention are those of the formula:

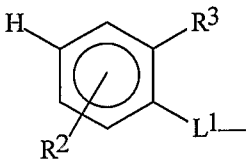


wherein:

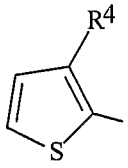
J is selected from the group consisting of



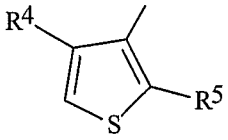
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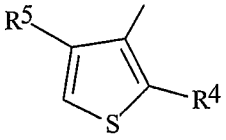
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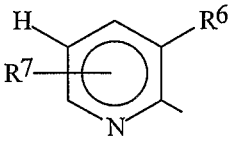
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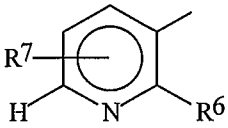
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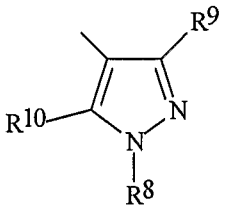
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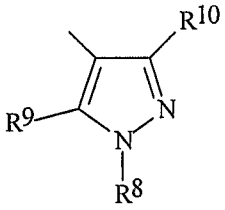
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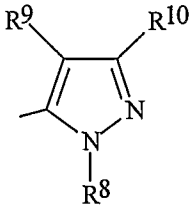
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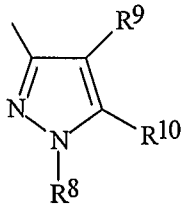
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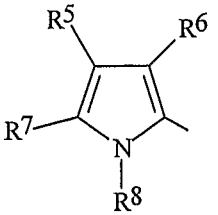
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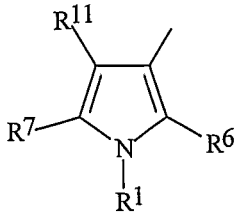
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J-11

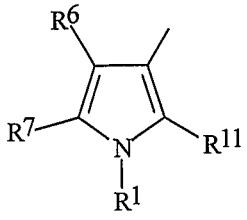


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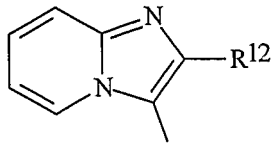
J-13

,



J-14

and



J-15

; or

J is $R^{13}SO_2N(CH_3)$-;

R is H or CH_3;

R^1 is F, Cl, Br, NO_2, C_1–C_4 alkyl, C_1–C_4 haloalkyl, C_3–C_4 cycloalkyl, C_2–C_4 haloalkenyl, C_1–C_4 alkoxy, C_1–C_4 haloalkoxy, C_2–C_4 alkoxyalkoxy, CO_2R^{14}, $C(O)NR^{15}R^{16}$, $SO_2NR^{17}R^{18}$, $S(O)_nR^{19}$, $C(O)R^{20}$, CH_2CN or L;

R^2 is H, F, Cl, Br, I, CN, CH_3, OCH_3, SCH_3, CF_3 or OCF_2H;

R^3 is Cl, NO_2, CO_2CH_3, $CO_2CH_2CH_3$, $C(O)CH_3$, $C(O)CH_2CH_3$, C(O)-cyclopropyl, $SO_2N(CH_3)_2$, SO_2CH_3, $SO_2CH_2CH_3$, OCH_3 or OCH_2CH_3;

R^4 is C_1–C_3 alkyl, C_1–C_2 haloalkyl, C_1–C_2 alkoxy, C_2–C_4 haloalkenyl, F, Cl, Br, NO_2, CO_2R^{14}, $C(O)NR^{15}R^{16}$, $SO_2NR^{17}R^{18}$, $S(O)_nR^{19}$, $C(O)R^{20}$ or L;

R^5 is H, F, Cl, Br or CH_3;

R^6 is C_1–C_3 alkyl optionally substituted with 0–3 F, 0–1 Cl and 0–1 C_3–C_4 alkoxyacetyloxy, or R^6 is C_1–C_2 alkoxy, C_2–C_4 haloalkenyl, F, Cl, Br, CO_2R^{14}, $C(O)NR^{15}R^{16}$, $SO_2NR^{17}R^{18}$, $S(O)_nR^{19}$, $C(O)R^{20}$ or L;

R^7 is H, F, Cl, CH_3 or CF_3;

R^8 is H, C_1–C_3 alkyl or pyridinyl;

R^9 is C_1–C_3 alkyl, C_1–C_2 alkoxy, F, Cl, Br, NO_2, CO_2R^{14}, $SO_2NR^{17}R^{18}$, $S(O)_nR^{19}$, OCF_2H, $C(O)R^{20}$, C_2–C_4 haloalkenyl or L;

R^{10} is H, Cl, F, Br, C_1–C_3 alkyl or C_1–C_2 alkoxy;

R^{11} is H, C_1–C_3 alkyl, C_1–C_2 alkoxy, C_2–C_4 haloalkenyl, F, Cl, Br, CO_2R^{14}, $C(O)NR^{15}R^{16}$, $SO_2NR^{17}R^{18}$, $S(O)_nR^{19}$, $C(O)R^{20}$ or L;

R^{12} is halogen, C_1–C_4 alkyl or C_1–C_3 alkylsulfonyl;

R^{13} is C_1–C_4 alkyl;

R^{14} is allyl, propargyl or oxetan-3-yl; or R^{14} is C_1–C_3 alkyl optionally substituted by at least one member independently selected from halogen, C_1–C_2 alkoxy and CN;

R^{15} is H, C_1–C_3 alkyl or C_1–C_2 alkoxy;

R^{16} is C_1–C_2 alkyl;

R^{17} is H, C_1–C_3 alkyl, C_1–C_2 alkoxy, allyl or cyclopropyl;

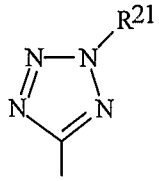
R^{18} is H or C_1–C_3 alkyl;

R^{19} is C_1–C_3 alkyl, C_1–C_3 haloalkyl, allyl or propargyl;

R^{20} is C_1–C_4 alkyl, C_1–C_4 haloalkyl or C_3–C_5 cycloalkyl optionally substituted by halogen;

n is 0, 1 or 2;

L is



;

L^1 is CH_2, NH or O;

R^{21} is H or C_1–C_3 alkyl;

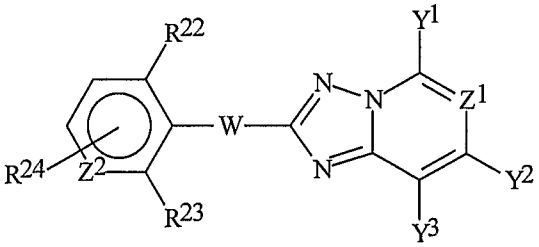
X is H, C_1–C_4 alkyl, C_1–C_4 alkoxy, C_1–C_4 haloalkoxy, C_1–C_4 haloalkyl, C_1–C_4 haloalkylthio, C_1–C_4 alkylthio, halogen, C_2–C_5 alkoxyalkyl, C_2–C_5 alkoxyalkoxy, amino, C_1–C_3 alkylamino or di(C_1–C_3 alkyl)amino;

Y is H, C_1–C_4 alkyl, C_1–C_4 alkoxy, C_1–C_4 haloalkoxy, C_1–C_4 alkylthio, C_1–C_4 haloalkylthio, C_2–C_5 alkoxyalkyl, C_2–C_5 alkoxyalkoxy, amino, C_1–C_3 alkylamino, di(C_1–C_3 alkyl)amino, C_3–C_4 alkenyloxy, C_3–C_4 alkynyloxy, C_2–C_5 alkylthioalkyl, C_2–C_5 alkylsulfinylalkyl, C_2–C_5 alkylsulfonylalkyl, C_1–C_4 haloalkyl, C_2–C_4 alkynyl, C_3–C_5 cycloalkyl, azido or cyano; and

Z is CH or N;

provided that (i) when one or both of X and Y is C_1 haloalkoxy, then Z is CH; and (ii) when X is halogen, then Z is CH and Y is OCH_3, OCH_2CH_3, $N(OCH_3)CH_3$, $NHCH_3$, $N(CH_3)_2$ or OCF_2H. Of note is the present single liquid herbicide composition comprising one or more sulfonylureas of Formula **I** wherein when R^6 is alkyl, said alkyl is unsubstituted.

Representative of the triazolopyrimidine herbicides contemplated for use in this invention are those of the formula:



wherein:

R^{22} and R^{23} each independently halogen, nitro, C_1–C_4 alkyl, C_1–C_4 haloalkyl, C_1–C_4 alkoxy, C_1–C_4 haloalkoxy or C_2–C_3 alkoxycarbonyl;

R^{24} is H, halogen, C_1–C_2 alkyl or C_1–C_2 alkoxy;

W is $-NHS(O)_2-$ or $-S(O)_2NH-$;

Y^1 is H, C_1–C_2 alkyl or C_1–C_2 alkoxy;

Y^2 is H, F, Cl, Br, C_1–C_2 alkyl or C_1–C_2 alkoxy;

Y^3 is H, F or methoxy;

Z^1 is CH or N; and

Z^2 is CH or N;

provided that at least one of Y^1 and Y^2 is other than H.

In the above Markush description of representative triazolopyrimidine herbicides, when W is -$NHS(O)_2$- the sulfonyl end of the sulfonamide moiety is connected to the [1,2,4]triazolopyrimidine ring system, and when W is -$S(O)_2NH$- the amino end of the sulfonamide moiety is connected to the [1,2,4]triazolopyrimidine ring system.

In the above recitations, the term "alkyl", used either alone or in compound words such as "alkylthio" or "haloalkyl" includes straight-chain or branched alkyl, such as, methyl, ethyl, *n*-propyl, *i*-propyl, or the different butyl isomers. "Cycloalkyl" includes, for example, cyclopropyl, cyclobutyl and cyclopentyl. "Alkenyl" includes straight-chain or branched alkenes such as ethenyl, 1-propenyl, 2-propenyl, and the different butenyl isomers. "Alkenyl" also includes polyenes such as 1,2-propadienyl and 2,4-butadienyl. "Alkynyl" includes straight-chain or branched alkynes such as ethynyl, 1-propynyl, 2-propynyl and the different butynyl isomers. "Alkynyl" can also include moieties comprised of multiple triple bonds such as 2,5-hexadiynyl. "Alkoxy" includes, for example, methoxy, ethoxy, *n*-propyloxy, isopropyloxy and the different butoxy isomers. "Alkoxyalkyl" denotes alkoxy substitution on alkyl. Examples of "alkoxyalkyl" include CH_3OCH_2, $CH_3OCH_2CH_2$, $CH_3CH_2OCH_2$, $CH_3CH_2CH_2CH_2OCH_2$ and $CH_3CH_2OCH_2CH_2$. "Alkoxyalkoxy" denotes alkoxy substitution on alkoxy. "Alkenyloxy" includes straight-chain or branched alkenyloxy moieties. Examples of "alkenyloxy" include $H_2C{=}CHCH_2O$, $(CH_3)CH{=}CHCH_2O$ and $CH_2{=}CHCH_2CH_2O$. "Alkynyloxy" includes straight-chain or branched alkynyloxy moieties. Examples of "alkynyloxy" include $HC{\equiv}CCH_2O$ and $CH_3C{\equiv}CCH_2O$. "Alkylthio" includes branched or straight-chain alkylthio moieties such as methylthio, ethylthio, and the different propylthio isomers. "Alkylthioalkyl" denotes alkylthio substitution on alkyl. Examples of "alkylthioalkyl" include CH_3SCH_2, $CH_3SCH_2CH_2$, $CH_3CH_2SCH_2$, $CH_3CH_2CH_2CH_2SCH_2$ and $CH_3CH_2SCH_2CH_2$; "alkylsulfinylalkyl" and "alkylsulfonylalkyl" include the corresponding sulfoxides and sulfones, respectively. Other substituents such as "alkylamino", "dialkylamino" are defined analogously.

The total number of carbon atoms in a substituent group is indicated by the "C_i–C_j" prefix where i and j are numbers from 1 to 5. For example, C_1–C_4 alkyl designates methyl through butyl, including the various isomers. As further examples, C_2 alkoxyalkyl designates CH_3OCH_2; C_3 alkoxyalkyl designates, for example, $CH_3CH(OCH_3)$, $CH_3OCH_2CH_2$ or $CH_3CH_2OCH_2$; and C_4 alkoxyalkyl designates the various isomers of an alkyl group

substituted with an alkoxy group containing a total of four carbon atoms, examples including $CH_3CH_2CH_2OCH_2$ and $CH_3CH_2OCH_2CH_2$.

The term "halogen", either alone or in compound words such as "haloalkyl", includes fluorine, chlorine, bromine or iodine. Further, when used in compound words such as "haloalkyl", said alkyl may be partially or fully substituted with halogen atoms which may be the same or different. Examples of "haloalkyl" include F_3C, $ClCH_2$, CF_3CH_2 and CF_3CCl_2. The terms "haloalkoxy", "haloalkylthio", and the like, are defined analogously to the term "haloalkyl". Examples of "haloalkoxy" include CF_3O, CCl_3CH_2O, $HCF_2CH_2CH_2O$ and CF_3CH_2O. Examples of "haloalkylthio" include CCl_3S, CF_3S, CCl_3CH_2S and $ClCH_2CH_2CH_2S$.

The following sulfonylurea herbicides illustrate the sulfonylureas useful for this invention: amidosulfuron (*N*-[[[[(4,6-dimethoxy-2-pyrimdinyl)amino]carbonyl]amino]-sulfonyl]-*N*-methylmethanesulfonamide), azimsulfuron (*N*-[[(4,6-dimethoxy-2-pyrimidinyl)-amino]carbonyl]-1-methyl-4-(2-methyl-2*H*-tetrazol-5-yl)-1*H*-pyrazole-5-sulfonamide), bensulfuron-methyl (methyl 2-[[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]-sulfonyl]methyl]benzoate), chlorimuron-ethyl (ethyl 2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoate), chlorsulfuron (2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide), cinosulfuron (*N*-[[(4,6-dimethoxy-1,3,5-triazin-2-yl)amino]carbonyl]-2-(2-methoxyethoxy)-benzenesulfonamide), cyclosulfamuron (*N*-[[[2-(cyclopropylcarbonyl)phenyl]amino]-sulfonyl]-N^1-(4,6-dimethoxypyrimidin-2-yl)urea), ethametsulfuron-methyl (methyl 2-[[[[[4-ethoxy-6-(methylamino)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]-benzoate), ethoxysulfuron (2-ethoxyphenyl [[(4,6-dimethoxy-2-pyrimidinyl)amino]-carbonyl]sulfamate), flazasulfuron (*N*-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-3-(trifluoromethyl)-2-pyridinesulfonamide), flucetosulfuron (1-[3-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-2-pyridinyl]-2-fluoropropyl methoxyacetate), flupyrsulfuron-methyl (methyl 2-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-amino]sulfonyl]-6-(trifluoromethyl)-3-pyridinecarboxylate), foramsulfuron (2-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-(formylamino)-*N,N*-dimethylbenzamide), halosulfuron-methyl (methyl 3-chloro-5-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-1-methyl-1*H*-pyrazole-4-carboxylate), imazosulfuron (2-chloro-*N*-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]imidazo[1,2-*a*]pyridine-3-sulfonamide), iodosulfuron-methyl (methyl 4-iodo-2-[[[[(4-methoxy-6-methyl-

1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate), mesosulfuron-methyl (methyl 2-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-4-[[(methylsulfonyl)amino]methyl]benzoate), metsulfuron-methyl (methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate), nicosulfuron (2-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-*N*,*N*-dimethyl-3-pyridinecarboxamide), oxasulfuron (3-oxetanyl 2-[[[[(4,6-dimethyl-2-pyrimidinyl)amino]-carbonyl]amino]sulfonyl]benzoate), primisulfuron-methyl (methyl 2-[[[[[4,6-bis(trifluoromethoxy)-2-pyrimidinyl]amino]carbonyl]amino]sulfonyl]benzoate), prosulfuron (*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]-2-(3,3,3-trifluoro-propyl)benzenesulfonamide), pyrazosulfuron-ethyl (ethyl 5-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-1-methyl-1*H*-pyrazole-4-carboxylate), rimsulfuron (*N*-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-3-(ethylsulfonyl)-2-pyridinesulfonamide), sulfometuron-methyl (methyl 2-[[[[(4,6-dimethyl-2-pyrimidinyl)-amino]carbonyl]amino]sulfonyl]benzoate), sulfosulfuron (*N*-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-2-(ethylsulfonyl)imidazo[1,2-*a*]pyridine-3-sulfonamide), thifensulfuron-methyl (methyl 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]-carbonyl]amino]sulfonyl]-2-thiophenecarboxylate), triasulfuron (2-(2-chloroethoxy)-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide), tribenuron-methyl (methyl 2-[[[[*N*-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-*N*-methylamino]carbonyl]amino]sulfonyl]benzoate), trifloxysulfuron (*N*-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-3-(2,2,2-trifluoroethoxy)-2-pyridinesulfonamide), triflusulfuron-methyl (methyl 2-[[[[[4-dimethylamino)-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]-3-methylbenzoate) and tritosulfuron (*N*-[[[4-methoxy-6-(trifluoromethyl)-1,3,5-triazin-2-yl]amino]carbonyl]-2-(trifluoromethyl)benzene-sulfonamide).

The following triazolopyrimidine herbicides illustrate the triazolopyrimidines useful for this invention: cloransulam-methyl (methyl 3-chloro-2-[[(5-ethoxy-7-fluoro[1,2,4]-triazolo[1,5-*c*]pyrimidin-2-yl)sulfonyl]amino]benzoate, diclosulam (*N*-(2,6-dichlorophenyl)-5-ethoxy-7-fluoro[1,2,4]triazolo[1,5-*c*]pyrimidine-2-sulfonamide, florasulam (*N*-(2,6-difluorophenyl)-8-fluoro-5-methoxy[1,2,4]triazolo[1,5-*c*]pyrimidine-2-sulfonamide), flumetsulam (*N*-(2,6-difluorophenyl)-5-methyl[1,2,4]triazolo[1,5-*a*]pyrimidine-2-sulfonamide), metosulam (*N*-(2,6-dichloro-3-methylphenyl)-5,7-dimethoxy[1,2,4]triazolo-[1,5-*a*]pyrimidine-2-sulfonamide), penoxsulam (2-(2,2-difluoroethoxy)-*N*-(5,8-dimethoxy-[1,2,4]triazolo[1,5-*c*]pyrimidin-2-yl)-6-(trifluoromethyl)benzenesulfonamide) and

pyroxsulam (*N*-(5,7-dimethoxy[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide).

The following sulfonylaminocarbonyltriazolinone herbicides illustrate the sulfonylaminocarbonyltriazolinones useful for this invention: flucarbazone (4,5-dihydro-3-methoxy-4-methyl-5-oxo-*N*-[[2-(trifluoromethoxy)phenyl]sulfonyl]-1*H*-1,2,4-triazole-1-carboxamide) and procarbazone (methyl 2-[[[(4,5-dihydro-4-methyl-5-oxo-3-propoxy-1*H*-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]benzoate).

Additional herbicides include phenmedipham, triazolinones, and the herbicides disclosed in WO2006/012981, herein incorporated by reference in its entirety.

The methods further comprise applying to the crop and the weeds in the field a sufficient amount of at least one herbicide to which the crop seeds or plants is tolerant, such as, for example, glyphosate, a hydroxyphenylpyruvatedioxygenase inhibitor (*e.g.*, mesotrione or sulcotrione), a phytoene desaturase inhibitor (*e.g.*, diflufenican), a pigment synthesis inhibitor, sulfonamide, imidazolinone, bialaphos, phosphinothricin, azafenidin, butafenacil, sulfosate, glufosinate, triazolopyrimidine, pyrimidinyloxy(thio)benzoate, or sulonylaminocarbonyltriazolinone, an acetyl Co-A carboxylase inhibitor such as quizalofop-P-ethyl, a synthetic auxin such as quinclorac, or a protox inhibitor to control the weeds without significantly damaging the crop plants.

b. Effective Amount of a Herbicide

Generally, the effective amount of herbicide applied to the field is sufficient to selectively control the weeds without significantly affecting the crop. "Weed" as used herein refers to a plant which is not desirable in a particular area. Conversely, a "crop plant" as used herein refers to a plant which is desired in a particular area, such as, for example, a soybean plant. Thus, in some embodiments, a weed is a non-crop plant or a non-crop species, while in some embodiments, a weed is a crop species which is sought to be eliminated from a particular area, such as, for example, an inferior and/or non-transgenic maize plant in a field planted with transgenic maize, or a soybean plant in a field planted with corn. Weeds can be either classified into two major groups: monocots and dicots.

Many plant species can be controlled (*i.e.*, killed or damaged) by the herbicides described herein. Accordingly, the methods of the invention are useful in controlling these plant species where they are undesirable (*i.e.*, where they are weeds). These plant species include crop plants as well as species commonly considered weeds, including but not limited to species such as: blackgrass (*Alopecurus myosuroides*), giant foxtail (*Setaria faberi*), large

crabgrass (*Digitaria sanguinalis*), Surinam grass (*Brachiaria decumbens*), wild oat (*Avena fatua*), common cocklebur (*Xanthium pensylvanicum*), common lambsquarters (*Chenopodium album*), morning glory (*Ipomoea coccinea*), pigweed (*Amaranthus spp.*), velvetleaf (*Abutilion theophrasti*), common barnyardgrass (*Echinochloa crus-galli*), bermudagrass (*Cynodon dactylon*), downy brome (*Bromus tectorum*), goosegrass (*Eleusine indica*), green foxtail (*Setaria viridis*), Italian ryegrass (*Lolium multiflorum*), Johnsongrass (*Sorghum halepense*), lesser canarygrass (*Phalaris minor*), windgrass (*Apera spica-venti*), wooly cupgrass (*Erichloa villosa*), yellow nutsedge (*Cyperus esculentus*), common chickweed (*Stellaria media*), common ragweed (*Ambrosia artemisiifolia*), *Kochia scoparia*, horseweed (*Conyza canadensis*), rigid ryegrass (*Lolium rigidum*), goosegrass (*Eleucine indica*), hairy fleabane (*Conyza bonariensis*), buckhorn plantain (*Plantago lanceolata*), tropical spiderwort (*Commelina benghalensis*), field bindweed (*Convolvulus arvensis*), purple nutsedge (*Cyperus rotundus*), redvine (*Brunnichia ovata*), hemp sesbania (*Sesbania exaltata*), sicklepod (*Senna obtusifolia*), Texas blueweed (*Helianthus ciliaris*), and Devil's claws (*Proboscidea louisianica*). In other embodiments, the weed comprises a herbicide-resistant ryegrass, for example, a glyphosate resistant ryegrass, a paraquat resistant ryegrass, a ACCase-inhibitor resistant ryegrass, and a non-selective herbicide resistant ryegrass. In some embodiments, the undesired plants are proximate the crop plants.

As used herein, by "selectively controlled" is intended that the majority of weeds in an area of cultivation are significantly damaged or killed, while if crop plants are also present in the field, the majority of the crop plants are not significantly damaged. Thus, a method is considered to selectively control weeds when at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more of the weeds are significantly damaged or killed, while if crop plants are also present in the field, less than 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 1% of the crop plants are significantly damaged or killed.

In some embodiments, a glyphosate/ALS inhibitor-tolerant plant of the invention is not significantly damaged by treatment with a particular herbicide applied to that plant at a dose equivalent to a rate of at least 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 150, 170, 200, 300, 400, 500, 600, 700, 800, 800, 1000, 2000, 3000, 4000, 5000 or more grams or ounces (1 ounce = 29.57ml) of active ingredient or commercial product or herbicide formulation per acre or per hectare, whereas an appropriate control plant is significantly damaged by the same treatment.

In specific embodiments, an effective amount of an ALS inhibitor herbicide comprises at least about 0.1, 1, 5, 10, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500,

600, 700, 750, 800, 850, 900, 950, 1000, 2000, 3000, 4000, 5000, or more grams or ounces (1 ounce = 29.57ml) of active ingredient per hectare. In other embodiments, an effective amount of an ALS inhibitor comprises at least about 0.1-50, about 25-75, about 50-100, about 100-110, about 110-120, about 120-130, about 130-140, about 140-150, about 150-200, about 200-500, about 500-600, about 600-800, about 800-1000, or greater grams or ounces (1 ounce = 29.57ml) of active ingredient per hectare. Any ALS inhibitor, for example, those listed in Table 1 can be applied at these levels.

In other embodiments, an effective amount of a sulfonylurea comprises at least 0.1, 1, 5, 10, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000 or more grams or ounces (1 ounce = 29.57ml) of active ingredient per hectare. In other embodiments, an effective amount of a sulfonylurea comprises at least about 0.1-50, about 25-75, about 50-100, about 100-110, about 110-120, about 120-130, about 130-140, about 140-150, about 150-160, about 160-170, about 170-180, about 190-200, about 200-250, about 250-300, about 300-350, about 350-400, about 400-450, about 450-500, about 500-550, about 550-600, about 600-650, about 650-700, about 700-800, about 800-900, about 900-1000, about 1000-2000, or more grams or ounces (1 ounce = 29.57ml) of active ingredient per hectare. Representative sulfonylureas that can be applied at this level are set forth in Table 1.

In other embodiments, an effective amount of a sulfonylaminocarbonyltriazolinones, triazolopyrimidines, pyrimidinyloxy(thio)benzoates, and imidazolinones can comprise at least about 0.1, 1, 5, 10, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1500, 1550, 1600, 1650, 1700, 1800, 1850, 1900, 1950, 2000, 2500, 3500, 4000, 4500, 5000 or greater grams or ounces (1 ounce = 29.57ml) active ingredient per hectare. In other embodiments, an effective amount of a sulfonyluminocarbonyltriazolines, triazolopyrimidines, pyrimidinyloxy(thio)benzoates, or imidazolinones comprises at least about 0.1-50, about 25-75, about 50-100, about 100-110, about 110-120, about 120-130, about 130-140, about 140-150, about 150-160, about 160-170, about 170-180, about 190-200, about 200-250, about 250-300, about 300-350, about 350-400, about 400-450, about 450-500, about 500-550, about 550-600, about 600-650, about 650-700, about 700-800, about 800-900, about 900-1000, about 1000-2000, or more grams or ounces (1 ounce = 29.57 ml) active ingredient per hectare.

Additional ranges of the effective amounts of herbicides can be found, for example, in various publications from University Extension services. See, for example, Bernards *et al.* (2006) *Guide for Weed Management in Nebraska* (www.ianrpubs.url.edu/sendlt/ec130);

Regher *et al.* (2005) *Chemical Weed Control for Fields Crops, Pastures, Rangeland, and Noncropland*, Kansas State University Agricultural Extension Station and Corporate Extension Service; Zollinger *et al.* (2006) *North Dakota Weed Control Guide*, North Dakota Extension Service, and the Iowa State University Extension at www.weeds.iastate.edu, each of which is herein incorporated by reference.

In some embodiments of the invention, glyphosate is applied to an area of cultivation and/or to at least one plant in an area of cultivation at rates between 8 and 32 ounces of acid equivalent per acre, or at rates between 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 ounces of acid equivalent per acre at the lower end of the range of application and between 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 ounces of acid equivalent per acre at the higher end of the range of application (1 ounce = 29.57 ml). In other embodiments, glyphosate is applied at least at 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or greater ounce of active ingredient per hectare (1 ounce = 29.57 ml). In some embodiments of the invention, a sulfonylurea herbicide is applied to a field and/or to at least one plant in a field at rates between 0.04 and 1.0 ounces of active ingredient per acre, or at rates between 0.1, 0.2, 0.4, 0.6, and 0.8 ounces of active ingredient per acre at the lower end of the range of application and between 0.2, 0.4, 0.6, 0.8, and 1.0 ounces of active ingredient per acre at the higher end of the range of application. (1 ounce = 29.57 ml)

As is known in the art, glyphosate herbicides as a class contain the same active ingredient, but the active ingredient is present as one of a number of different salts and/or formulations. However, herbicides known to inhibit ALS vary in their active ingredient as well as their chemical formulations. One of skill in the art is familiar with the determination of the amount of active ingredient and/or acid equivalent present in a particular volume and/or weight of herbicide preparation.

In some embodiments, an ALS inhibitor herbicide is employed. Rates at which the ALS inhibitor herbicide is applied to the crop, crop part, seed or area of cultivation can be any of the rates disclosed herein. In specific embodiments, the rate for the ALS inhibitor herbicide is about 0.1 to about 5000 g ai/hectare, about 0.5 to about 300 g ai/hectare, or about 1 to about 150 g ai/hectare.

Generally, a particular herbicide is applied to a particular field (and any plants growing in it) no more than 1, 2, 3, 4, 5, 6, 7, or 8 times a year, or no more than 1, 2, 3, 4, or 5 times per growing season.

By "treated with a combination of" or "applying a combination of" herbicides to a crop, area of cultivation or field" is intended that a particular field, crop or weed is treated

with each of the herbicides and/or chemicals indicated to be part of the combination so that desired effect is achieved, *i.e.*, so that weeds are selectively controlled while the crop is not significantly damaged. In some embodiments, weeds which are susceptible to each of the herbicides exhibit damage from treatment with each of the herbicides which is additive or synergistic. The application of each herbicide and/or chemical may be simultaneous or the applications may be at different times, so long as the desired effect is achieved. Furthermore, the application can occur prior to the planting of the crop.

The proportions of herbicides used in the methods of the invention with other herbicidal active ingredients in herbicidal compositions are generally in the ratio of 5000:1 to 1:5000, 1000:1 to 1:1000, 100:1 to 1:100, 10:1 to 1:10 or 5:1 to 1:5 by weight. The optimum ratios can be easily determined by those skilled in the art based on the weed control spectrum desired. Moreover, any combinations of ranges of the various herbicides disclosed in Table 3 can also be applied in the methods of the invention.

Thus, in some embodiments, the invention provides improved methods for selectively controlling weeds in a field wherein the total herbicide application may be less than 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 1% of that used in other methods. Similarly, in some embodiments, the amount of a particular herbicide used for selectively controlling weeds in a field may be less than 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 1% of the amount of that particular herbicide that would be used in other methods, *i.e.*, methods not utilizing a plant of the invention.

In some embodiments, a glyphosate/ALS inhibitor-tolerant plant of the invention benefits from a synergistic effect wherein the herbicide tolerance conferred by a polypeptide that confers resistance to glyphosate (i.e., GAT) and at least an ALS inhibitor-tolerant polypeptide is greater than expected from simply combining the herbicide tolerance conferred by each gene separately to a transgenic plant containing them individually. See, *e.g.*, McCutchen *et al.* (1997) *J. Econ. Entomol.* 90: 1170-1180; Priesler *et al.* (1999) *J. Econ. Entomol.* 92: 598-603. As used herein, the terms "synergy," "synergistic," "synergistically" and derivations thereof, such as in a "synergistic effect" or a "synergistic herbicide combination" or a "synergistic herbicide composition" refer to circumstances under which the biological activity of a combination of herbicides, such as at least a first herbicide and a second herbicide, is greater than the sum of the biological activities of the individual herbicides. Synergy, expressed in terms of a "Synergy Index (SI)," generally can be determined by the method described by F. C. Kull, et al., *Applied Microbiology* 9, 538

(1961). See also Colby, S. R., "Calculating Synergistic and Antagonistic Responses of Herbicide Combinations," *Weeds* 15, 20-22 (1967).

In other instances, the herbicide tolerance conferred on a glyphosate/ALS inhibitor-tolerant plant of the invention is additive; that is, the herbicide tolerance profile conferred by the herbicide tolerance genes is what would be expected from simply combining the herbicide tolerance conferred by each gene separately to a transgenic plant containing them individually. Additive and/or synergistic activity for two or more herbicides against key weed species will increase the overall effectiveness and/or reduce the actual amount of active ingredient(s) needed to control said weeds. Where such synergy is observed, the plant of the invention may display tolerance to a higher dose or rate of herbicide and/or the plant may display tolerance to additional herbicides or other chemicals beyond those to which it would be expected to display tolerance. For example, a plant containing a GAT gene and an HRA gene may show tolerance to organophosphate compounds such as insecticides and/or inhibitors of 4-hydroxyphenylpyruvate dioxygenase.

Thus, for example, glyphosate/ALS inhibitor-tolerant plants of the invention can exhibit greater than expected tolerance to various herbicides, including but not limited to glyphosate, ALS inhibitor chemistries, and sulfonylurea herbicides. The glyphosate/ALS inhibitor-tolerant plants of the invention may show tolerance to a particular herbicide or herbicide combination that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 12%, 15%, 17%, 20%, 22%, 25%, 27%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, 200%, 300%, 400%, or 500% or more higher than the tolerance of an appropriate control plant that contains only a single herbicide tolerance gene which confers tolerance to the same herbicide or herbicide combination. Thus, glyphosate/ALS inhibitor-tolerant plants of the invention may show decreased damage from the same dose of herbicide in comparison to an appropriate control plant, or they may show the same degree of damage in response to a much higher dose of herbicide than the control plant. Accordingly, in specific embodiments, a particular herbicide used for selectively containing weeds in a field is more than 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 100% or greater than the amount of that particular herbicide that would be used in other methods, i.e., methods not utilizing a plant of the invention.

In the same manner, in some embodiments, a glyphosate/ALS inhibitor-tolerant plant of the invention shows improved tolerance to a particular formulation of a herbicide active ingredient in comparison to an appropriate control plant. Herbicides are sold commercially

as formulations which typically include other ingredients in addition to the herbicide active ingredient; these ingredients are often intended to enhance the efficacy of the active ingredient. Such other ingredients can include, for example, safeners and adjuvants (see, *e.g.*, Green and Foy (2003) "Adjuvants: Tools for Enhancing Herbicide Performance," in *Weed Biology and Management*, ed. Inderjit (Kluwer Academic Publishers, The Netherlands)). Thus, a glyphosate/ALS inhibitor-tolerant plant of the invention can show tolerance to a particular formulation of a herbicide (*e.g.*, a particular commercially available herbicide product) that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 12%, 15%, 17%, 20%, 22%, 25%, 27%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, 1100%, 1200%, 1300%, 1400%, 1500%, 1600%, 1700%, 1800%, 1900%, or 2000% or more higher than the tolerance of an appropriate control plant that contains only a single herbicide tolerance gene which confers tolerance to the same herbicide formulation.

In some embodiments, a glyphosate/ALS inhibitor-tolerant plant of the invention shows improved tolerance to a herbicide or herbicide class to which the at least one other herbicide tolerance gene confers tolerance as well as improved tolerance to at least one other herbicide or chemical which has a different mechanism or basis of action than either glyphosate or the herbicide corresponding to said at least one other herbicide tolerance gene. This surprising benefit of the invention finds use in methods of growing crops that comprise treatment with various combinations of chemicals, including, for example, other chemicals used for growing crops. Thus, for example, a glyphosate/ALS inhibitor-tolerant maize plant of the invention (i.e., a GAT/HRA plant) may also show improved tolerance to chlorpyrifos, a systemic organophosphate insecticide which interferes with the ability of maize to metabolize herbicide via interference with the cytochrome P450 gene. Thus, the invention also provides a transgenic plant comprising a sequence that confers tolerance to glyphosate (i.e., a GAT gene) and a sulfonylurea herbicide tolerance gene which shows improved tolerance to chemicals which affect the cytochrome P450 gene, and methods of use thereof. In some embodiments, glyphosate/ALS inhibitor-tolerant plants of the invention comprising, for example, a GAT gene and a sulfonylurea herbicide tolerance gene also show improved tolerance to dicamba. In these embodiments, the improved tolerance to dicamba may be evident in the presence of glyphosate and a sulfonylurea herbicide.

In other methods, a herbicide combination is applied over a glyphosate/ALS inhibitor-tolerant plant of the invention, where the herbicide combination produces either an additive or a synergistic effect for controlling weeds. Such combinations of herbicides can allow the

application rate to be reduced, a broader spectrum of undesired vegetation to be controlled, improved control of the undesired vegetation with fewer applications, more rapid onset of the herbicidal activity, or more prolonged herbicidal activity.

An "additive herbicidal composition" has a herbicidal activity that is about equal to the observed activities of the individual components. A "synergistic herbicidal combination" has a herbicidal activity higher than what can be expected based on the observed activities of the individual components when used alone. Accordingly, the presently disclosed subject matter provides a synergistic herbicide combination, wherein the degree of weed control of the mixture exceeds the sum of control of the individual herbicides. In some embodiments, the degree of weed control of the mixture exceeds the sum of control of the individual herbicides by any statistically significant amount including, for example, about 1% to 5%, about 5% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to 40%, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 80% to about 90%, about 90% to about 100%, about 100% to 120% or greater. Further, a "synergistically effective amount" of a herbicide refers to the amount of one herbicide necessary to elicit a synergistic effect in another herbicide present in the herbicide composition. Thus, the term "synergist," and derivations thereof, refer to a substance that enhances the activity of an active ingredient (ai), i.e., a substance in a formulation from which a biological effect is obtained, for example, a herbicide.

Accordingly, in some embodiments, the presently disclosed subject matter provides a method for controlling weeds in an area of cultivation. In some embodiments, the method comprises: (a) planting the area with crop seeds or crop plants, wherein the crop seeds or crop plants comprise: (i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in the crop seeds or crop plants; and (ii) a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide operable linked to a promoter active in the crop seeds or crop plants; and (b) applying to the weed, the crop plants, a crop part, the area of cultivation, or a combination thereof, an effective amount of a herbicide composition comprising at least one of a synergistically effective amount of glyphosate and a synergistically effective amount of an ALS inhibitor (for example, but not limited to, a sulfonylurea herbicide), or agriculturally suitable salts thereof, wherein at least one of: (i) the synergistically effective amount of the glyphosate is lower than an amount of glyphosate required to control the weeds in the absence of the sulfonylurea herbicide; (ii) the synergistically effective amount of the ALS inhibitor herbicide is lower than an amount of the ALS inhibitor required to control the weeds in the absence of glyphosate; and (iii)

combinations thereof; and wherein the effective amount of the herbicide composition is tolerated by the crop seeds or crop plants and controls the weeds in the area of cultivation.

As described in more detail hereinabove, in some embodiments, the first polynucleotide encodes a glyphosate-N-acetyltransferase. More particularly, in some embodiments, the first polynucleotide encodes a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase or a glyphosate-tolerant glyphosate oxido-reductase. Further, as also described in more detail hereinabove, the ALS inhibitor-tolerant polypeptide comprises a mutated acetolactate synthase polypeptide. In some embodiments, the mutated acetolactate synthase polypeptide comprises HRA.

In some embodiments, the herbicide composition used in the presently disclosed method for controlling weeds comprises a synergistically effective amount of glyphosate and a sulfonylurea herbicide. In further embodiments, the presently disclosed synergistic herbicide composition comprises glyphosate and a sulfonylurea herbicide selected from the group consisting of metsulfuron-methyl, chlorsulfuron, and triasulfuron.

In particular embodiments, the synergistic herbicide combination further comprises an adjuvant such as, for example, an ammonium sulfate-based adjuvant, e.g., ADD-UP® (Wenkem S.A., Halfway House, Midrand, South Africa). In additional embodiments, the presently disclosed synergistic herbicide compositions comprise an additional herbicide, for example, an effective amount of a pyrimidinyloxy(thio)benzoate herbicide. In some embodiments, the pyrimidinyloxy(thio)benzoate herbicide comprises bispyribac, e.g., (VELOCITY®, Valent U.S.A. Corp., Walnut Creek, California, United States of America), or an agriculturally suitable salt thereof.

In some embodiments of the presently disclosed method for controlling undesired plants, the glyphosate is applied pre-emergence, post-emergence or pre- and post-emergence to the undesired plants or plant crops; and the ALS inhibitor herbicide (i.e., the sulfonylurea herbicide) is applied pre-emergence, post-emergence or pre- and post-emergence to the undesired plants or plant crops. In other embodiments, the glyphosate and the ALS inhibitor herbicide (i.e., the sulfonylurea herbicide) are applied together or are applied separately. In yet other embodiments, the synergistic herbicide composition is applied, e.g. step (b) above, at least once prior to planting the crop(s) of interest, e.g., step (a) above.

While the glyphosate/ALS inhibitor-tolerant plants of the invention are tolerant to many herbicides, they are not tolerant to several herbicides, such as, for example, dinitroaniline, ACCase, and chloracetamide herbicides. Thus, methods of the invention that comprise the control of weeds may also make use of these treatments to control

glyphosate/ALS inhibitor-tolerant plants, such as, for example, "volunteer" glyphosate/ALS inhibitor-tolerant plants crops that arise in a field that has been planted or replanted with a different crop.

Weeds that can be difficult to control with glyphosate alone in fields where a crop is grown (such as, for example, a soybean crop) include but are not limited to the following: horseweed (*e.g., Conyza canadensis*); rigid ryegrass (*e.g., Lolium rigidum*); goosegrass (*e.g., Eleusine indica*); Italian ryegrass (*e.g., Lolium multiflorum*); hairy fleabane (*e.g., Conyza bonariensis*); buckhorn plantain (*e.g., Plantago lanceolata*); common ragweed (*e.g., Ambrosia artemisifolia*); morning glory (*e.g., Ipomoea spp.*); waterhemp (*e.g., Amaranthus spp.*); field bindweed (*e.g., Convolvulus arvensis*); yellow nutsedge (*e.g., Cyperus esculentus*); common lambsquarters (*e.g., Chenopodium album*); wild buckwheat (*e.g., Polygonium convolvulus*); velvetleaf (*e.g., Abutilon theophrasti*); kochia (*e.g., Kochia scoparia*); and Asiatic dayflower (*e.g., Commelina spp.*). In areas where such weeds are found, the glyphosate/ALS inhibitor-tolerant plants of the invention (GAT-HRA plants) are particularly useful in allowing the treatment of a field (and therefore any crop growing in the field) with combinations of herbicides that would cause unacceptable damage to crop plants that did not contain both of these polynucleotides. Plants of the invention that are tolerant to glyphosate and other herbicides such as, for example, sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinyl(thio)benzoate, and/or sulfonylaminocarbonyltriazolinone herbicides in addition to being tolerant to at least one other herbicide with a different mode of action or site of action are particularly useful in situations where weeds are tolerant to at least two of the same herbicides to which the plants are tolerant. In this manner, plants of the invention make possible improved control of weeds that are tolerant to more than one herbicide.

For example, some commonly used treatments for weed control in fields where current commercial crops (including, for example, soybeans) are grown include glyphosate and, optionally, 2,4-D; this combination, however, has some disadvantages. Particularly, there are weed species that it does not control well and it also does not work well for weed control in cold weather. Another commonly used treatment for weed control in soybean fields is the sulfonylurea herbicide chlorimuron-ethyl, which has significant residual activity in the soil and thus maintains selective pressure on all later-emerging weed species, creating a favorable environment for the growth and spread of sulfonylurea-resistant weeds. However, the glyphosate/ALS inhibitor-tolerant plants (i.e., GAT-HRA plants of the invention), including glyphosate/ALS inhibitor-tolerant soybean plants (i.e., GAT-HRA soybean plants),

can be treated with herbicides (*e.g.*, chlorimuron-ethyl) and combinations of herbicides that would cause unacceptable damage to standard plant varieties. Thus, for example, fields containing the glyphosate/ALS inhibitor-tolerant soybean plant (i.e., GAT-HRA soybean plants) can be treated with sulfonylurea, imidazolinone, triazolopyrimidines, pyrimidiny(thio)benzoates, and/or sulfonylaminocarbonyltriazonlinone such as the sulfonylurea chlorimuron-ethyl, either alone or in combination with other herbicides. For example, fields containing soybean plants of the invention can be treated with a combination of glyphosate and tribenuron-methyl (available commercially as Express®). This combination has several advantages for weed control under some circumstances, including the use of herbicides with different modes of action and the use of herbicides having a relatively short period of residual activity in the soil. A herbicide having a relatively short period of residual activity is desirable, for example, in situations where it is important to reduce selective pressure that would favor the growth of herbicide-tolerant weeds. Of course, in any particular situation where weed control is required, other considerations may be more important, such as, for example, the need to prevent the development of and/or appearance of weeds in a field prior to planting a crop by using a herbicide with a relatively long period of residual activity. The glyphosate/ALS inhibitor-tolerant soybean plants can also be treated with herbicide combinations that include at least one of nicosulfuron, metsulfuron-methyl, tribenuron-methyl, thifensulfuron-methyl, and/or rimsulfuron. Treatments that include both tribenuron-methyl and thifensulfuron-methyl may be particularly useful.

Other commonly used treatments for weed control in fields where current commercial varieties of crops (including, for example, soybeans) are grown include the sulfonylurea herbicide thifensulfuron-methyl (available commercially as Harmony GT®). However, one disadvantage of thifensulfuron-methyl is that the higher application rates required for consistent weed control often cause injury to a crop growing in the same field. The glyphosate/ALS inhibitor-tolerant plants of the invention, including soybean plants, can be treated with a combination of glyphosate and thifensulfuron-methyl, which has the advantage of using herbicides with different modes of action. Thus, weeds that are resistant to either herbicide alone are controlled by the combination of the two herbicides, and the glyphosate/ALS inhibitor-tolerant plants of the invention are not significantly damaged by the treatment.

Other herbicides which are used for weed control in fields where current commercial varieties of crops (including, for example, soybeans) are grown are the triazolopyrimidine herbicide cloransulam-methyl (available commercially as FirstRate®) and the imidazolinone

herbicide imazaquin (available commercially as Sceptor®). When these herbicides are used individually they may provide only marginal control of weeds. However, fields containing the glyphosate/ALS inhibitor-tolerant plants of the invention, including soybean plants, can be treated, for example, with a combination of glyphosate (*e.g.*, Roundup® (glyphosate isopropylamine salt)), imazapyr (currently available commercially as Arsenal®), chlorimuron-ethyl (currently available commercially as Classic®), quizalofop-P-ethyl (currently available commercially as Assure II®), and fomesafen (currently available commercially as Flexstar®). This combination has the advantage of using herbicides with different modes of action. Thus, weeds that are tolerant to just one or several of these herbicides are controlled by the combination of the five herbicides, and the glyphosate/ALS inhibitor-tolerant plants of the invention are not significantly damaged by treatment with this herbicide combination. This combination provides an extremely broad spectrum of protection against the type of herbicide-tolerant weeds that might be expected to arise and spread under current weed control practices.

Fields containing the glyphosate/ALS inhibitor-tolerant plants of the invention (i.e., GAT/HRA plants), including soybean plants, may also be treated, for example, with a combination of herbicides including glyphosate, rimsulfuron, and dicamba or mesotrione. This combination may be particularly useful in controlling weeds which have developed some tolerance to herbicides which inhibit ALS. Another combination of herbicides which may be particularly useful for weed control includes glyphosate and at least one of the following: metsulfuron-methyl (commercially available as Ally®), imazapyr (commercially available as Arsenal®), imazethapyr, imazaquin, and sulfentrazone. It is understood that any of the combinations discussed above or elsewhere herein may also be used to treat areas in combination with any other herbicide or agricultural chemical.

Some commonly-used treatments for weed control in fields where current commercial crops (including, for example, maize) are grown include glyphosate (currently available commercially as Roundup®), rimsulfuron (currently available commercially as Resolve® or Matrix®), dicamba (commercially available as Clarity®), atrazine, and mesotrione (commercially available as Callisto®). These herbicides are sometimes used individually due to poor crop tolerance to multiple herbicides. Unfortunately, when used individually, each of these herbicides has significant disadvantages. Particularly, the incidence of weeds that are tolerant to individual herbicides continues to increase, rendering glyphosate less effective than desired in some situations. Rimsulfuron provides better weed control at high doses which can cause injury to a crop, and alternatives such as dicamba are often more expensive

than other commonly-used herbicides. However, glyphosate/ALS inhibitor-tolerant plants (i.e., GAT-HRA plants) of the invention, including glyphosate/ALS inhibitor-tolerant maize plants, can be treated with herbicides and combinations of herbicides that would cause unacceptable damage to standard plant varieties, including combinations of herbicides that comprise rimsulfuron and/or dicamba. Other suitable combinations of herbicides for use with glyphosate/ALS inhibitor-tolerant plants of the invention include glyphosate, sulfonylurea, imidazolinone, triazolopyrimidine, pryimidinyloxy(thio)benzoates, and/or sulfonylaminocarbonyltriazonlinone herbicides, including, for example, and at least one of the following: metsulfuron-methyl, tribenuron-methyl, chlorimuron-ethyl, imazethapyr, imazapyr, and imazaquin.

For example, glyphosate/ALS inhibitor-tolerant maize plants (i.e. GAT/HRA plants) can be treated with a combination of glyphosate and rimsulfuron, or a combination or rimsulfuron and at least one other herbicide. Glyphosate/ALS inhibitor plants (i.e., GAT-HRA plants) can also be treated with a combination of glyphosate, rimsulfuron, and dicamba, or a combination of glyphosate, rimsulfuron, and at least one other herbicide. In some embodiments, the at least one other herbicide has a different mode of action than both glyphosate and rimsulfuron. The combination of glyphosate, rimsulfuron, and dicamba has the advantage that these herbicides have different modes of action and short residual activity, which decreases the risk of incidence and spread of herbicide-tolerant weeds.

Some commonly-used treatments for weed control in fields where current commercial crops (including, for example, cotton) are grown include glyphosate (currently available commercially as Roundup®), chlorimuron-ethyl, tribenuron-methyl, rimsulfuron (currently available commercially as Resolve® or Matrix®), imazethapyr, imazapyr, and imazaquin. Unfortunately, when used individually, each of these herbicides has significant disadvantages. Particularly, the incidence of weeds that are tolerant to individual herbicides continues to increase, rendering each individual herbicide less effective than desired in some situations. However, glyphosate/ALS inhibitor-tolerant plants of the invention, including cotton plants, can be treated with a combination of herbicides that would cause unacceptable damage to standard plant varieties, including combinations of herbicides that include at least one of those mentioned above.

c. Methods of Herbicide Application

In the methods of the invention, a herbicide may be formulated and applied to an area of interest such as, for example, a field or area of cultivation, in any suitable manner. A

herbicide may be applied to a field in any form, such as, for example, in a liquid spray or as solid powder or granules. In specific embodiments, the herbicide or combination of herbicides that are employed in the methods comprise a tankmix or a premix. A herbicide may also be formulated, for example, as a "homogenous granule blend" produced using blends technology (see, *e.g.*, U.S. Pat. No. 6,022,552, entitled "Uniform Mixtures of Pesticide Granules"). The blends technology of U.S. Patent No. 6,022,552 produces a nonsegregating blend (*i.e.*, a "homogenous granule blend") of formulated crop protection chemicals in a dry granule form that enables delivery of customized mixtures designed to solve specific problems. A homogenous granule blend can be shipped, handled, subsampled, and applied in the same manner as traditional premix products where multiple active ingredients are formulated into the same granule.

Briefly, a "homogenous granule blend" is prepared by mixing together at least two extruded formulated granule products. In some embodiments, each granule product comprises a registered formulation containing a single active ingredient which is, for example, a herbicide, a fungicide, and/or an insecticide. The uniformity (homogeneity) of a "homogenous granule blend" can be optimized by controlling the relative sizes and size distributions of the granules used in the blend. The diameter of extruded granules is controlled by the size of the holes in the extruder die, and a centrifugal sifting process may be used to obtain a population of extruded granules with a desired length distribution (see, *e.g.*, U.S. Pat. No. 6,270,025).

A homogenous granule blend is considered to be "homogenous" when it can be subsampled into appropriately sized aliquots and the composition of each aliquot will meet the required assay specifications. To demonstrate homogeneity, a large sample of the homogenous granule blend is prepared and is then subsampled into aliquots of greater than the minimum statistical sample size (see Example 4).

In non-limiting embodiments, the glyphosate/ALS inhibitor-tolerant plant (i.e., a GAT-HRA plant), including a soybean plant, can be treated with herbicides (*e.g.*, chlorimuron-ethyl and combinations of other herbicides that without the glyphosate/ALS inhibitor-tolerant crop would have caused unacceptable crop response to plant varieties without the glyphosate/ALS inhibitor genetics). Thus, for example, fields planted with and containing glyphosate/ALS inhibitor-tolerant soybean, corn or cotton varieties (i.e., GAT/HRA plants) can be treated with sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinyl(thio)benzoate, and/or sulfonylaminocarbonyltriazonlinone herbicides, either alone or in combination with other herbicides. Since ALS inhibitor chemistries have

different herbicidal attributes, blends of ALS plus other chemistries will provide superior weed management strategies including varying and increased weed spectrum, the ability to provide specified residual activity (SU/ALS inhibitor chemistry with residual activity leads to improved foliar activity which leads to a wider window between glyphosate applications, as well as, an added period of control if weather conditions prohibit timely application).

Blends also afford the ability to add other agrochemicals at normal, labeled use rates such as additional herbicides (a 3rd/4th mechanism of action), fungicides, insecticides, plant growth regulators and the like thereby saving costs associated with additional applications.

Any herbicide formulation applied over the glyphosate/ALS inhibitor-tolerant plant can be prepared as a "tank-mix" composition. In such embodiments, each ingredient or a combination of ingredients can be stored separately from one another. The ingredients can then be mixed with one another prior to application. Typically, such mixing occurs shortly before application. In a tank-mix process, each ingredient, before mixing, typically is present in water or a suitable organic solvent. For additional guidance regarding the art of formulation, see T. S. Woods, "The Formulator's Toolbox--Product Forms for Modern Agriculture" *Pesticide Chemistry and Bioscience, The Food-Environment Challenge*, T. Brooks and T. R. Roberts, Eds., Proceedings of the 9th International Congress on Pesticide Chemistry, The Royal Society of Chemistry, Cambridge, 1999, pp. 120-133. See also U.S. Pat. No. 3,235,361, Col. 6, line 16 through Col. 7, line 19 and Examples 10-41; U.S. Pat. No. 3,309,192, Col. 5, line 43 through Col. 7, line 62 and Examples 8, 12, 15, 39, 41, 52, 53, 58, 132, 138-140, 162-164, 166, 167 and 169-182; U.S. Pat. No. 2,891,855, Col. 3, line 66 through Col. 5, line 17 and Examples 1-4; Klingman, *Weed Control as a Science*, John Wiley and Sons, Inc., New York, 1961, pp 81-96; and Hance *et al., Weed Control Handbook*, 8th Ed., Blackwell Scientific Publications, Oxford, 1989, each of which is incorporated herein by reference in their entirety.

The methods of the invention further allow for the development of herbicide combinations to be used in with the glyphosate/ALS inhibitor-tolerant plants. In such methods, the environmental conditions in an area of cultivation are evaluated. Environmental conditions that can be evaluated include, but are not limited to, ground and surface water pollution concerns, intended use of the crop, crop tolerance, soil residuals, weeds present in area of cultivation, soil texture, pH of soil, amount of organic matter in soil, application equipment, and tillage practices. Upon the evaluation of the environmental conditions, an effective amount of a combination of herbicides can be applied to the crop, crop part, seed of the crop or area of cultivation.

d. Timing of Herbicide Application

In some embodiments, the herbicide applied to the glyphosate/ALS inhibitor-tolerant plants of the invention serves to prevent the initiation of growth of susceptible weeds and/or serve to cause damage to weeds that are growing in the area of interest. In some embodiments, the herbicide or herbicide mixture exert these effects on weeds affecting crops that are subsequently planted in the area of interest (*i.e.*, field or area of cultivation). In the methods of the invention, the application of the herbicide combination need not occur at the same time. So long as the field in which the crop is planted contains detectable amounts of the first herbicide and the second herbicide is applied at some time during the period in which the crop is in the area of cultivation, the crop is considered to have been treated with a mixture of herbicides according to the invention. Thus, methods of the invention encompass applications of herbicide which are "preemergent," "postemergent," "preplant incorporation" and/or which involve seed treatment prior to planting.

In one embodiment, methods are provided for coating seeds. The methods comprise coating a seed with an effective amount of a herbicide or a combination of herbicides (as disclosed elsewhere herein). The seeds can then be planted in an area of cultivation. Further provided are seeds having a coating comprising an effective amount of a herbicide or a combination of herbicides (as disclosed elsewhere herein).

"Preemergent" refers to a herbicide which is applied to an area of interest (*e.g.*, a field or area of cultivation) before a plant emerges visibly from the soil. "Postemergent" refers to a herbicide which is applied to an area after a plant emerges visibly from the soil. In some instances, the terms "preemergent" and "postemergent" are used with reference to a weed in an area of interest, and in some instances these terms are used with reference to a crop plant in an area of interest. When used with reference to a weed, these terms may apply to only a particular type of weed or species of weed that is present or believed to be present in the area of interest. While any herbicide may be applied in a preemergent and/or postemergent treatment, some herbicides are known to be more effective in controlling a weed or weeds when applied either preemergence or postemergence. For example, rimsulfuron has both preemergence and postemergence activity, while other herbicides have predominately preemergence (metolachlor) or postemergence (glyphosate) activity. These properties of particular herbicides are known in the art and are readily determined by one of skill in the art. Further, one of skill in the art would readily be able to select appropriate herbicides and application times for use with the transgenic plants of the invention and/or on areas in which

transgenic plants of the invention are to be planted. "Preplant incorporation" involves the incorporation of compounds into the soil prior to planting.

Thus, the invention provides improved methods of growing a crop and/or controlling weeds such as, for example, "pre-planting burn down," wherein an area is treated with herbicides prior to planting the crop of interest in order to better control weeds. The invention also provides methods of growing a crop and/or controlling weeds which are "no-till" or "low-till" (also referred to as "reduced tillage"). In such methods, the soil is not cultivated or is cultivated less frequently during the growing cycle in comparison to traditional methods; these methods can save costs that would otherwise be incurred due to additional cultivation, including labor and fuel costs.

The methods of the invention encompass the use of simultaneous and/or sequential applications of multiple classes of herbicides. In some embodiments, the methods of the invention involve treating a plant of the invention and/ or an area of interest (*e.g.*, a field or area of cultivation) and/or weed with just one herbicide or other chemical such as, for example, a sulfonylurea herbicide.

The time at which a herbicide is applied to an area of interest (and any plants therein) may be important in optimizing weed control. The time at which a herbicide is applied may be determined with reference to the size of plants and/or the stage of growth and/or development of plants in the area of interest, *e.g.*, crop plants or weeds growing in the area. The stages of growth and/or development of plants are known in the art. For example, soybean plants normally progress through vegetative growth stages known as V_E (emergence), V_C (cotyledon), V_1 (unifoliate), and V_2 to V_N. Soybeans then switch to the reproductive growth phase in response to photoperiod cues; reproductive stages include R_1 (beginning bloom), R_2 (full bloom), R_3 (beginning pod), R_4 (full pod), R_5 (beginning seed), R_6 (full seed), R_7 (beginning maturity), and R_8 (full maturity). Corn plants normally progress through the following vegetative stages VE (emergence); V1 (first leaf); V2 (second leaf); V3 (third leaf); V(n) (Nth/leaf); and VT (tasseling). Progression of maize through the reproductive phase is as follows: R1 (silking); R2 (blistering); R3 (milk); R4 (dough); R5 (dent); and R6 (physiological maturity). Cotton plants normally progress through V_E (emergence), V_C (cotyledon), V_1 (first true leaf), and V_2 to V_N. Then, reproductive stages beginning around V_{14} include R_1 (beginning bloom), R_2 (full bloom), R_3 (beginning boll), R_4 (cutout, boll development), R_5 (beginning maturity, first opened boll), R_6 (maturity, 50% opened boll), and R_7 (full maturity, 80-90% open bolls). Thus, for example, the time at which a herbicide or other chemical is applied to an area of interest in which plants are

growing may be the time at which some or all of the plants in a particular area have reached at least a particular size and/or stage of growth and/or development, or the time at which some or all of the plants in a particular area have not yet reached a particular size and/or stage of growth and/or development.

In some embodiments, the glyphosate/ALS inhibitor-tolerant plants of the invention show improved tolerance to postemergence herbicide treatments. For example, plants of the invention may be tolerant to higher doses of herbicide, tolerant to a broader range of herbicides (i.e., tolerance to more ALS inhibitor chemistries), and/or may be tolerant to doses of herbicide applied at earlier or later times of development in comparison to an appropriate control plant. For example, in some embodiments, the glyphosate/ALS inhibitor-tolerant plants of the invention show an increased resistance to morphological defects that are known to result from treatment at particular stages of development. Thus, for example, a phenomenon known as "ear pinch" often results when maize plants are treated with herbicide at a stage later than V5, V6, V7, V8, V9, V10, V11, V12, V13, or a later stage, whereas the glyphosate/ALS inhibitor-tolerant plants of the invention show a decreased incidence of "ear pinch" when treated at the same stage. Thus, the glyphosate/ALS inhibitor-tolerant plants of the invention find use in methods involving herbicide treatments at later stages of development than were previously feasible. Thus, plants of the invention may be treated with a particular herbicide that causes morphological defects in a control plant treated at the same stage of development, but the glyphosate/ALS inhibitor-tolerant plants of the invention will not be significantly damaged by the same treatment.

Different chemicals such as herbicides have different "residual" effects, *i.e.*, different amounts of time for which treatment with the chemical or herbicide continues to have an effect on plants growing in the treated area. Such effects may be desirable or undesirable, depending on the desired future purpose of the treated area (*e.g.*, field or area of cultivation). Thus, a crop rotation scheme may be chosen based on residual effects from treatments that will be used for each crop and their effect on the crop that will subsequently be grown in the same area. One of skill in the art is familiar with techniques that can be used to evaluate the residual effect of a herbicide; for example, generally, glyphosate has very little or no soil residual activity, while herbicides that act to inhibit ALS vary in their residual activity levels. Residual activities for various herbicides are known in the art, and are also known to vary with various environmental factors such as, for example, soil moisture levels, temperature, pH, and soil composition (texture and organic matter). The glyphosate/ALS inhibitor-

tolerant plants of the invention find particular use in methods of growing a crop where improved tolerance to residual activity of a herbicide is beneficial.

For example, in one embodiment, the glyphosate/ALS inhibitor-tolerant plants of the invention have an improved tolerance to glyphosate as well as to ALS inhibitor chemistries (such as sulfonylurea herbicides) when applied individually, and further provide improved tolerance to combinations of herbicides such as glyphosate and/or ALS inhibitor chemistries. Moreover, the transgenic plants of the invention provide improved tolerance to treatment with additional chemicals commonly used on crops in conjunction with herbicide treatments, such as safeners, adjuvants such as ammonium sulfonate and crop oil concentrate, and the like.

e. Safeners and Adjuvants

The term "safener" refers to a substance that when added to a herbicide formulation eliminates or reduces the phytotoxic effects of the herbicide to certain crops. One of ordinary skill in the art would appreciate that the choice of safener depends, in part, on the crop plant of interest and the particular herbicide or combination of herbicides included in the synergistic herbicide composition. Exemplary safeners suitable for use with the presently disclosed herbicide compositions include, but are not limited to, those disclosed in U.S. Patent Nos. 4,808,208; 5,502,025; 6,124,240 and U.S. Patent Application Publication Nos. 2006/0148647; 2006/0030485; 2005/0233904; 2005/0049145; 2004/0224849; 2004/0224848; 2004/0224844; 2004/0157737; 2004/0018940; 2003/0171220; 2003/0130120; 2003/0078167, the disclosures of which are incorporated herein by reference in their entirety. The methods of the invention can involve the use of herbicides in combination with herbicide safeners such as benoxacor, BCS (1-bromo-4-[(chloromethyl) sulfonyl]benzene), cloquintocet-mexyl, cyometrinil, dichlormid, 2-(dichloromethyl)-2-methyl-1,3-dioxolane (MG 191), fenchlorazole-ethyl, fenclorim, flurazole, fluxofenim, furilazole, isoxadifen-ethyl, mefenpyr-diethyl, methoxyphenone ((4-methoxy-3-methylphenyl)(3-methylphenyl)-methanone), naphthalic anhydride (1,8-naphthalic anhydride) and oxabetrinil to increase crop safety. Antidotally effective amounts of the herbicide safeners can be applied at the same time as the compounds of this invention, or applied as seed treatments. Therefore an aspect of the present invention relates to the use of a mixture comprising glyphosate, at least one other herbicide, and an antidotally effective amount of a herbicide safener.

Seed treatment is particularly useful for selective weed control, because it physically restricts antidoting to the crop plants. Therefore a particularly useful embodiment of the

present invention is a method for selectively controlling the growth of weeds in a field comprising treating the seed from which the crop is grown with an antidotally effective amount of safener and treating the field with an effective amount of herbicide to control weeds. Antidotally effective amounts of safeners can be easily determined by one skilled in the art through simple experimentation. An antidotally effective amount of a safener is present where a desired plant is treated with the safener so that the effect of a herbicide on the plant is decreased in comparison to the effect of the herbicide on a plant that was not treated with the safener; generally, an antidotally effective amount of safener prevents damage or severe damage to the plant treated with the safener. One of skill in the art is capable of determining whether the use of a safener is appropriate and determining the dose at which a safener should be administered to a crop.

In specific embodiments, the herbicide or herbicide combination applied to the plant of the invention acts as a safener. In this embodiment, a first herbicide or a herbicide mixture is applied at an antidotally effect amount to the plant. Accordingly, a method for controlling weeds in an area of cultivation is provided. The method comprises planting the area with crop seeds or plants which comprise a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in a plant; and, a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide operably linked to a promoter active in a plant. A combination of herbicides comprising at least an effective amount of a first and a second herbicide is applied to the crop, crop part, weed or area of cultivation thereof. The effective amount of the herbicide combination controls weeds; and, the effective amount of the first herbicide is not tolerated by the crop when applied alone when compared to a control crop that has not been exposed to the first herbicide; and, the effective amount of the second herbicide is sufficient to produce a safening effect, wherein the safening effect provides an increase in crop tolerance upon the application of the first and the second herbicide when compared to the crop tolerance when the first herbicide is applied alone.

In specific embodiments, the combination of safening herbicides comprises a first ALS inhibitor and a second ALS inhibitor. In other embodiments, the safening effect is achieved by applying an effective amount of a combination of glyphosate and at least one ALS inhibitor chemistry. In still other embodiments, a safening affect is achieved when the glyphosate/ALS inhibitor-tolerant crops, crop part, crop seed, weed, or area of cultivation is treated with at least one herbicide from the sulfonylurea family of chemistries in combination with at least one herbicide from the ALS family of chemistries (such as, for example, an imidazolinone).

Such mixtures provides increased crop tolerance (i.e., a decrease in herbicidal injury). This method allows for increased application rates of the chemistries post or pre-treatment. Such methods find use for increased control of unwanted or undesired vegetation. In still other embodiments, a safening affect is achieved when the glyphosate/ALS inhibitor-tolerant crops, crop part, crop seed, weed, or area of cultivation is treated with at least one herbicide from the sulfonylurea family of chemistry in combination with at least one herbicide from the imidazolinone family. This method provides increased crop tolerance (i.e., a decrease in herbicidal injury). In specific embodiments, the sulfonylurea comprises rimsulfuron and the imidazolinone comprises imazethapyr. In other embodiments, glyphosate is also applied to the crop, crop part, or area of cultivation.

As used herein, an "adjuvant" is any material added to a spray solution or formulation to modify the action of an agricultural chemical or the physical properties of the spray solution. See, for example, Green and Foy (2003) "Adjuvants: Tools for Enhancing Herbicide Performance," in *Weed Biology and Management*, ed. Inderjit (Kluwer Academic Publishers, The Netherlands). Adjuvants can be categorized or subclassified as activators, acidifiers, buffers, additives, adherents, antiflocculants, antifoamers, defoamers, antifreezes, attractants, basic blends, chelating agents, cleaners, colorants or dyes, compatibility agents, cosolvents, couplers, crop oil concentrates, deposition agents, detergents, dispersants, drift control agents, emulsifiers, evaporation reducers, extenders, fertilizers, foam markers, formulants, inerts, humectants, methylated seed oils, high load COCs, polymers, modified vegetable oils, penetrators, repellants, petroleum oil concentrates, preservatives, rainfast agents, retention aids, solubilizers, surfactants, spreaders, stickers, spreader stickers, synergists, thickeners, translocation aids, uv protectants, vegetable oils, water conditioners, and wetting agents.

f. Additional Agricultural Chemicals

In addition, methods of the invention can comprise the use of a herbicide or a mixture of herbicides, as well as, one or more other insecticides, fungicides, nematocides, bactericides, acaricides, growth regulators, chemosterilants, semiochemicals, repellents, attractants, pheromones, feeding stimulants or other biologically active compounds or entomopathogenic bacteria, virus, or fungi to form a multi-component mixture giving an even broader spectrum of agricultural protection. Examples of such agricultural protectants which can be used in methods of the invention include: insecticides such as abamectin, acephate, acetamiprid, amidoflumet (S-1955), avermectin, azadirachtin, azinphos-methyl, bifenthrin,

bifenazate, buprofezin, carbofuran, cartap, chlorfenapyr, chlorfluazuron, chlorpyrifos, chlorpyrifos-methyl, chromafenozide, clothianidin, cyflumetofen, cyfluthrin, beta-cyfluthrin, cyhalothrin, lambda-cyhalothrin, cypermethrin, cyromazine, deltamethrin, diafenthiuron, diazinon, dieldrin, diflubenzuron, dimefluthrin, dimethoate, dinotefuran, diofenolan, emamectin, endosulfan, esfenvalerate, ethiprole, fenothiocarb, fenoxycarb, fenpropathrin, fenvalerate, fipronil, flonicamid, flubendiamide, flucythrinate, tau-fluvalinate, flufenerim (UR-50701), flufenoxuron, fonophos, halofenozide, hexaflumuron, hydramethylnon, imidacloprid, indoxacarb, isofenphos, lufenuron, malathion, metaflumizone, metaldehyde, methamidophos, methidathion, methomyl, methoprene, methoxychlor, metofluthrin, monocrotophos, methoxyfenozide, nitenpyram, nithiazine, novaluron, noviflumuron (XDE-007), oxamyl, parathion, parathion-methyl, permethrin, phorate, phosalone, phosmet, phosphamidon, pirimicarb, profenofos, profluthrin, pymetrozine, pyrafluprole, pyrethrin, pyridalyl, pyriprole, pyriproxyfen, rotenone, ryanodine, spinosad, spirodiclofen, spiromesifen (BSN 2060), spirotetramat, sulprofos, tebufenozide, teflubenzuron, tefluthrin, terbufos, tetrachlorvinphos, thiacloprid, thiamethoxam, thiodicarb, thiosultap-sodium, tralomethrin, triazamate, trichlorfon and triflumuron; fungicides such as fungicides such as acibenzolar, aldimorph, amisulbrom, azaconazole, azoxystrobin, benalaxyl, benomyl, benthiavalicarb, benthiavalicarb-isopropyl, binomial, biphenyl, bitertanol, blasticidin-S, Bordeaux mixture (Tribasic copper sulfate), boscalid/nicobifen, bromuconazole, bupirimate, buthiobate, carboxin, carpropamid, captafol, captan, carbendazim, chloroneb, chlorothalonil, chlozolinate, clotrimazole, copper oxychloride, copper salts such as copper sulfate and copper hydroxide, cyazofamid, cyflunamid, cymoxanil, cyproconazole, cyprodinil, dichlofluanid, diclocymet, diclomezine, dicloran, diethofencarb, difenoconazole, dimethomorph, dimoxystrobin, diniconazole, diniconazole-M, dinocap, discostrobin, dithianon, dodemorph, dodine, econazole, etaconazole, edifenphos, epoxiconazole, ethaboxam, ethirimol, ethridiazole, famoxadone, fenamidone, fenarimol, fenbuconazole, fencaramid, fenfuram, fenhexamide, fenoxanil, fenpiclonil, fenpropidin, fenpropimorph, fentin acetate, fentin hydroxide, ferbam, ferfurazoate, ferimzone, fluazinam, fludioxonil, flumetover, fluopicolide, fluoxastrobin, fluquinconazole, fluquinconazole, flusilazole, flusulfamide, flutolanil, flutriafol, folpet, fosetyl-aluminum, fuberidazole, furalaxyl, furametapyr, hexaconazole, hymexazole, guazatine, imazalil, imibenconazole, iminoctadine, iodicarb, ipconazole, iprobenfos, iprodione, iprovalicarb, isoconazole, isoprothiolane, kasugamycin, kresoxim-methyl, mancozeb, mandipropamid, maneb, mapanipyrin, mefenoxam, mepronil, metalaxyl, metconazole, methasulfocarb, metiram, metominostrobin/fenominostrobin, mepanipyrim,

metrafenone, miconazole, myclobutanil, neo-asozin (ferric methanearsonate), nuarimol, octhilinone, ofurace, orysastrobin, oxadixyl, oxolinic acid, oxpoconazole, oxycarboxin, paclobutrazol, penconazole, pencycuron, penthiopyrad, perfurazoate, phosphonic acid, phthalide, picobenzamid, picoxystrobin, polyoxin, probenazole, prochloraz, procymidone, propamocarb, propamocarb-hydrochloride, propiconazole, propineb, proquinazid, prothioconazole, pyraclostrobin, pryazophos, pyrifenox, pyrimethanil, pyrifenox, pyrolnitrine, pyroquilon, quinconazole, quinoxyfen, quintozene, silthiofam, simeconazole, spiroxamine, streptomycin, sulfur, tebuconazole, techrazene, tecloftalam, tecnazene, tetraconazole, thiabendazole, thifluzamide, thiophanate, thiophanate-methyl, thiram, tiadinil, tolclofos-methyl, tolyfluanid, triadimefon, triadimenol, triarimol, triazoxide, tridemorph, trimoprhamide tricyclazole, trifloxystrobin, triforine, triticonazole, uniconazole, validamycin, vinclozolin, zineb, ziram, and zoxamide; nematocides such as aldicarb, oxamyl and fenamiphos; bactericides such as streptomycin; acaricides such as amitraz, chinomethionat, chlorobenzilate, cyhexatin, dicofol, dienochlor, etoxazole, fenazaquin, fenbutatin oxide, fenpropathrin, fenpyroximate, hexythiazox, propargite, pyridaben and tebufenpyrad; and biological agents including entomopathogenic bacteria, such as *Bacillus thuringiensis* subsp. *Aizawai, Bacillus thuringiensis* subsp. *Kurstaki,* and the encapsulated delta-endotoxins of *Bacillus thuringiensis* (e.g., Cellcap, MPV, MPVII); entomopathogenic fungi, such as green muscardine fungus; and entomopathogenic virus including baculovirus, nucleopolyhedro virus (NPV) such as HzNPV, AfNPV; and granulosis virus (GV) such as CpGV. The weight ratios of these various mixing partners to other compositions (*e.g.*, herbicides) used in the methods of the invention typically are between 100:1 and 1:100, or between 30:1 and 1:30, between 10:1 and 1:10, or between 4:1 and 1:4.

The present invention also pertains to a composition comprising a biologically effective amount of a herbicide of interest or a mixture of herbicides, and an effective amount of at least one additional biologically active compound or agent and can further comprise at least one of a surfactant, a solid diluent or a liquid diluent. Examples of such biologically active compounds or agents are: insecticides such as abamectin, acephate, acetamiprid, amidoflumet (S-1955), avermectin, azadirachtin, azinphos-methyl, bifenthrin, binfenazate, buprofezin, carbofuran, chlorfenapyr, chlorfluazuron, chlorpyrifos, chlorpyrifos-methyl, chromafenozide, clothianidin, cyfluthrin, beta-cyfluthrin, cyhalothrin, lambda-cyhalothrin, cypermethrin, cyromazine, deltamethrin, diafenthiuron, diazinon, diflubenzuron, dimethoate, diofenolan, emamectin, endosulfan, esfenvalerate, ethiprole, fenothicarb, fenoxycarb, fenpropathrin, fenvalerate, fipronil, flonicamid, flucythrinate, tau-fluvalinate, flufenerim

(UR-50701), flufenoxuron, fonophos, halofenozide, hexaflumuron, imidacloprid, indoxacarb, isofenphos, lufenuron, malathion, metaldehyde, methamidophos, methidathion, methomyl, methoprene, methoxychlor, monocrotophos, methoxyfenozide, nithiazin, novaluron, noviflumuron (XDE-007), oxamyl, parathion, parathion-methyl, permethrin, phorate, phosalone, phosmet, phosphamidon, pirimicarb, profenofos, pymetrozine, pyridalyl, pyriproxyfen, rotenone, spinosad, spiromesifin (BSN 2060), sulprofos, tebufenozide, teflubenzuron, tefluthrin, terbufos, tetrachlorvinphos, thiacloprid, thiamethoxam, thiodicarb, thiosultap-sodium, tralomethrin, trichlorfon and triflumuron; fungicides such as acibenzolar, azoxystrobin, benomyl, blasticidin-S, Bordeaux mixture (tribasic copper sulfate), bromuconazole, carpropamid, captafol, captan, carbendazim, chloroneb, chlorothalonil, copper oxychloride, copper salts, cyflufenamid, cymoxanil, cyproconazole, cyprodinil, (*S*)-3,5-dichloro-*N*-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-4-methylbenzamide (RH 7281), diclocymet (S-2900), diclomezine, dicloran, difenoconazole, (*S*)-3,5-dihydro-5-methyl-2-(methylthio)-5-phenyl-3-(phenyl-amino)-4*H*-imidazol-4-one (RP 407213), dimethomorph, dimoxystrobin, diniconazole, diniconazole-M, dodine, edifenphos, epoxiconazole, famoxadone, fenamidone, fenarimol, fenbuconazole, fencaramid (SZX0722), fenpiclonil, fenpropidin, fenpropimorph, fentin acetate, fentin hydroxide, fluazinam, fludioxonil, flumetover (RPA 403397), flumorf/flumorlin (SYP-L190), fluoxastrobin (HEC 5725), fluquinconazole, flusilazole, flutolanil, flutriafol, folpet, fosetyl-aluminum, furalaxyl, furametapyr (S-82658), hexaconazole, ipconazole, iprobenfos, iprodione, isoprothiolane, kasugamycin, kresoxim-methyl, mancozeb, maneb, mefenoxam, mepronil, metalaxyl, metconazole, metomino-strobin/fenominostrobin (SSF-126), metrafenone (AC375839), myclobutanil, neo-asozin (ferric methane-arsonate), nicobifen (BAS 510), orysastrobin, oxadixyl, penconazole, pencycuron, probenazole, prochloraz, propamocarb, propiconazole, proquinazid (DPX-KQ926), prothioconazole (JAU 6476), pyrifenox, pyraclostrobin, pyrimethanil, pyroquilon, quinoxyfen, spiroxamine, sulfur, tebuconazole, tetraconazole, thiabendazole, thifluzamide, thiophanate-methyl, thiram, tiadinil, triadimefon, triadimenol, tricyclazole, trifloxystrobin, triticonazole, validamycin and vinclozolin; nematocides such as aldicarb, oxamyl and fenamiphos; bactericides such as streptomycin; acaricides such as amitraz, chinomethionat, chlorobenzilate, cyhexatin, dicofol, dienochlor, etoxazole, fenazaquin, fenbutatin oxide, fenpropathrin, fenpyroximate, hexythiazox, propargite, pyridaben and tebufenpyrad; and biological agents including entomopathogenic bacteria, such as *Bacillus thuringiensis* subsp. *Aizawai, Bacillus thuringiensis* subsp. *Kurstaki,* and the encapsulated delta-endotoxins of *Bacillus thuringiensis* (*e.g.*, Cellcap, MPV, MPVII);

entomopathogenic fungi, such as green muscardine fungus; and entomopathogenic virus including baculovirus, nucleopolyhedro virus (NPV) such as HzNPV, AfNPV; and granulosis virus (GV) such as CpGV. Methods of the invention may also comprise the use of plants genetically transformed to express proteins toxic to invertebrate pests (such as *Bacillus thuringiensis* delta-endotoxins). In such embodiments, the effect of exogenously applied invertebrate pest control compounds may be synergistic with the expressed toxin proteins.

General references for these agricultural protectants include *The Pesticide Manual, 13th Edition*, C. D. S. Tomlin, Ed., British Crop Protection Council, Farnham, Surrey, U.K., 2003 and *The BioPesticide Manual, 2nd Edition*, L. G. Copping, Ed., British Crop Protection Council, Farnham, Surrey, U.K., 2001.

In certain instances, combinations with other invertebrate pest control compounds or agents having a similar spectrum of control but a different mode of action will be particularly advantageous for resistance management. Thus, compositions of the present invention can further comprise a biologically effective amount of at least one additional invertebrate pest control compound or agent having a similar spectrum of control but a different mode of action. Contacting a plant genetically modified to express a plant protection compound (*e.g.*, protein) or the locus of the plant with a biologically effective amount of a compound of this invention can also provide a broader spectrum of plant protection and be advantageous for resistance management.

Thus, methods of the invention employ a herbicide or herbicide combination and may further comprise the use of insecticides and/or fungicides, and/or other agricultural chemicals such as fertilizers. The use of such combined treatments of the invention can broaden the spectrum of activity against additional weed species and suppress the proliferation of any resistant biotypes.

Methods of the invention can further comprise the use of plant growth regulators such as aviglycine, *N*-(phenylmethyl)-1*H*-purin-6-amine, ethephon, epocholeone, gibberellic acid, gibberellin A_4 and A_7, harpin protein, mepiquat chloride, prohexadione calcium, prohydrojasmon, sodium nitrophenolate and trinexapac-methyl, and plant growth modifying organisms such as *Bacillus cereus* strain BP01.

VI. Use as Selectable Markers and Methods of Transformation

In some embodiments of the invention, a construct of the invention comprising a GAT polynucleotide or an ALS inhibitor-tolerant polypeptide functions as a selectable marker,

e.g., in a plant, bacteria, actinomycete, yeast, algae or other fungi. For example, an organism that has been transformed with a vector including a GAT polynucleotide can be selected based on its ability to grow in the presence of glyphosate. Alternatively, an organism that has been transformed with a vector comprising an ALS-inhibitor-tolerant polynucleotide can be selected based on its ability to grown in the presence of an ALS inhibitor. In some embodiments of the invention, a construct of the invention comprising a GAT polynucleotide and another herbicide-tolerance polynucleotide (i.e., an polynucleotide encoding an ALS inhibitor-tolerant polypeptide, a polynucleotide encoding an HRA polypeptide, etc.) functions as a selectable marker, *e.g.*, in a plant, bacteria, actinomycete, yeast, algae or other fungi. For example, an organism that has been transformed with a vector including a GAT polynucleotide and another herbicide-tolerance polynucleotide can be selected based on its ability to grow in the presence of glyphosate and the appropriate other herbicide. As demonstrated in Example 10 and Figure 7, such methods of selection allow one to evaluate expression of any polynucleotide of interest at, for example, early stages in the transformation process in order to identify potential problems with expression. While any polynucleotide of interest can be employed, in specific embodiments, an insecticidal gene is used.

A construct of the invention comprising a GAT polynucleotide and/or a polynucleotide encoding an ALS inhibitor-tolerant polypeptide may exhibit a very high transformation efficiency, such as an efficiency of at least 20%, 30%, 40%, 50%, or 60% or higher. In this manner, improved methods of transformation are provided. Moreover, when a construct of the invention comprises a GAT polynucleotide and/or ALS inhibitor-tolerant polynucleotide, the transformants that are obtained may exhibit a very high frequency of tolerance to glyphosate or ALS inhibitor, so that, for example, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the transformants are tolerant to glyphosate and/or an ALS inhibitor. As used herein "transformation efficacy" is defined as the percentage of the T0 events that were resistant to a specific concentration of selection agent, such as glyphosate and/or an ALS inhibitor chemistry. When a construct of the invention comprises a GAT polynucleotide and/or ALS inhibitor-tolerant polynucleotide operably linked to an enhancer such as, for example, a 35S enhancer, the transformants that are obtained may exhibit a very high frequency of tolerance to glyphosate and/or ALS inhibitor, so that for example, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the transformants are tolerant to glyphosate or ALS inhibitor. In addition, when a construct of the invention comprises a GAT polynucleotide and/or an ALS inhibitor-tolerant polynucleotide, the frequency of

transformation events in which only a single copy of the construct is inserted into the genome may be as high as at least 35%, 40%, 50%, 60%, 70%, 80%, 90%, or higher. When a construct of the invention comprises a GAT polynucleotide and/or ALS inhibitor-tolerant polynucleotide operably linked to an enhancer such as, for example, a 35S enhancer, the frequency of transformation events in which only a single copy of the construct is inserted into the genome may be as high as at least 35%, 40%, 50%, 60%, 70%, 80%, 90%, or higher. In this manner, the invention also provides improved methods of transformation. It is recognized that when an enhancer is employed in the construct (such as S^{35} enhancer) multiple copies could be used, including 1, 2, 3, 4, 5, 6 or more. In such methods, the transformants may be selected using glyphosate and/or an ALS inhibitor, or they may be selected using another compound, such as another herbicide for which the transformed construct contains a tolerance trait.

VII. Kits

The invention further provides a kit comprising at least one nucleic acid construct which comprises a polynucleotide which encodes a polypeptide that can confer glyphosate tolerance and/or a polynucleotide encoding an ALS inhibitor-tolerant polypeptide for use in creating a glyphosate/ALS inhibitor plant of the invention. In specific embodiments, the kit can comprise a polynucleotide encoding GAT or the kit can comprise a polynucleotide encoding GAT and a polynucleotide encoding an ALS inhibitor-tolerant polynucleotide (i.e., HRA). In some aspects a construct of the invention will comprise a T-DNA sequence. The construct can optionally include a regulatory sequence (*e.g.*, a promoter) operably linked to the polynucleotide conferring glyphosate resistance, where the promoter is heterologous with respect to the polynucleotide and effective to cause sufficient expression of the encoded polypeptide to enhance the glyphosate tolerance of a plant cell transformed with the nucleic acid construct.

The article "a" and "an" are used herein to refer to one or more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each

individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

EXPERIMENTAL

Example 1—GAT-HRA Maize Plants are Tolerant of Various Herbicides and Agricultural Chemicals

For *Agrobacterium*-mediated transformation of maize with an expression cassette containing a GAT polynucleotide (SEQ ID NO:4) and an HRA polynucleotide (SEQ ID NO:66) operably linked to a constitutive promoter, the method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Expression cassettes were made that comprised a GAT polynucleotide and an HRA polynucleotide. In some expression cassettes, the GAT and HRA polynucleotides were operably linked to at least one copy of a 35S enhancer [SEQ ID NO:72]. In some expression cassettes, the GAT and HRA polynucleotides were operably linked to two or three copies of the 35S enhancer of SEQ ID NO:1. In some expression cassettes, the GAT polynucleotide was operably linked to a ubiquitin promoter and the HRA polynucleotide was operably linked to the native maize acetolactate synthase (ALS) promoter.

Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the GAT and HRA sequence to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). The immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). The immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered

(step 4: the selection step). The immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and calli grown on selective medium are cultured on solid medium to regenerate the plants.

Evaluation of herbicide tolerance

GAT-HRA maize plants were evaluated for tolerance to glyphosate and other herbicides. One protocol used in this evaluation was as follows. At the V2 stage (Ritchie and Hanway (1982), "How a corn plant develops" *Spec. Rep. 48.* (Coop. Ext. Ser., Iowa State Univ., Ames, Iowa)), plant heights were measured and herbicides were applied by spray application. Ten to fourteen days after the spray application, the transgenic plants were evaluated for injury symptoms and measured for plant height again in order to determine plant growth rates. In one series of tests, GAT-HRA maize and control plants were treated (postemergence) with one of the following herbicides: tribenuron (as Express®, at application rates of 0 and 200 grams active ingredient per hectare (g ai/ha)); chlorimuron (as Classic®, at 0 and 200 g ai/ha); imazapyr (as Arsenal®, at 0 and 200 g ai/ha); metsulfuron-methyl (as Ally®, at 0.5 oz ai/ac (36.9 ml ai/ha) or 35 g ai/ha). For each treatment, the GAT-HRA maize showed no significant damage from the treatment, while the control maize was severely damaged or killed.

GAT-HRA maize plants were also treated with various other herbicides and combinations of herbicides as well as other agricultural chemicals, including: glyphosate (as WeatherMax®, at an application rate of 64.4 oz ai/ac (4.7 L ai/ha)); rimsulfuron (as Matrix®, at an application rate of 1.9 oz ai/ac (140 ml ai/ha)); sulfometuron-methyl (as Oust®, at an application rate of 4.5 oz ai/ac (332 ml ai/ha)); Basis® (a combination of rimsulfuron and thifensulfuron-methyl, at an application rate of 1.4 oz ai/ac (103 ml ai/ha)); and chlorpyrifos (as Lorsban®, at an application rate of 14.4 oz ai/ac (1.06 L ai/ha)). Plants were then evaluated at various times after treatment, such as 10 or 14 days after treatment. Tolerant plants were those which showed little or no damage following treatment with a herbicide or herbicide combination. This evaluation identified GAT-HRA plants which were tolerant to multiple sulfonylurea and glyphosate chemistries. GAT-HRA plants did not exhibit any significant differences in growth or seed set (*i.e.*, yield) in comparison to control plants. Leaf samples were taken from GAT-HRA plants and used in quantitative PCR analysis and other analyses to determine the number and arrangement of copies of the GAT and HRA polynucleotides that had been integrated into the plant genome.

Other protocols for evaluation included treatment with imidazolinone herbicides, such as the commercial herbicide Lightning® (a combination of imazapyr and imazethapyr), which was applied to GAT-HRA maize at the V2 leaf stage at four times the field label rate (250 g ai/ha). Plants were evaluated fourteen days after the herbicide application, and four of the six transgenic events tested showed no symptoms of injury from the herbicide. Another protocol for evaluation included treatment with imidazolinone herbicides as well as sulfonylurea and glyphosate herbicides. In these tests, GAT-HRA maize was treated with various combinations of Lightning® (imazapyr and imazethapyr), Basis® (rimsulfuron and thifensulfuron-methyl) and WeatherMAX® (glyphosate potassium salt). These herbicides were applied at the V2 stage as follows: Lightning® at twice the field label rate (1.8 oz ai/ac (133 ml ai/ha)), Basis® at twice the field label rate (0.5 oz ai/ac (36.9 ml ai/ha)) and WeatherMax® at four times the field label rate (43 oz ai/ac). Fourteen days after the treatment application, the plants were evaluated. Plants containing eleven of the twelve transgenic events showed no herbicide injury symptoms. "Field label rate" refers to the application rate specified on the product label. Where the application rate for a particular situation is a range of rates, as used herein, the term "field label rate" indicates the upper end of that range. Product label information for pesticides is available from the U.S. Environmental Protection Agency and is updated online at the url oaspub.epa.gov/pestlabl/ppls.own; product label information is also available online at the url www.cdms.net.

Further tests confirmed that the multiple herbicide tolerance of these GAT-HRA maize plants was stably inherited. T1 seed was harvested from 20 transgenic T0 plants that showed excellent herbicide tolerance in greenhouse evaluations. As is known in the art, the tolerance of plants to herbicide can vary with the environment, and herbicide treatments in a greenhouse environment can have a greater impact on treated plants than herbicide treatments in the field. Accordingly, the T1 seed was further evaluated under field conditions. T1 plants were sprayed at the V4 leaf stage with four different herbicide treatment combinations including sulfonylurea and glyphosate herbicides. In some tests, a transgenic control line was used that was known to be tolerant of glyphosate but susceptible to other herbicides. The tested T1 plants also showed excellent herbicide tolerance under field conditions, confirming the stable inheritance of the herbicide tolerance.

Evaluation of tolerance to range of herbicides

GAT-HRA maize plants were then evaluated to determine whether they were also tolerant to other herbicides. A test of both preemergent and postemergent herbicide application was conducted. Specifically, seven corn seeds of each line to be evaluated were planted 1 cm deep in 5.5 inch square plastic containers of Tama silt loam soil. Treatments described in Tables 3 and 4 were applied before any watering. A week after emergence, seedlings were thinned so that each container had two uniform plants. Corn seedlings were watered and fertilized for rapid growth and grown with a 16-h light photoperiod. When the natural light intensity fell below 500 $\mu E/m^2/s$, it was supplemented by metal halide lights with 160 $\mu E/m^2/s$ photosynthetically active radiation. Temperature was maintained at 28 ± 2 °C during the day and 22 ± 2 °C at night. Relative humidity generally ranged from 50 to 90%.

The studies of preemergence herbicide application used commercial herbicide formulations. Spray mixtures were prepared using deionized water at room temperature and were stirred for at least 15 minutes. Treatments were sprayed 1 to 2 h after preparation. All treatments were applied in a spray volume of 374 L/ha with a flat fan nozzle at 51 cm with spray pressure set at 138 kPa with a high pH, basic blend adjuvant to ensure solubilization. Plants were visually evaluated for injury and fresh shoots were weighed 4 weeks after treatment (results shown in Table 1). Crop injury was also estimated visually on a scale ranging from 0% to 100%, where 0% signifies no injury and 100% signifies plant death. Results are shown in Table 3 and are expressed as the mean of four replications.

Table 3. Preemergence Effect of 34 ALS Herbicides on Fresh Weight of GAT-HRA and Parent Inbred Corn

ALS Inhibitor Class	Herbicide Treatment*	patent elite stiff stalk inbred	Hetero-zygous GAT-HRA
		Fresh Weight (g)	
Untreated		36.7	47.0
Imidazolinone	200 g/ha Imazamethabenz-methyl	36.2	48.5
	200 g/ha Imazethapyr	24.4	50.0
	200 g/ha Imazamox	0.8	47.5
	200 g/ha Imazapyr	0.3	44.2
	200 g/ha Imazaquin	1.5	45.4
Pyrimidinylthio-benzoate	200 g/ha Pyriminobac methyl	30.4	39.9
	200 g/ha Pyrithiobac Na^+	0.0	48.6
Sulfonylaminocar-bonyltriazolinone	200 g/ha Flucarbazone Na^+	0.0	48.8
	200 g/ha Propoxycarbazone Na^+	0.6	46.7
Sulfonylurea	200 g/ha Amidosulfuron	14.2	47.7
	200 g/ha Azimsulfuron	0.0	49.1
	200 g/ha Bensulfuron-methyl	28.4	39.9
	200 g/ha Chlorimuron-ethyl	9.3	39.4
	200 g/ha Chlorsulfuron	0.0	48.4
	200 g/ha Ethametsulfuron-methyl	1.6	50.1
	200 g/ha Ethoxysulfuron	28.1	40.0
	200 g/ha Flupyrsulfuron-methyl Na^+	23.6	45.8
	200 g/ha Foramsulfuron	30.6	39.1
	200 g/ha Halosulfuron-methyl	28.9	40.1
	200 g/ha Iodosulfuron-methyl Na^+	24.4	52.5
	200 g/ha Metsulfuron-methyl	0.0	41.1
	200 g/ha Nicosulfuron	33.6	50.3
	200 g/ha Primisulfuron-methyl	19.5	37.9
	200 g/ha Prosulfuron	20.9	43.3
	200 g/ha Rimsulfuron	29.8	45.3
	200 g/ha Sulfometuron-methyl	0.0	47.3
	200 g/ha Sulfosulfuron	1.0	50.6
	200 g/ha Thifensulfuron-methyl	15.2	36.0
	200 g/ha Triasulfuron	14.1	47.7
	200 g/ha Tribenuron-methyl	32.1	46.8
	200 g/ha Trifloxysulfuron Na^+	0.0	50.8
	200 g/ha Triflusulfuron-methyl	26.3	53.5
Triazolopyrimidine	200 g/ha Chloransulam-methyl	5.5	44.9
	200 g/ha Flumetsulam	24.6	39.1

Table 4. Preemergence Effect of 34 ALS Herbicides on Visual Injury to GAT-HRA and Parent Inbred Corn

ALS Class	Herbicide Treatment*	Patent elite stiff stalk inbred	Hetero-zygous GAT-HRA
		% Visual Injury	
Imidazolinone	200 g/ha Imazamethabenz-methyl	61	15
	200 g/ha Imazethapyr	69	20
	200 g/ha Imazamox	99	13
	200 g/ha Imazapyr	99	30
	200 g/ha Imazaquin	99	28
Pyrimidinylthio-benzoate	200 g/ha Pyriminobac-methyl	83	20
	200 g/ha Pyrithiobac Na^+	100	0
Sulfonylaminocar-bonyltriazolinone	200 g/ha Flucarbazone Na^+	100	40
	200 g/ha Propoxycarbazone Na^+	100	100
Sulfonylurea	200 g/ha Amidosulfuron	97	25
	200 g/ha Azimsulfuron	100	40
	200 g/ha Bensulfuron-methyl	23	10
	200 g/ha Chlorimuron-ethyl	100	10
	200 g/ha Chlorsulfuron	100	33
	200 g/ha Ethametsulfuron-methyl	100	88
	200 g/ha Ethoxysulfuron	63	25
	200 g/ha Flupyrsulfuron-methyl Na^+	87	90
	200 g/ha Foramsulfuron	5	7
	200 g/ha Halosulfuron-methyl	30	15
	200 g/ha Iodosulfuron-methyl Na^+	92	5
	200 g/ha Metsulfuron-methyl	100	0
	200 g/ha Nicosulfuron	43	0
	200 g/ha Primisulfuron-methyl	68	7
	200 g/ha Prosulfuron	68	9
	200 g/ha Rimsulfuron	74	0
	200 g/ha Sulfometuron-methyl	100	6
	200 g/ha Sulfosulfuron	100	18
	200 g/ha Thifensulfuron-methyl	88	3
	200 g/ha Triasulfuron	89	12
	200 g/ha Tribenuron-methyl	66	25
	200 g/ha Trifloxysulfuron Na^+	100	0
	200 g/ha Triflusulfuron-methyl	100	5
Triazolopyrimidine	200 g/ha Chloransulam-methyl	3	3
	200 g/ha Flumetsulam	13	13

The studies of postemergence herbicide application also used commercial herbicide formulations. Specifically, four corn seeds of each line to be evaluated were planted 1 cm deep in 5.5 inch square plastic containers of synthetic growth medium. Very young transgenic seedlings were pretreated with glyphosate to eliminate segregants which were sensitive to glyphosate. Plants injured by the glyphosate treatment were removed and containers were thinned to two uniform plants each. Extra containers were planted so that only containers with two uniform and uninjured plants were used in the experiment. Corn seedlings were watered and fertilized for rapid growth and grown with a 16-h light photoperiod. When the natural light intensity fell below 500 $\mu E/m^2/s$, it was supplemented by metal halide lights with 160 $\mu E/m^2/s$ photosynthetically active radiation. Temperature was maintained at 28± 2 °C during the day and 22 ± 2 °C at night. Relative humidity generally ranged from 50 to 90%.

Postemergence studies used commercial herbicide formulations and were applied two weeks after planting. Spray mixtures of herbicides were prepared using deionized water at room temperature and were stirred for at least 15 minutes. Treatments were sprayed 1 to 2 h after preparation. All ALS herbicide treatments were applied in a spray volume of 374 L/ha with a flat fan nozzle at 51 cm with spray pressure set at 138 kPa and included a high pH, basic blend adjuvant to ensure solubilization and foliar penetration. Glyphosate and glufosinate herbicide preparations included adjuvant systems in their commercial formulations to ensure high foliar activity. Fresh shoots were weighed 2 weeks after treatment (data shown in Table 5), and plants were visually evaluated for injury on a scale from 0% to 100% on which 0% indicates no injury and 100% indicates plant death (data shown in Table 6). Results are expressed in Tables 5 and 6 as the mean of four replications.

Table 5. Postemergence Effect of 34 ALS Herbicides on Fresh Weight of GAT-HRA and Parent Inbred Corn

ALS Class	Herbicide Treatment*	Parent elite stiff stalk inbred	Hetero-zygous GAT-HRA
		Fresh Weight (g)	
Untreated		76.4	98.8
Imidazolinone	200 g/ha Imazamethabenz-methyl	66.9	95.0
	200 g/ha Imazethapyr	6.4	93.4
	200 g/ha Imazamox	2.2	90.9
	200 g/ha Imazapyr	2.3	97.8
	200 g/ha Imazaquin	4.4	113.3
Pyrimidinylthio-benzoate	200 g/ha Pyriminobac-methyl	7.6	95.2
	200 g/ha Pyrithiobac Na^+	2.8	87.9
Sulfonylaminocar-bonyltriazolinone	200 g/ha Flucarbazone Na^+	3.1	86.0
	200 g/ha Propoxycarbazone Na^+	1.9	101.2
Sulfonylurea	200 g/ha Amidosulfuron	32.0	107.4
	200 g/ha Azimsulfuron	1.5	87.9
	200 g/ha Bensulfuron-methyl	9.0	103.3
	200 g/ha Chlorimuron-ethyl	10.1	95.5
	200 g/ha Chlorsulfuron	1.7	105.2
	200 g/ha Ethametsulfuron-methyl	5.6	98.0
	200 g/ha Ethoxysulfuron	24.3	79.3
	200 g/ha Flupyrsulfuron-methyl Na^+	5.2	95.5
	200 g/ha Foramsulfuron	62.5	80.6
	200 g/ha Halosulfuron-methyl	58.1	94.5
	200 g/ha Iodosulfuron-methyl Na^+	3.5	95.0
	200 g/ha Metsulfuron-methyl	1.9	75.5
	200 g/ha Nicosulfuron	62.7	94.7
	200 g/ha Primisulfuron-methyl	16.6	101.0
	200 g/ha Prosulfuron	53.6	91.3
	200 g/ha Rimsulfuron	12.6	99.2
	200 g/ha Sulfometuron-methyl	2.0	104.5
	200 g/ha Sulfosulfuron	4.0	105.5
	200 g/ha Thifensulfuron-methyl	4.2	86.6
	200 g/ha Triasulfuron	6.4	90.3
	200 g/ha Tribenuron-methyl	1.8	90.8
	200 g/ha Trifloxysulfuron Na^+	1.0	86.6
	200 g/ha Triflusulfuron-methyl	2.1	110.9
Triazolopyrimidine	200 g/ha Chloransulam-methyl	6.8	104.4
	200 g/ha Flumetsulam	12.8	103.8
Glycine	3472 g ae/ha Glyphosate	1.0	97.4
Phosphinic Acid	1870 g/ha Glufosinate ammonium	2.4	112.3

Table 6. Postemergence Effect of 34 ALS Herbicides on GAT-HRA and Parent Inbred Corn

ALS Class	Herbicide Treatment*	Parent elite stiff stalk inbred	Hetero-zygous GAT-HRA
		% Visual Injury	
Imidazolinone	200 g/ha Imazamethabenz-methyl	11	16
	200 g/ha Imazethapyr	97	9
	200 g/ha Imazamox	100	4
	200 g/ha Imazapyr	100	1
	200 g/ha Imazaquin	100	2
Pyrimidinylthio-benzoate	200 g/ha Pyriminobac-methyl	93	4
	200 g/ha Pyrithiobac Na^+	100	9
Sulfonylaminocar-bonyltriazolinone	200 g/ha Flucarbazone Na^+	100	14
	200 g/ha Propoxycarbazone Na^+	100	9
Sulfonylurea	200 g/ha Amidosulfuron	71	8
	200 g/ha Azimsulfuron	100	20
	200 g/ha Bensulfuron-methyl	93	14
	200 g/ha Chlorimuron-ethyl	93	15
	200 g/ha Chlorsulfuron	100	13
	200 g/ha Ethametsulfuron-methyl	96	6
	200 g/ha Ethoxysulfuron	88	15
	200 g/ha Flupyrsulfuron-methyl Na^+	95	4
	200 g/ha Foramsulfuron	28	8
	200 g/ha Halosulfuron-methyl	14	8
	200 g/ha Iodosulfuron-methyl Na^+	100	1
	200 g/ha Metsulfuron-methyl	100	14
	200 g/ha Nicosulfuron	24	0
	200 g/ha Primisulfuron-methyl	75	5
	200 g/ha Prosulfuron	25	29
	200 g/ha Rimsulfuron	91	10
	200 g/ha Sulfometuron-methyl	100	2
	200 g/ha Sulfosulfuron	97	3
	200 g/ha Thifensulfuron-methyl	98	10
	200 g/ha Triasulfuron	97	6
	200 g/ha Tribenuron-methyl	100	8
	200 g/ha Trifloxysulfuron Na^+	100	29
	200 g/ha Triflusulfuron-methyl	100	8
Triazolopyrimidine	200 g/ha Chloransulam-methyl	96	3
	200 g/ha Flumetsulam	85	3
Glycine	3472 g ae/ha Glyphosate	100	10
Phosphinic Acid	1870 g/ha Glufosinate ammonium	99	1

Additional tests including other agricultural chemicals

Other protocols were used to evaluate whether GAT-HRA plants, in addition to being more tolerant to various herbicides than control plants, were also more tolerant to other agricultural chemicals than control plants. For example, one protocol included treatment of GAT-HRA maize with the sulfonylurea herbicides rimsulfuron and thifensulfuron-methyl in addition to treatment with the organophosphate insecticide chlorpyrifos (Lorsban®). In this test, plants were evaluated 14 days after treatment, and all 20 GAT-HRA transgenic plants showed good to excellent tolerance to these chemicals while the control plants showed significant damage as a result of the treatments (see Table 8). Herbicide injury scores (also called tolerance scores) were assigned on a plot basis on a 1 to 9 scale with a rating of 9 indicating that plants exhibited no injury symptoms and a rating of 1 indicating complete plant death. A rating of 5 indicates moderate leaf injury. The scale used here is further explained in Table 7 below.

Table 7. Herbicide injury scale (1 to 9 scale scoring system) for maize

Rating	Main categories	Detailed description
9	No Effect	No crop reduction or injury
8	Slight Effect	Slight crop discoloration or stunting
7		Some crop discoloration, stunting, or stunt loss
6		Crop injury more pronounced, but not lasting
5	Moderate Effect	Moderate injury, crop usually recovers
4		Crop injury more lasting, recovery doubtful
3		Lasting crop injury, no recovery
2	Severe Effect	Heavy crop injury and stand loss
1		Crop nearly destroyed - A few surviving plants

Table 8. GAT HRA Transgenic Maize Plants Show Excellent Tolerance to an Herbicide as Measured by Tolerance Scores

		V4 Tolerance Scores			
Transgenic Event	Expression cassette	Basis® (rimsulfuron and thifensulfuron-methyl), 1.0 oz ai/ac (73.9 ml ai/ha); WeatherMax® (glyphosate) 10.7 oz ai/ac (790 ml ai/ha)	Basis® (rimsulfuron and thifensulfuron-methyl), 1.0 oz ai/ac (73.9 ml ai/ha); Lorsban® (chlorpyrifos), 14.4 oz ai/ac (1.06 L ai/ha); WeatherMax® (glyphosate) 10.7 oz ai/ac (790 ml ai/ha)	WeatherMax® (glyphosate) 10.7 oz ai/ac (790 ml ai/ha)	WeatherMax® (glyphosate) 42.9 oz ai/ac (3.17 L ai/ha)
2	4	9	9	9	7
4	3	9	9	9	9
3	3	9	9	9	5
10	3	8	9	9	7
Glyphosate tolerant non-GAT HRA Control		5.5	2.5	9	8.5

Fourteen days after the spray application, the 20 transgenic plants were also measured for plant height on a plot basis. Plant heights of the same four most tolerant GAT HRA events along with the non-GAT HRA control were collected. The four transgenic events showed uniform plant growth on all the herbicide treatments. The non-GAT HRA control showed a reduction in plant growth with the sulfonylurea treatments. The average plot height results from these measurements are shown in Table 9.

Table 9. GAT HRA Transgenic Maize Plants Show Excellent Tolerance to an Insecticide as Measured by Average Plant Height

		V4 Average Plant Heights (inches)			
Transgenic Event	Expression cassette	Basis® (rimsulfuron and thifensulfuron-methyl), 1.0 oz ai/ac (73.9 ml ai/ha); WeatherMax® (glyphosate) 10.7 oz ai/ac (790 ml ai/ha)	Basis® (rimsulfuron and thifensulfuron-methyl), 1.0 oz ai/ac (73.9 ml ai/ha); Lorsban® (chlorpyrifos), 14.4 oz ai/ac (1.06 L ai/ha); WeatherMax® (glyphosate) 10.7 oz ai/ac (790 ml ai/ha)	WeatherMax® (glyphosate) 10.7 oz ai/ac (790 ml ai/ha)	WeatherMax® (glyphosate) 42.9 oz ai/ac (3.17L ai/ha)
2	4	16	14	14	13
4	3	15	14	15	14
3	3	15	14	14	14
10	3	15	15	15	14
Glyphosate tolerant non-GAT HRA Control		10	7.5	14.5	14

Comparison of response of GAT-HRA plants to a range of herbicide doses

GAT-HRA maize plants were produced using various expression cassettes and assayed as described above for tolerance to multiple sulfonylurea chemistries in combination with glyphosate (see Table 10).

Table 10. GAT HRA Maize Produced Using Various Expression Cassettes Is Tolerant to Multiple Sulfonylurea Chemistries

Spray Treatment	Expression cassette	Percentage of highly tolerant events	Average growth of sprayed plants (inches)	Average growth of non-sprayed plants (inches)	Average number inserts integrated in highly tolerant events	Total number of events evaluated	Number of events with no injury
Matrix® (rimsulfuron) 1.9 oz ai/ac (140 ml ai/ha), WeatherMax® (glyphosate) 64.4 oz ai/ac (4.7 L ai/ha)	3	25%	5.2	5.1	2.0	56	14
Matrix® (rimsulfuron) 1.9 oz ai/ac (140 ml ai/ha), WeatherMax® (glyphosate) 64.4 oz ai/ac (4.7 L ai/ha)	4	31%	5.2	5.1	1.6	51	16
Matrix® (rimsulfuron) 1.9 oz ai/ac (140 ml ai/ha), WeatherMax® (glyphosate) 64.4 oz ai/ac (4.7 L ai/ha)	5	27%	4.8	5.3	1.7	63	17
Matrix® (rimsulfuron) 1.9 oz ai/ac (140 ml ai/ha), WeatherMax® (glyphosate) 64.4 oz ai/ac (4.7 L ai/ha)	6	41%	4.5	5.0	1.4	182	75
Basis® (rimsulfuron and thifensulfuron-methyl) 1.4 oz ai/ac (103 ml ai/ha), Lorsban® (chlorpyrifos) 14.4 oz ai/ac (1.06 L ai/ha), WeatherMax® (glyphosate) 64.4 oz ai/ac (4.7 L ai/ha)	9	59%	2.6	3.1	1.4	27	16

Oust® (sulfometuron-methyl) 4.5 oz ai/ac (322 ml ai/ac), WeatherMax® (glyphosate) 64.4 oz ai/ac (4.7 L ai/ha)	9	71%	4.2	4.8	1.1	146	104

Example 2—GAT-HRA Soybean Plants are Tolerant of Various Herbicides and Agricultural Chemicals

Transformation and Regeneration of Transgenic Plants

Soybean embryos were bombarded with an expression cassette containing GAT (SEQ ID NO:68) and HRA polynucleotides (SEQ ID NO:65) operably linked to a constitutive promoter, as follows. The promoter used for the GAT sequence is the SCP1 promoter, and the promoter used for the HRA sequence is the SAMS promoter. The 2 promoter/gene combinations are arranged in a tandem orientation with the HRA promoter/gene downstream of the GAT promoter/gene. To induce somatic embryos, cotyledons less than 4 mm in length dissected from surface-sterilized, immature seeds of the soybean variety Jack were cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos were then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions were maintained as described below. Here, the herbicide-tolerance traits that should be possessed by the transformed plants were used as selectable markers.

Soybean embryogenic suspension cultures were maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with fluorescent lights on a 16:8 hour day/night schedule. Cultures were subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

Soybean embryogenic suspension cultures were then transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature (London)* 327: 70-73, U.S. Patent No. 4,945,050).

To 50 µl of a 60 mg/ml 1 µm gold particle suspension was added (in order): 5 µl DNA (1 µg/µl), 20 µl spermidine (0.1 M), and 50 µl $CaCl_2$ (2.5 M). The particle preparation was then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant

removed. The DNA-coated particles were then washed once in 400 µl 70% ethanol and resuspended in 40 µl of anhydrous ethanol. The DNA/particle suspension were sonicated three times for one second each. Five microliters of the DNA-coated gold particles were then loaded on each macro carrier disk.

Approximately 150-200 mg of a two-week-old suspension culture was placed in an empty 60x15 mm Petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue were normally bombarded. Membrane rupture pressure was set at 1100 psi (77.356 kg/cm), and the chamber was evacuated to a vacuum of 28 inches mercury. The tissue was placed approximately 8.89 cm away from the retaining screen and bombarded three times. Following bombardment, the tissue was divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media was exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 30-50 mg/L hygromycin and 100 ng/ml chlorsulfuron was used as a selection agent. This selective media was refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue was observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue was removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new culture derived from a separate area of transformed tissue was treated as an independent transformation event; the individual culture as well as the initial (T0) plant(s) derived from a single area of transformed tissue as well as its descendants were generally to represent a single "event." These suspensions were then subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

Herbicide treatments

Young regenerated transgenic plants were sprayed with 1x glyphosate (1X rate of glyphosate is 1120 g/ha of glyphosate isopropylamine) to cull segregants. Four replications were performed for each treatment. Plants were then treated with various herbicides, including glyphosate and ALS-inhibitor herbicides. When treated with tribenuron-methyl herbicide, the GAT- and HRA-containing transgenic soybeans treated at 0 and 35 g/ha showed no significant damage, while herbicide-treated non-transgenic control soybeans were killed by the treatment. Plants were then treated with glyphosate at 8x and tribenuron-methyl at 35 g/ha as well as rimsulfuron at 35 g/ha, and similar results were obtained.

Six soybean seeds from each variety to be assayed were planted 1 cm deep in 5.5 (13.97 cm) inch square plastic container of a synthetic growth medium. Very young transgenic seedlings were pretreated with glyphosate to eliminate segregants that were not tolerant to glyphosate; plants injured by this treatment were removed. When possible, containers were thinned to two uniform plants. Non-transgenic lines were thinned to two uniform plants per container. Soybean seedlings were watered and fertilized for rapid growth. The plants were grown with a 16-h light photoperiod, and when natural light intensity fell below 500 $\mu E/m^2/s$ it was supplemented with metal halide lights with 160 $\mu E/m^2/s$ photosynthetically active radiation. Temperature was maintained at 28± 2 °C during the day and 22 ± 2 °C at night. Relative humidity generally ranged from 50 to 90%.

These postemergence studies used commercial herbicide formulations and were applied approximately two weeks after planting. Spray mixtures were made using deionized water at room temperature and were stirred for at least 15 minutes. Treatments were sprayed 1 to 2 h after preparation. The 35 g/ha rimsulfuron and tribenuron-methyl herbicide treatments were applied in a spray volume of 374 L/ha with a flat fan nozzle at 51 cm with spray pressure set at 138 kPa and included a high pH, basic blend adjuvant to ensure solubilization and foliar penetration. The commercial formulation of glyphosate treatment was also applied in a spray volume of 374 L/ha with a flat fan nozzle at 51 cm with spray pressure set at 138 kPa. Plants were visually evaluated for injury on a scale of 0% being no injury and 100% being plant death. Results are expressed below in Table 11 as the mean of four replications.

Table 11. Postemergence Effect of Glyphosate and Two Sulfonylurea Herbicides on GAT-HRA Soybeans

Event #	Line # (and mean)	Glyphosate (8960 g/ha)	Tribenuron -methyl (35 g/ha)	Rimsulfuron (35 g/ha)
		------- *% Visual Injury* -------		
64	**Mean**	**14**	**43**	**73**
	47	10	34	73
	41	20	55	73
60	**Mean**	**15**	**55**	**83**
	41	13	38	80
	70	9	40	80
	35	9	49	85
	11	11	71	83
	22	11	45	76
	75	14	69	86
	79	18	69	86
	07	19	46	84
	77	19	56	90
	46	20	49	78
	82	20	61	90
	84	22	71	83
61	**Mean**	**20**	**54**	**82**
	99	10	34	79
	47	15	36	78
	62	6	43	71
	49	9	54	84
	02	10	41	80
	14	10	65	90
	18	10	69	89
	51	10	60	83
	02B	10	40	70
	05	11	55	78
	60	16	45	73
	34	23	76	90
	24	60	63	90
	33	84	80	95
59	**Mean**	**38**	**59**	**87**
	28	37	60	86
	21	40	58	88
STS ®	**Mean**	**100**	**87**	**97**
	25	100	87	97
	46	100	88	97
Wild Type	**Mean**	**100**	**97**	**97**
	82	100	97	97
	Jack	100	97	97

Example 3—Transgenic GAT-HRA Cotton is Tolerant of Several Herbicides

Cotton (*Gossypium hirsutum*) Coker 312 was transformed with a GAT polynucleotide (*gat 4621*) together with an *Hra* polynucleotide (SEQ ID NO:86) both operably linked to

strong, constitutive plant viral promoters. The promoter linked to *gat4621* contains a duplicated portion of the Strawberry Vein Banding Virus transcript promoter (Wang et al. Virus Genes 20: 11-17, 2000; Genbank X97304). The *Hra* gene was driven by a duplicated portion of the Mirabilis Mosaic Caulimovirus full-length transcript promoter (US patent 6,420,547; Dey and Maiti, Transgenics 3:61-70, 1999). The transformation procedure used *Agrobacterium tumefaciens* containing an expression cassette with the genes to be transferred and cotyledon explant derived callus with some capacity to undergo embryogenesis. The callus was exposed to *Agrobacterium* for 48-72 hours. The callus was then exposed to selection for transformed cells on 50-200 μg/l chlorosulfuron and/or 50-450 μM glyphosate in solid medium. Glyphosate can also be used in a concentration range of about 5 to about 450 μM. Selection was applied until somatic embryos formed, and selection was again applied at the embryo germination step, and optionally during rooting of plantlets. Over 450 GAT-HRA transformation events were produced using selection with both chlorsulfuron and glyphosate.

Transformed cotton plants were rooted and then transferred to soil for further growth. Plants were then subjected to herbicide spray treatments in a greenhouse. In one treatment, glyphosate was sprayed over the top of plants at the 4-6 leaf stage of development at an application rate of 1.5 lb acid equivalent per acre (2.58 Kg acid equivalent per hectare). Untransformed Coker 312 control plants were dead 2 weeks after glyphosate application. In contrast, approximately 50% of the GAT-HRA transgenic plants (each corresponding to a separate transformation event) showed no deleterious symptoms from the glyphosate treatment 14 days after application. Plants transformed with both GAT and HRA were further subjected to an "over the top" application of the sulfonylurea herbicide rimsulfuron at a rate of 16 g active ingredient/hectare. Again, approximately 35% of the transgenic plants showed no deleterious symptoms from the dual herbicide application 14 days after the rimsulfuron application and 28 days after the glyphosate application. Untransformed Coker 312 plants showed severe damage from the rimsulfuron application, even in the absence of any glyphosate application. In further experiments 32 g ai/ha rimsulfuron were applied to the cotton.

The presence of each of the GAT and HRA polynucleotides was further confirmed in the herbicide-resistant transgenic plants using polymerase chain reaction ("PCR") assays and using Western blot analysis to detect the expressed GAT and HRA polypeptides.

Example 4—Formulation of Homogenous Granule Blends

A herbicidal composition useful for the present invention may be formulated in any suitable manner, for example, as a "homogeneous granule blend" (see, *e.g.*, U.S. Pat. No. 6,022,552, entitled "Uniform Mixtures of Pesticide Granules"). A herbicidal composition formulated as a homogeneous granule blend according to U.S. Pat. No. 6,022,552 can be prepared by shaking or otherwise mixing two or more groups of substantially cylindrical granules typically made by extrusion or pelletization, wherein one group has an active ingredient content comprising at least one herbicide, and one or more other groups have a different active ingredient content or inert content, the granules within each group having substantially uniform diameters and longitudinal lengths of from 1 to 8 times the diameter with the average length of the granules being from 1.5 to 4 times the diameter, and the average diameter of each group differing from another group by no more than 30%. In some embodiments, each granule group comprises a registered formulation product containing a single active ingredient, which is for example, a herbicide, fungicide, and/or an insecticide. "Substantially cylindrical" is rod like or tubular wherein the cross-sectional shape may be circular, octagonal, rectangular, or any other conceivable shape and wherein the longitudinal surface is spiral, curved, or straight. The difference in average diameter is calculated by subtracting the average diameter of the granules in the group having the smaller diameter from the average diameter of the granules in the group having the larger diameter, then dividing the calculated difference by the average diameter of the granules in the group having the smaller diameter, and finally multiplying the calculated quotient by 100%.

The uniformity of a "homogenous granule blend" can be optimized by controlling the relative sizes and size distributions of the granules used in the blend. Density differences are comparatively unimportant (see, *e.g.*, Rhodes (1990) *Principles of Powder Technology*, pp. 71-76 (John Wiley & Sons)). The diameter of extruded granules is controlled by the size of the holes in the extruder die, and a centrifugal sifting process may be used to obtain a population of extruded granules with a desired length distribution (see, *e.g.*, U.S. Pat. No. 6,270,025). Preferably the average diameter of each granule group differs from another group by no more than 20%, more preferably by no more than 10%. Also preferably the longitudinal length of each group is form 1.5 to 4 times the diameter of the granules.

The active ingredient in each formulation has an associated tolerance for variability based on guidelines of the Food and Agriculture Organization of the United Nations (FAO), as shown below in Table 12.

Table 12: FAO Nominal Concentration Guidelines for Active Ingredient in a Formulation

Nominal Concentration (= N)	FAO Range (as % of Nominal)
$N \leq 2.5\%$	± 25%
$2.5\% \leq N \leq 10\%$	± 10%
$10\% \leq N \leq 25\%$	± 6%
$25\% \leq N \leq 50\%$	± 5%
$N > 50\%$	± 25 g/kg

The active ingredient content in a homogenous granule blend is determined based on the active ingredient content of the component granules and the ratio in which the component granules are mixed. Homogenous granule blends are manufactured assuming that the nominal values of active ingredients of the blend components are correct. Because of the real-life variability associated with assays of active ingredients as well as variability in mixing and sampling, procedures were developed to calculate ranges for the active ingredient content in a homogenous granule blend, as follows.

1. Define the registered FAO specifications for each of the blend components ("%AI in Component").
2. Apply the FAO tolerance to establish manufacturing limits for the amount of each component in the blend ("% Component in Blend").
3. Calculate the maximum limit for the active ingredient in the blend ("%AI in the Blend") by multiplying the maximum limit for "% AI in Component" with the maximum limit for "%Component in Blend." The minimum and nominal calculations are similarly done.

Examples of the calculations for several homogenous granule blend products follow. The last column in each example table shows the standard FAO assay range that would apply to a traditional premix product containing the same active ingredient content as the homogenous granule blend in the example. The broader range for the homogenous granule blend product ("%AI in Blend") allows for the variability introduced by using a registered product with an associated range of active ingredient content as a component in the product.

Table 13: Calculations for product "DPX-CDQ73 39.1WG" Homogenous Granule Blend*			
%AI in Components (A)	% Component in Blend (B)	%AI in Blend (A x B)	Compare to FAO tolerance for a premix
% metsulfuro-methyl in Ally 20PX	*%Ally 20 PX in DPX-CDQ73 Blend*	*% metsulfuron-methyl in DPX-CDQ73 Blend*	*% metsulfuron-methyl in traditional premix*
21.2 max	66.7 max	14.4 max	13.8 max
20.0 ± 6%	65.2 ± 25 g/kg	13.0 nominal	13.0 nominal
18.8 min	62.7 min	11.8 min	12.3 min
% tribenuron-methyl in Quantum 75PX	*% Quantum 75PX in DPX-CDQ73 Blend*	*% tribenuron-methyl in DPX-CDQ73 Blend*	*% tribenuron-methyl in traditional premix*
77.5 max	36.5 max	28.3 max	27.4 max
75 ± 25 g/kg	34.8 ± 5%	26.1 nominal	26.1 nominal
72.5 min	33.1 min	24.0 min	24.8 min

* = A blend of 65.2% Ally 20PX and 34.8% Quantum 75PX. This blend is sold commercially as BiPlay and DP911.

Table 14: Calculations for "DPX-CDQ74 51.5WG" Homogenous Granule Blend **

% AI in Components (A)	% Component in Blend (B)	% AI in Blend (A x B)	Compare to FAO tolerance for a premix
%metsulfuron-methyl in Ally 20PX	*%Ally 20 PX in DPX-CDQ73 Blend*	*%metsulfuron-methyl in DPX-CDQ73 Blend*	*%metsulfuron-methyl in traditional premix*
21.2 max	44.9 max	9.5 max	9.4 max
20.0 ± 6%	42.8 ± 5%	8.6 nominal	8.6 nominal
18.8 min	40.7 min	7.7 min	7.7 min
% thifensulfuron-methyl in Harmony 75PX	*% Harmony 75PX in DPX-CDQ74 Blend*	*% thifensulfuron-methyl in DPX-CDQ74 Blend*	*% thifensulfuron-methyl in traditional premix*
77.5 max	59.7 max	46.3 max	45.0 max
75 ± 25 g/kg	57.2 ± 25 g/kg	42.9 nominal	42.9 nominal
72.5 min	54.7 min	39.7 min	40.8 min

** = A blend of 42.8% Ally 20PX and 57.2% Harmony 75PX. This blend is sold commercially as Finish and DP928.

Table 15: "DPX-FKU22 60WG" Homogenous Granule Blend ***			
% AI in Components (A)	% Component in Blend (B)	% AI in Blend (A x B)	Compare to FAO tolerance for a premix
% flupyrsulfuron methyl in Lexus 50PX	*% Lexus 50PX in DPX-FKU22 Blend*	*% flupyrsulfuron methyl in DPX-FKU22 Blend*	*% flupyrsulfuron methyl in traditional premix*
52.5 max	62.5 max	32.8 max	31.5 max
50.0 ± 5%	60.0 ± 25 g/kg	30.0 nominal	30.0 nominal
47.5 min	57.5 min	27.3 min	28.5 min
% tribenuron-methyl in Quantum 75PX	*% Quantum 75PX in DPX-FKU22 Blend*	*% tribenuron-methyl in DPX-FKU22 Blend*	*% tribenuron-methyl in traditional premix*
77.5 max	42.0 max	32.6 max	31.5 max
75 ± 25 g/kg	40.0 ± 5%	30.0 nominal	30.0 nominal
72.5 min	38.0 min	27.6 min	28.5 min

*** = A blend of 60% Lexus 50PX and 40% Quantum 75PX. This blend is sold commercially as DP953.

Procedures were also developed to determine whether a particular homogenous granule blend falls within the desired ranges. Homogenous granule blends are random mixtures of granules; therefore, in order to accurately represent the composition, a certain number of granules must be evaluated. The minimum number of granules for the sample can be estimated using a statistical equation (see Rhodes (1990), *Principles of Powder Technology* (John Wiley & Sons), pp. 71-76),

$$s^2 = P(100-P)/n$$

where s = standard deviation for the component proportion in the blend, P = weight percent of the component, and n = number of granules in the sample (~ 400 1-mm diameter granules = 1 gram). The sample size required to represent the blend composition for a particular chosen level of variability can be obtained by solving for n and converting this value to grams by dividing 'n' by the average number of 1-mm paste extruded granules in a 1-gram sample (*e.g.*, 400).

If the standard deviation in this calculation is based on the FAO tolerance for the amount of a component in a particular granule blend, a minimum sample size for that granule blend can be calculated. For 95% confidence, the tolerance around the "% component in the blend" is set as 2 standard deviations. It is understood that this is a theoretical statistical estimate.

For a granule blend to be a feasible commercial product, the statistical sample size must be equivalent to or smaller than the smallest amount that would be measured by a farmer or applicator, typically a hectare dose. Examples of a sample size calculation for the granule blends discussed above is shown below.

Table 16: Minimum statistical sample size calculation for DPX-CDQ73 39.1WG Blend

Blend Component	P (% component in Blend)	FOA tolerance for % component	Statistical Minimum Sample Size
Ally 20PX	65.2	± 25 g/kg (± 3.8% relative)	4 grams
Quantum 75PX	34.8	± 5% relative	8 grams

Detailed calculation for minimum sample size for DPX-CDQ73 39.1WG Blend:

For the minor blend component (Quantum 75PX) the FAO tolerance of ± 5% relative gives a range of: 5% x 34.8 = 1.7

For 95% confidence, 2 standard deviations are set at = 1.7 giving a standard deviation of s = 0.85 for the calculation:

$s^2 = P(1-P)/n = (0.85)2 = (65.2 \times 34.8)/n$

n = 3140 granules = 7.8 grams (based on 400 granules/gram)

The calculated minimum statistical sample size of about 8 grams is less than the product use rate of 38 g/ha.

A homogenous granule blend is considered to be "homogenous" when it can be subsampled into appropriately sized aliquots and the composition of each aliquot will meet the required assay specifications. To demonstrate homogeneity, a large sample of the homogenous granule blend is prepared and is then subsampled into aliquots of greater than the minimum statistical sample size. A second sample of the blend is prepared and subjected to a simulated transportation test (ASTM D 4728-87, Standard Method for Random Vibration Testing of Shipping Containers) and then subsampled into aliquots. The aliquots are

analyzed for active ingredient using liquid chromatography. The simulated transportation test shakes the bottle at specific frequencies for a standard amount of time, giving the granules an opportunity to move about. When the granule blend components are not appropriately sized, segregation occurs and the aliquot compositions vary unacceptably.

Aliquot analysis data from the homogeneity tests for DPX-CDQ73 39.1WG Blend are shown below in Table 17. All data points tested in the granule blend aliquots fell within their respective calculated specification ranges, indicating that the blend was homogenous.

Table 17: Analysis of DPX-CDQ73 39.1WG Blend as made after simulated shipment.

Aliquot (20 grams)	% metsulfuron-methyl in DPX-CDQ73 Blend	% tribenuron-methyl in DPX-CDQ73 Blend	% metsulfuron-methyl in DPX-CDQ73 Blend after simulated shipment	% tribenuron-methyl in DPX-CDQ73 Blend after simulated shipment
Proposed assay range	11.8-14.4	24.0-28.3	11.8-14.4	24.0-28.3
1	13.43	25.95	13.83	26.84
2	13.87	25.05	13.84	24.43
3	13.79	25.88	13.28	27.2
4	13.36	26.8	13.67	26.25
5	13.49	27.37	13.57	27.33

Homogenous granule blends have been manufactured in a batch process using a roll-type mixer. Once mixed, the granule blend is dispensed into appropriate containers (bottles, bags, *etc.*) for commercial sale. Testing of the manufactured homogenous granule blend DPX-CDQ73 WG showed that all data for the different batches of blend were within the respective proposed assay ranges for active ingredient.

Example 5. Evaluation of Levels of Glyphosate and ALS inhibitor herbicide Efficacy Among Soybean Plants Carrying Different GAT and HRA Sequences

Three GAT7 events in soybean were compared to four selected GAT11 soybean events to determine if differences could be detected for tolerance to high rates of glyphosate + sulfonylurea treatments. Across the treatment combinations, the GAT7 construct had significantly less spray response compared to the GAT11 constructs at 7, 14, and 28 DAS.

Within the 8X Touchdown® + 1X Resolve® + 2X Express® treatment, and the 8X Touchdown® + 2X Resolve® + 4X Express® treatment, GAT7 event EAFS 3560.4.3 had the lowest spray response scores compared to all other events at 7, 14, and 28 DAS.

Materials and Methods

For each treatment, three replications of two 12 foot plots of three selected GAT7 events and four selected GAT11 events were grown in a RCB design (blocked by treatment) at two locations in Hawaii. Individual lines, events, and construct tested are listed below in Table 18.

Table 18

Entry Name	GAT	Event	Construct	Construct Description
JH12862353	GAT11	EAFS 3861.2.3	PHP22021A	H2B:GAT4618::3(35Senh) +:SCP:HRA (DV)
JH12862357	GAT11	EAFS 3862.2.5	PHP22021A	H2B:GAT4618::3(35Senh) +:SCP:HRA (DV)
JH12862359	GAT11	EAFS 3862.2.5	PHP22021A	H2B:GAT4618::3(35Senh) +:SCP:HRA (DV)
JH12862360	GAT11	EAFS 3862.2.5	PHP22021A	H2B:GAT4618::3(35Senh) +:SCP:HRA (DV)
JH12862361	GAT11	EAFS 3862.2.5	PHP22021A	H2B:GAT4618::3(35Senh) +:SCP:HRA (DV)
JH12862364	GAT11	EAFS 3862.4.2	PHP22021A	H2B:GAT4618::3(35Senh) +:SCP:HRA (DV)
JH12862365	GAT11	EAFS 3862.4.2	PHP22021A	H2B:GAT4618::3(35Senh) +:SCP:HRA (DV)
JH12862405	GAT11	EAFS 3876.8.15	PHP22117A	H2B:GAT4621::3(35Senh)+:SCP:HRA (DV)
JH12862406	GAT11	EAFS 3876.8.15	PHP22117A	H2B:GAT4621::3(35Senh)+:SCP:HRA (DV)
JH12862528	GAT7	EAFS 3560.4.3	PHP20163A	SCP:GAT4601::SAMS:HRA
JH12862529	GAT7	EAFS 3559.2.1	PHP20163A	SCP:GAT4601::SAMS:HRA
JH12862531	GAT7	EAFS 3561.1.1	PHP20163A	SCP:GAT4601::SAMS:HRA

The three different treatments applied at the V3 growth stage were; 1. 8X Touchdown® Hi-Tech (8630.55 g/ha a.i. glyphosate) + 1X Resolve® (35.0 g/ha a.i. rimsulfuron) + 2X Express® (17.5 g/ha tribenuron), 2. 8X Touchdown® Hi-Tech (8630.55 g/ha a.i. glyphosate) + 2X Resolve® (70.0 g/ha a.i. rimsulfuron) + 4X Express® (35.0 g/ha tribenuron), and 3. Unsprayed Control. All spray treatments also contained a 1X non-ionic surfactant and ammonium sulfate. At 7, 14, and 28 days after spraying, plots were given a visual spray damage rating based upon observed chlorosis, necrosis, and/or plant stunting (0% = no observed effect to 100% = entire plot deceased). Visual rating data were subject to ANOVA and mean separation using SAS.

Results and Discussion

Across the three treatments, the round of GAT (7 vs. 11), DNA construct, event, treatment, GAT*treatment, construct*treatment, and event*treatment were significantly different at 7 DAS and 14 DAS (data not shown). At 28 DAS, the GAT round, construct, event, treatment, GAT*treatment, and event*treatment effects were significantly different

(data not shown). The GAT7 lines had significantly less response noted across the three treatments at 7, 14, and 28 DAS (data not shown).

Within the 8X Touchdown® + 1X Resolve® + 2X Express® treatment, the GAT7 construct PHP20163A had significantly lower spray response ratings compared to GAT11 construct PHP22021A at 7, 14, and 28 DAS (data not shown). PHP20163A had significantly more tolerance to this treatment compared to PHP22117A only at 28 DAS (data not shown). Among the events compared, GAT7 event EAFS 3560.4.3 had the most initial tolerance observed at 7 and 14 DAS, and had the best recovery score at 28 DAS (data not shown). In examining the differences between least squares means (LSMeans), each of the three GAT7 events had significantly less spray response at 7 DAS compared to GAT11 EAFS 3861.2.3 and GAT11 EAFS 3862.2.5, and were rated statistically similar to GAT 11 EAFS 3862.2.5 and GAT11 EAFS 3876.8.1 (data not shown). At 14 DAS, the 3 GAT7 events had significantly less response scores compared to GAT11 EAFS 3862.4.2, but only GAT7 EAFS 3560.4.3 had significantly less response compared to EAFS 3861.2.3 and EAFS 3862.2.5 (data not shown). At 28 DAS, all three GAT7 events had significantly better recovery compared to GAT11 events EAFS 3861.2.3, EAFS 3862.4.2, and EAFS 3876.8.1 (data not shown). In addition, GAT7 event EAFS 3560.4.3 had significantly less spray response compared to GAT11 event EAFS 3862.2.5 at 28 DAS (data not shown).

In examining the 8X Touchdown® + 2X Resolve® + 4X Express® treatment, GAT7 PHP20163A had significantly less response compared to GAT11 PHP22021 at 7, 14, and 28 DAS, and PHP20163A had significantly less response compared to GAT11 PHP22117A at 28 DAS (data not shown). Among the events, GAT7 EAFS 3560.4.3 had significantly better tolerance compared to all other events at 7 and 14 DAS, and GAT7 EAFS 3559.2.1 and EAFS 3560.4.3 had significantly lower spray response compared to all other events at 28 DAS (data not shown). Comparing the differences in LSMeans, at 7 DAS and 14 DAS, GAT7 EAFS 3560.4.3 had significantly less response compared to all the GAT 11 events (data not shown). The other 2 GAT7 events were significantly lower in response compared to GAT 11 event EAFS 3862.4.2 at 7 and 14 DAS (data not shown). At 28 DAS, GAT7 events EAFS 3559.2.1 and EAFS 3560.4.3 had significantly better recovery compared to all the GAT11 events (data not shown). GAT7 event EAFS 3561.1.1 was only significantly better than GAT11 event EAFS 3862.4.2 (data not shown).

Example 6. Robustness Trial Data Analysis in Soybean

The interactions between sulfonylurea and imidazolinone under field trial conditions have been studied. Antagonism between the sulfonylurea and imidazolinone chemistries has been seen in the past on commercial STS® soybean varieties. An SU like thifensulfuron on STS® soy is normally safe. Add an IMI like imazethapyr (Pursuit) and the mixture becomes less safe (antagonized the crop safety). In the case of GAT Rd7, rimsulfuron causes crop phyto. Add an IMI like Pursuit and the mixture became more safe (antagonized the crop injury). Th e filed trials described below show there is an increased crop safety when normally injurious amounts of, for example, rimsulfuron were mixed with, for example, imazethapyr, a normally "safe" imidazolinone herbicide. Example 6A comprises a field trial that demonstrates this effect. Example 6B provides greenhouse data that confirms the field trial data.

Example 6A

The T6 generation of soybean of the lead GAT7 event (SEQ ID NO:68) also having HRA (SEQ ID NO:65) [SCP:GAT7::SAMS:ALS] was compared to 92B25 to determine levels of robustness when sprayed with different combinations and rates of sulfonylurea, chlorpyrifos, and/or imazethapyr chemistries. Across all the treatment combinations except 16X Harmony® (no significance detected), GAT7 had significantly lower spray response compared to STS® at 7, 14, and 28 days after spraying (DAS). Application of 1X Resolve® with and without Lorsban® created a large response from GAT7 and STS® at 7 DAS. The GAT7 was able to significantly recover from these treatments at 14 and 28 DAS, while the STS® line remained heavily damaged. The GAT7 line had significantly less spray response to 4X Pursuit® compared to the STS® line at 7 DAS, while both lines were not statistically different at 14 and 28 DAS. When 1X Resolve® + 4X Pursuit® was applied, the GAT7 line had significantly higher tolerance compared to the STS® line at 7, 14, and 28 DAS. At 7, 14, and 28 DAS 16X Harmony®, there was not a large response observed from either the GAT7 or STS® line. Data from the treatments combining 16X Harmony® with 4X Pursuit® or 16X Harmony® with 4X Express® indicated GAT7 had significantly lower spray response compared to the STS® line at 7, 14, and 28 DAS. In addition, the 7, 14, and 28 DAS data from 0.25X Resolve® + 1.5X Express® treatment showed GAT7 had significantly higher tolerance compared to STS®. In general, the data from this study indicate GAT7 provides significantly higher tolerance compared to STS® across multiple herbicide and insecticide chemistries.

Materials and Methods

For each treatment, three replications of two 12 foot plots of the lead GAT7 event (EAFS 3560.4.3; GEID=JH12862528) and 92B25 (STS®) were grown in a RCB design (blocked by treatment) at two locations in Hawaii. The nine different treatments applied at the V3 growth stage were; 1. 1X Resolve® (2 oz) (35.0 g/ha a.i. rimsulfuron), 2. 1X Resolve® (35.0 g/ha a.i. rimsulfuron) + 1X Lorsban® 4E (560.0 g/ha a.i. chlorpyrifos), 3. 4X Pursuit® (211.8 g/ha a.i. imazethapyr), 4. 1X Resolve® (35.0 g/ha a.i. rimsulfuron) + 4X Pursuit® (211.8 g/ha a.i. imazethapyr), 5. 16X Harmony® GT (70.0 gms/ha a.i. thifensulfuron), 6. 16X Harmony® (70.0 gms/ha a.i. thifensulfuron) + 4X Pursuit® (211.8 g/ha a.i. imazethapyr), 7. 16X Harmony® (70.0 gms/ha a.i. thifensulfuron) + 4X Express® (35.0 g/ha a.i. tribenuron) 8. 0.25X Resolve® (2,157 g/ha a.i. glyphosate) + 1.5X Express® (13.1 g/ha a.i. tribenuron) 9. Unsprayed Control. All spray treatments also contained a 1X non-ionic surfactant and ammonium sulfide. At 7, 14, and 28 days after spraying, plots were given a visual spray damage rating based upon observed chlorosis, necrosis, and/or plant stunting (0% = no observed effect to 100% = entire plot deceased). Visual rating data were subject to ANOVA and mean separation using SAS.

Results and Discussion

Across all treatments at 7 and 14 DAS, the location, GEID, treatments, and GEID*treatment effects were significantly different (data not shown). The GAT7 line was scored significantly lower than the STS® line across all treatments at 7 and 14 DAS (data not shown). At 28 DAS, the GEID, loc*GEID, treatments, and GEID*treatment effects were significantly different (data not shown). Over all the treatments, the GAT7 line was scored with significantly less spray damage compared to the STS® line at 28 DAS (data not shown).

The 1X Resolve® (rimsulfuron) treatment created a large response from both the GAT7 (70%); and STS® (83%) lines at 7 DAS (data not shown). At 14 DAS, the GAT7 line (74%) had significantly less response compared to the STS® line (93%) (data not shown). By 28 DAS, the GAT7 was able to recover and was scored with significantly less damage compared to the STS® line (32% vs. 93%) (data not shown).

When date from the Lorsban® (chlorpyrifos) + 1X Resolve® treatment are examined, the GAT7 line did not have significantly less response compared to the response of the STS® line until 14 and 28 DAS (data not shown). At 28 DAS, the GAT7 line sprayed with

Lorsban® and Resolve® (56%) did not recover as quickly compared to only the 1X Resolve® (32%) treatment on the GAT7 line (data not shown).

Application of 4X Pursuit® (imazethapyr) provided a large response from the STS® line (72%) at 7 DAS, while the GAT7 line (29%) had significantly less response (data not shown). At 14 DAS and 28 DAS, the difference between GAT7 and STS® was not significant (data not shown).

A tank mix of 1X Resolve® + 4X Pursuit® resulted in significantly less spray response for the GAT7 line compared to the STS® line at 7, 14, and 28 DAS (data not shown). The 1X Resolve® + 4X Pursuit® treatment on GAT7 was scored with less damage overall at 7, 14, and 28 DAS compared to the 1X Resolve® only and 1X Resolve® + Lorsban® treatment (data not shown). Using a pairwise comparison of these treatments on only the GAT7 line, the 1X Resolve® + 4X Pursuit® treatment was scored significantly less than the 1X Resolve® + Lorsban® treatment at 14 and 28 DAS. In addition, a pairwise comparison of the 1X Resolve® + 4X Pursuit® treatment compared to the 1X Resolve® treatment on GAT7 was only significantly less at 14 DAS.

Application of 16X Harmony® did not create a large response from the GAT7 or STS® lines at the three rating dates (data not shown). GAT7 was not significantly different from STS® for all the 16X Harmony® scores (data not shown). The treatment applying 4X Pursuit® mixed with 16X Harmony® did create significantly lower response scores for the GAT7 line compared to the STS® line at 7, 14, and 28 DAS (data not shown). The mixture of 4X Express® with 16X Harmony® was scored significantly lower for the GAT7 line compared to the STS® line at 7, 14, and 28 DAS (data not shown). In general, the GAT7 line provided excellent overall tolerance to the 16X Harmony®, 16X Harmony® + 4X Pursuit®, and 16X Harmony® + 4X Express® treatments at all the scoring dates.

A mixture of 0.25X Resolve® + 1.5X Express® created a crop response from the GAT7 line at 7 DAS (53%) and 14 DAS (47%) that was not as evident at 28 DAS (12%) (data not shown). The STS® line had significantly higher response compared to GAT7 at 7 DAS (79%) and 14 DAS (90%) (data not shown). The STS® line did not recover at 28 DAS (87%) and had significantly more response compared to the GAT7 line (data not shown). Examining only the GAT7 line, a pairwise comparison of the 0.25X Resolve® + 1.5X Express® treatment had significantly less response observed compared to the 1X Resolve® treatment at 7, 14, and 28 DAS.

Table 19 Summary of Treatment Protocols

TIMINGS:

00	=	UNTRCHK, UNTREATED TIMING
01	=	POSPOS , POSTEMERGENCE V2-3

TRT		TREATMENT COMPONENT	FORMULATION	RATE	UNIT	TIMING
1		Resolve 2 oz				
	A	>DPX-E9636 (WG 25.00 PC)	WG 25.00 PC	2.00	OMA	01 POSPOS
	B	SURFACTANT - NON-IONIC (SL)	SL 1.00 PR	0.25	PMV	01 POSPOS
	C	>AMSUL (GR 100 PC)	GR 100.00 PC	2.00	LMA	01 POSPOS
2		Resolve + Lorsban				
	A	>DPX-E9636 (WG 25.00 PC)	WG 25.00 PC	2.00	OMA	01 POSPOS
	B	LORSBAN 4E (EC)	EC 4.00 LG	1.00	PMA	01 POSPOS
	C	SURFACTANT - NON-IONIC (SL)	SL 1.00 PR	0.25	PMV	01 POSPOS
	D	>AMSUL (GR 100 PC)	GR 100.00 PC	2.00	LMA	01 POSPOS
3		Pursuit				
	A	PURSUIT DG (70WG)	WG 70.00 PC	4.32	OMA	01 POSPOS
	B	SURFACTANT - NON-IONIC (SL)	SL 1.00 PR	0.25	PMV	01 POSPOS
	C	>AMSUL (GR 100 PC)	GR 100.00 PC	2.00	LMA	01 POSPOS
4		Resolve + Pursuit				
	A	>DPX-E9636 (WG 25.00 PC)	WG 25.00 PC	2.00	OMA	01 POSPOS
	B	PURSUIT DG (70WG)	WG 70.00 PC	4.32	OMA	01 POSPOS
	C	SURFACTANT - NON-IONIC (SL)	SL 1.00 PR	0.25	PMV	01 POSPOS
	D	>AMSUL (GR 100 PC)	GR 100.00 PC	2.00	LMA	01 POSPOS
5		Harmony GT 1.33 oz				
	A	>HARMONY GT PX (75% EXTRUDED WG)	WG 75.00 PC	1.33	OMA	01 POSPOS
	B	SURFACTANT - NON-IONIC (SL)	SL 1.00 PR	0.25	PMV	01 POSPOS
	C	>AMSUL (GR 100 PC)	GR 100.00 PC	2.00	LMA	01 POSPOS
6		GT + Pursuit				
	A	>HARMONY GT PX (75% EXTRUDED WG)	WG 75.00 PC	1.33	OMA	01 POSPOS
	B	PURSUIT DG (70WG)	WG 70.00 PC	2.16	OMA	01 POSPOS
	C	SURFACTANT - NON-IONIC (SL)	SL 1.00 PR	0.25	PMV	01 POSPOS
	D	>AMSUL (GR 100 PC)	GR 100.00 PC	2.00	LMA	01 POSPOS
7		Harmony GT 1.33 + EX 0.67				
	A	>HARMONY GT PX (75% EXTRUDED WG)	WG 75.00 PC	1.33	OMA	01 POSPOS
	B	>EXPRESS PX (75% EXTRUDED WG)	WG 75.00 PC	0.67	OMA	01 POSPOS
	C	SURFACTANT - NON-IONIC (SL)	SL 1.00 PR	0.25	PMV	01 POSPOS
	D	>AMSUL (GR 100 PC)	GR 100.00 PC	2.00	LMA	01 POSPOS
8		Resolve 0.5 + Express 0.25				
	A	>DPX-E9636 (WG 25.00 PC)	WG 25.00 PC	0.50	OMA	01 POSPOS
	B	>EXPRESS PX (75% EXTRUDED WG)	WG 75.00 PC	0.25	OMA	01 POSPOS
	C	SURFACTANT - NON-IONIC (SL)	SL 1.00 PR	0.25	PMV	01 POSPOS
	D	>AMSUL (GR 100 PC)	GR 100.00 PC	2.00	LMA	01 POSPOS
999		UNTREATED CHECK				
	A	UNTREATED CHECK	NA 0.00 NA	0.00	NA	00

UNTRCHK

> = SUPPLEMENTAL CHEMICAL

RATE UNITS:

LMA	=	POUNDS MATERIAL/ACRE
NA	=	NOT APPLICABLE
OMA	=	OZ (DRY) MATERIAL/ACRE
PMA	=	PINTS MATERIAL/ACRE
PMV	=	% MATERIAL VOL TO VOL

DESIGN:	RANDOMIZED COMPLETE BLOCK DESIGN
NO. REPS:	3
PLOT SIZE:	5 X 10 FEET
PLOT AREA:	50 SQUARE FEET

OBSERVATIONS/RATING:

Crop Response 7, 14 & 28 DAT

Example 6B

Example 6B provides greenhouse data that confirms the field trial data provided above in Example 6A. Soybeans comprising the lead GAT7 event and also having HRA were used in the studies.

Table 20. Summary of treatment conditions

Test Plant	Rimsulfuron Rate (g ai/ha)	Imazethapyr Rate (g ai/ha)	Treatment #	Replicates				Mean
				A	B	C	D	
GAT/HRA Soybeans	0	0	1	53.91		53.16	59.21	55.43
		140	2	48.8	52.97	54.45	61.67	54.47
		280	3	49.06	53.65	39.74	61.06	50.88
		560	4	56.46	52.98	57.76	55.76	55.74
		1024	5	51.34	53.89	45.64	51.47	50.59
	35	0	6	21.09	20.45	27.11	20.83	22.37
		140	7	18.18	18.79	26.03	22.15	21.29
		280	8	26.45	15.59	14.53	26.5	20.77
		560	9	22.2	22.97	26.8	20.65	23.16
		1024	10	24.56	30.53	28.04	19.95	25.77
	70	0	11	18.33	24.56	15.83	19.75	19.62
		140	12	16.71	23.1	27.27	21.44	22.13
		280	13	27.43	34.8	32.26	21.65	29.04
		560	14	28.84	26.17	28.12	31.95	28.77
		1024	15	32.51	25.19	34.18	29.96	30.46
	140	0	16	20.09	12.5	13.96	23.32	17.47
		140	17	21.69	24.38	16.15	17.27	19.87
		280	18	23.77	21.72	27.16	26.22	24.72
		560	19	30.39	34.67	21.46	28.41	28.73
		1024	20	35.19	30.68	26	35.19	31.77
	280	0	21	18.14	17.76	20.01	14.3	17.55
		140	22	15.67	16.62	18.29	15.91	16.62
		280	23	15.83	21.36	20.3	24.67	20.52
		560	24	23.9	19.22	28.67	24.7	24.12
		1024	25	30.93	28.76	24.69	36.65	30.26
Jack Soybeans	0	0	1	50.93	60.34	53.87	46.89	53.01
		70	2	45.88	42.49	41.6	37.47	41.86
		140	3	38.09	38.57	28.06	42.33	36.76
		280	4	35.25	33.01	42.97	41.4	38.16
		560	5	39.2	37.15	38.6	29.67	36.16
	0.5	0	6	26.27	18.24	21.48	16.09	20.52
		70	7	15.12	13.93	11.01	7.12	11.80
		140	8	12.68	6.92	10.82	12.51	10.73
		280	9.	12.86	7.14	12.16	9.61	10.44
		560	10	12.74	10.68	12.71	11.06	11.80
	1	0	11	5.8	7.64	7.13	9.21	7.45
		70	12	8.05	6.76	8.42	7.48	7.68
		140	13	7.57	6.54	7.49	10	7.90
		280	14	6.84	7.92	7.25	8.37	7.60
		560	15	12.51	10.59	9.57	7.16	9.96
	2	0	16	4.09	5.37	4.3	11.58	6.34
		70	17	3.36	6.57	4.83	7.67	5.61
		140	18	6.12	6.55	5.59	6.09	6.09
		280	19	5.87	4.89	5.48	6.24	5.62
		560	20	5.35	7.73	8.9	5.48	6.87
	4	0	21	4.12	4.48	2.93	3.46	3.75
		70	22	2.75	2.94	11.64	4.21	5.39
		140	23	3.29	3.23	4.24		3.59
		280	24	4.46	5.9	3.92	3.65	4.48
		560	25	4.67	5.01	2.77	4.1	4.14

Table 21. Summary of greenhouse results.

Test Plant GAT/HRA Soybeans RimsulfuronR Rate (g ai/ha)	Imazethapyr Rate (g ai/ha)	Treatment #	Replicates A	B	C	D	Mean	Standard Deviation	Mean - Initial Weight	% Growth Reduction
0	0	1	53.91		53.16	59.21	55.43	3.30	49.28	0
	140	2	48.8	52.97	54.45	61.67	54.47	5.36	48.33	2
	280	3	49.06	53.65	39.74	61.06	50.88	8.92	44.73	9
	560	4	56.46	52.98	57.76	55.76	55.74	2.02	49.60	-1
	1024	5	51.34	53.89	45.64	51.47	50.59	3.50	44.44	10
35	0	6	21.09	20.45	27.11	20.83	22.37	3.17	16.23	67
	140	7	18.18	18.79	26.03	22.15	21.29	3.61	15.14	69
	280	8	26.45	15.59	14.53	26.5	20.77	6.60	14.62	70
	560	9	22.2	22.97	26.8	20.65	23.16	2.61	17.01	65
	1024	10	24.56	30.53	28.04	19.95	25.77	4.59	19.63	60
70	0	11	18.33	24.56	15.83	19.75	19.62	3.67	13.47	73
	140	12	16.71	23.1	27.27	21.44	22.13	4.37	15.99	68
	280	13	27.43	34.8	32.26	21.65	29.04	5.80	22.89	54
	560	14	28.84	26.17	28.12	31.95	28.77	2.40	22.63	54
	1024	15	32.51	25.19	34.18	29.96	30.46	3.92	24.32	51
140	0	16	20.09	12.5	13.96	23.32	17.47	5.10	11.32	77
	140	17	21.69	24.38	16.15	17.27	19.87	3.84	13.73	72
	280	18	23.77	21.72	27.16	26.22	24.72	2.46	18.57	62
	560	19	30.39	34.67	21.46	28.41	28.73	5.51	22.59	54
	1024	20	35.19	30.68	26	35.19	31.77	4.39	25.62	48
280	0	21	18.14	17.76	20.01	14.3	17.55	2.38	11.41	77
	140	22	15.67	16.62	18.29	15.91	16.62	1.18	10.48	79
	280	23	15.83	21.36	20.3	24.57	20.52	3.61	14.37	71
	560	24	23.9	19.22	28.67	24.7	24.12	3.88	17.98	64
	1024	25	30.93	28.75	24.69	36.65	30.26	4.99	24.11	51

Example 7. Methods of Transformation Employing a GAT sequence in Maize

I. Preparation of Agrobacterium master plate

1. Obtain engineered *Agrobacterium tumefaciens* strain with GAT components (SEQ ID NO: 70 or SEQ ID NO:55) and stored in –80°C degree freezer as a 50% glycerol stock. The transcriptional control region used was the 3X35S ENH (-) operably linked to the ZmUbi PRO-5UTR-ZmUbi intron 1 promoter (SEQ ID NO:78). This transcriptional control region (SEQ ID NO:78) is set forth below denoting the location of the various regions of the regulatory region: a) the 35S enhancer (3X) in the reverse direction has a single underline; b) the UBI promoter has a double underline, and c) the UBI intron is in italics.

atcacatcaatccacttgctttgaagacgtggttggaacgtcttcttttttccacgatgctcctcgtgggtgggggtccatctttgggaccact
gtcggcagaggcatcttcaacgatggcctttcctttatcgcaatgatggcatttgtaggagccaccttcctttccactatcttcacaataaa
gtgacagatagctgggcaatggaatccgaggaggtttccggatattacccttttgttgaaaagtctcaattgccctttggtcttctgagactg
tatctttgatatttttggagtagacaagcgtgtcgtgctccaccatgttgacgaagattttcttcttgtcattgagtcgtaagagactctgtatg
aactgttcgccagtctttacggcgagttctgttaggtcctctatttgaatctttgactccatggacggtatcgataagctagcttgatatcaca
tcaatccacttgctttgaagacgtggttggaacgtcttcttttttccacgatgctcctcgtgggtgggggtccatctttgggaccactgtcgg
cagaggcatcttcaacgatggcctttcctttatcgcaatgatggcatttgtaggagccaccttcctttccactatcttcacaataaagtgac

cagaggcatcttcaacgatggcctttcctttatcgcaatgatggcatttgtaggagccaccttccttttccactatcttcacaataaagtgac
agatagctgggcaatggaatccgaggaggtttccggatattacccttgttgaaaagtctcaattgccctttggtcttctgagactgtatctt
tgatatttttggagtagacaagcgtgtcgtgctccaccatgttgacgaagattttcttcttgtcattgagtcgtaagagactctgtatgaactg
ttcgccagtctttacggcgagttctgttaggtcctctatttgaatctttgactccatgatcgaattatcacatcaatccacttgctttgaagacg
tggttggaacgtcttcttttccacgatgctcctcgtgggtgggggtccatctttgggaccactgtcggcagaggcatcttcaacgatggc
ctttcctttatcgcaatgatggcatttgtaggagccaccttcctttccactatcttcacaataaagtgacagatagctgggcaatggaatcc
gaggaggtttccggatattacccttgttgaaaagtctcaattgccctttggtcttctgagactgtatctttgatatttttggagtagacaagc
gtgtcgtgctccaccatgttgacgaagattttcttcttgtcattgagtcgtaagagactctgtatgaactgttcgccagtctttacggcgagtt
ctgttaggtcctctatttgaatctttgactccatgggaattcctgcagcccagcttgcatgcctgcagtgcagcgtgacccggtcgtgccc
ctctctagagataatgagcattgcatgtctaagttataaaaaattaccacatatttttttttgtcacacttgtttgaagtgcagtttatctatctttat
acatatatttaaactttactctacgaataatataatctatagtactacaataatatcagtgttttagagaatcatataaatgaacagttagacat
ggtctaaaggacaattgagtattttgacaacaggactctacagttttatcttttagtgtgcatgtgttctccttttttttgcaaatagcttcacc
tatataatacttcatccatttttattagtacatccatttagggtttagggttaatggtttttatagactaatttttttagtacatctattttattctatttta
gcctctaaattaagaaaactaaaactctatttttagttttttttatttaataatttagatataaaatagaataaaataaagtgactaaaaattaaaca
aataccctttaagaaattaaaaaaactaaggaaacatttttcttgtttcgagtagataatgccagcctgttaaacgccgtcgacgagtctaa
cggacaccaaccagcgaaccagcagcgtcgcgtcgggccaagcgaagcagacggcacggcatctctgtcgctgcctctggaccc
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tatgcagcagctatatgtggatttttttagccctgccttcatacgctatttatttgcttggtactgtttcttttgtcgatgctcaccctgttgtttg
gtgttacttctgca (SEQ ID NO:78)

2. Prepare master plate from a glycerol stock by streaking the bacteria to produce single colonies on #800 medium and incubate the bacteria at 28°C in the dark for 3-4 days.

3. Prepare a working plate by streaking 1 colony from the master plate across #810 media. Incubate bacteria at 28°C in the dark for 1-2 days.

II. Preparation of bacteria for embryo infection

1. Prepare liquid culture of *Agrobacterium* 1 day prior to embryo isolation. Set up a flask with 30 mls of 557A medium, 30 µl of 2% acetosyringone and 30 µl of 5% spectinomycin.
2. Inoculate with 1 loopful of *Agrobacterium* from 810 medium and place on shaker (200 rpm) in dark room at 28°C overnight.
3. On morning of infection, take samples of the liquid culture of *Agrobacterium* and make a ¼ dilution with 557A. Use the diluted liquid culture to take OD reading using visible light at 550 nm.
4. Make dilutions to *Agrobacterium* culture as appropriate according the OD reading to maintain OD reading between 0.2-0.8 during embryo isolation.
5. When preparing *Agrobacterium* for infection, repeat OD reading of liquid culture. Using the OD reading calculate the number of mls required to obtain 5 E10 cfu/ml (cfu=colony forming unit) by using the formula EXPONENT (1.755 *(lnOD) + 21.77) as derived from a standard curve. Pipet the calculated amount of *Agrobacterium* liquid culture into 14 ml tube and centrifuge at 4500 rpm at 4 – 20 °C for ten minutes. Remove the supernatant and resuspend *Agrobacterium* in appropriate amount of 100 uM acetosyringone solution in 561Q.

III. Immature embryo isolation

1. Harvest GS3 ears at 9-11 days after pollination with embryo size of 1-2 mm in length.
2. Sterilize ear in 50% bleach and 1 drop Tween for 20-30 minutes. Rinse 3-5 times in sterile water
3. Isolate embryos from kernels and place in microtube containing 2 mls 561Q.

VI. Agrobacterium infection of embryos

1. Remove 561Q with pipette from the microtube with isolated embryos and add 1 ml of Agrobacterium suspension at OD described above.
2. Mix by vortexing for about 30 seconds.
3. Allow 5 minutes for infection at room temperature.

V. Co-cultivation

1. After removing liquid medium, transfer embryos and orient the embryos with embryonic axis down on the surface of 562P co-cultivation medium.
2. Place embryos in 20°C incubator for 3 days. Transfer to 28°C for 3 additional days.

VI. Selection of transgenic putative callus events

1. After co-cultivation, transfer embryos to 563I selection medium containing 1 mM glyphosate. Culture the embryos at 28°C in dark.
2. Every 14-21 days transfer embryos to fresh 563I medium. The selection process may last about 2 months until actively growing putative callus events can be identified. Maintain putative callus events on 563I medium and sample callus for PCR.

VII. Regeneration of T0 plants

1. Transfer callus events to 287I medium containing 0.1 mM Glyphosate until somatic embryos mature. Culture the callus at 28°C in dark.
2. Transfer mature embryos to 273I embryo germination medium containing 0.1 mM glyphosate in plates. Culture the plates at 28°C in light.
3. When shoots and roots emerge, transfer individual plants to 273I containing 0.1 mM Glyphosate in tubes. Culture the tubes at 28°C in light.
4. Plantlets with established shoots and roots shall be transferred to greenhouse for further growth and production of T1 seed.

Example 8. Effect of 35S enhancer on Transformation Efficiency and Efficacy of GAT and ALS in Maize

Materials and Methods

Four 35S enhancer constructs (PHP20118, PHP20120, PHP20122, PHP20124) and one non-35S construct (PHP19288) were used to produce events to evaluate the effect of 35S enhancer on transformation efficiency and efficacy of GAT (SEQ ID NO:70) (Fig. 1). The differences between the four 35S enhancer constructs are the copy numbers of the 35S enhancer and the orientations of the 35S enhancer in the constructs. A summary of each 35S enhancer construct is provided below.

PHP20118 comprises 35S ENH(+):ZmUBI PRO-5UTR-UBI INTRON1 (+ denotes forward direction of 35S enhancer). This transcriptional control region (SEQ ID NO:80) is set forth below denoting the location of the various regions of the regulatory region: a) the 35S enhancer in the forward direction has a single underline; b) the UBI promoter has a double underline, and c) the UBI intron is in italics.

cccatggagtcaaagattcaaatagaggacctaacagaactcgccgtaaagactggcgaacagttcatacagagtctcttacgactca
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a (SEQ ID NO:80)

PHP20122 comprises 3X35S ENH (+):ZmUBI PRO-5UTR-UBI INTRON1 (+ denotes forward direction of 35S enhancer). This transcriptional control region (SEQ ID NO:81) is set forth below denoting the location of the various regions of the regulatory

region: a) the 35S enhancer in the forward direction has a single underline; b) the UBI promoter has a double underline, and c) the UBI intron is in italics.

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PHP20120 comprises 35S ENH (-):ZmUBI PRO-5UTR-UBI INTRON1 (- denotes reverse direction of 35S enhancer). This transcriptional control region (SEQ ID NO:82) is set forth below denoting the location of the various regions of the regulatory region: a) the 35S enhancer in the reverse direction has a single underline; b) the UBI promoter has a double underline, and c) the UBI intron is in italics.

atcacatcaatccacttgctttgaagacgtggttggaacgtcttcttttttccacgatgctcctcgtgggtgggggtccatctttgggaccact gtcggcagaggcatcttcaacgatggcctttcctttatcgcaatgatggcatttgtaggagccaccttccttttccactatcttcacaataaa gtgacagatagctgggcaatggaatccgaggaggtttccggatattacccttttgttgaaaagtctcaattgccctttggtcttctgagactg tatctttgatatttttggagtagacaagcgtgtcgtgctccaccatgttgacgaagattttcttcttgtcattgagtcgtaagagactctgtatg aactgttcgccagtctttacggcgagttctgttaggtcctctatttgaatctttgactccatgggaattcctgcagcccagcttgcatgcctg cagtgcagcgtgacccggtcgtgcccctctctagagataatgagcattgcatgtctaagttataaaaaattaccacatattttttttgtcaca cttgtttgaagtgcagtttatctatctttatacatatatttaaactttactctacgaataatataatctatagtactacaataatatcagtgttttaga gaatcatataaatgaacagttagacatggtctaaaggacaattgagtattttgacaacaggactctacagttttatcttttttagtgtgcatgtg ttctccttttttttttgcaaatagcttcacctatataatacttcatccattttattagtacatccatttagggtttagggttaatggtttttatagactaa ttttttttagtacatctattttattctatttttagcctctaaattaagaaaactaaaactctatttttagtttttttatttaataatttagatataaaatagaat aaaataaagtgactaaaaattaaacaaataccctttaagaaattaaaaaaactaaggaaacatttttcttgtttcgagtagataatgccagc ctgttaaacgccgtcgacgagtctaacggacaccaaccagcgaaccagcagcgtcgcgtcgggccaagcgaagcagacggcacg gcatctctgtcgctgcctctggacccctctcgagagttccgctccaccgttggacttgctccgctgtcggcatccagaaattgcgtggcg gagcggcagacgtgagccggcacggcaggcggcctcctcctcctctcacggcaccggcagctacgggggattcctttcccaccgct ccttcgctttcccttcctcgcccgccgtaataaatagacacccccctccacaccctctttccccaacctcgtgttgttcggagcgcacacac acacaaccagatctcccccaaatccacccgtcggcacctccgcttcaag*gtacgccgctcgtcctcccccccccccctctctaccttct ctagatcggcgttccggtccatggttagggcccggtagttctacttctgttcatgtttgtgttagatccgtgtttgtgttagatccgtgctgc tagcgttcgtacacggatgcgacctgtacgtcagacacgttctgattgctaacttgccagtgtttctctttggggaatcctgggatggct ctagccgttccgcagacgggatcgatttcatgatttttttgtttcgttgcatagggtttggtttgccctttttcctttatttcaatatatgccgtg cacttgtttgtcgggtcatcttttcatgctttttttgtcttggttgtgatgatgtggtctggttgggcggtcgttctagatcggagtagaattc tgtttcaaactacctggtggatttattaattttggatctgtatgtgtgtgccatacatattcatagttacgaattgaagatgatggatgga

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PHP20124 comprises 3X35S ENH (-): ZmUBI PRO-5UTR-UBI INTRON1 (- denotes reverse direction of 35S enhancer). This transcriptional control region (SEQ ID NO:83) is set forth below denoting the location of the various regions of the regulatory region: a) the 35S enhancer in the reverse direction has a single underline; b) the UBI promoter has a double underline, and c) the UBI intron is in italics.

atcacatcaatccacttgctttgaagacgtggttggaacgtcttcttttttccacgatgctcctcgtgggtgggggtccatctttgggaccact gtcggcagaggcatcttcaacgatggcctttccttttatcgcaatgatggcatttgtaggagccaccttccttttccactatcttcacaataaa gtgacagatagctgggcaatggaatccgaggaggtttccggatattacccttttgttgaaaagtctcaattgccctttggtcttctgagactg tatctttgatatttttggagtagacaagcgtgtcgtgctccaccatgttgacgaagattttcttcttgtcattgagtcgtaagagactctgtatg aactgttcgccagtctttacggcgagttctgttaggtcctctatttgaatctttgactccatggacggtatcgataagctagcttgatatcaca tcaatccacttgctttgaagacgtggttggaacgtcttcttttttccacgatgctcctcgtgggtgggggtccatctttgggaccactgtcgg cagaggcatcttcaacgatggcctttccttttatcgcaatgatggcatttgtaggagccaccttccttttccactatcttcacaataaagtgac agatagctgggcaatggaatccgaggaggtttccggatattacccttttgttgaaaagtctcaattgccctttggtcttctgagactgtatctt tgatattttggagtagacaagcgtgtcgtgctccaccatgttgacgaagattttcttcttgtcattgagtcgtaagagactctgtatgaactg ttcgccagtctttacggcgagttctgttaggtcctctatttgaatctttgactccatgatcgaattatcacatcaatccacttgctttgaagacg tggttggaacgtcttcttttttccacgatgctcctcgtgggtgggggtccatctttgggaccactgtcggcagaggcatcttcaacgatggc ctttcctttatcgcaatgatggcatttgtaggagccaccttccttttccactatcttcacaataaagtgacagatagctgggcaatggaatcc gaggaggtttccggatattacccttttgttgaaaagtctcaattgccctttggtcttctgagactgtatctttgatattttggagtagacaagc gtgtcgtgctccaccatgttgacgaagattttcttcttgtcattgagtcgtaagagactctgtatgaactgttcgccagtctttacggcgagtt ctgttaggtcctctatttgaatctttgactccatgggaattcctgcagcccagcttgcatgcctgcagtgcagcgtgacccggtcgtgccc ctctctagagataatgagcattgcatgtctaagttataaaaaattaccacatattttttttgtcacacttgtttgaagtgcagtttatctatctttat acatatatttaaactttactctacgaataatataatctatagtactacaataatatcagtgttttagagaatcatataaatgaacagttagacat ggtctaaaggacaattgagtattttgacaacaggactctacagttttatcttttttagtgtgcatgtgttctccttttttttttgcaaatagcttcacc tatataatacttcatccattttattagtacatccatttagggtttagggttaatggtttttatagactaatttttttagtacatctattttattctatttta gcctctaaattaagaaaactaaaactctattttagtttttttatttaataatttagatataaaatagaataaaataaagtgactaaaaattaaaca aataccctttaagaaattaaaaaaactaaggaaacatttttcttgtttcgagtagataatgccagcctgttaaacgccgtcgacgagtctaa

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gtttaagatggatggaaatatcgatctaggataggtatacatgttgatgtgggttttactgatgcatatacatgatggcatatgcagca
tctattcatatgctctaaccttgagtacctatctattataataaacaagtatgttttataattattttgatcttgatatacttggatgatggca
tatgcagcagctatatgtggatttttttagccctgccttcatacgctatttatttgcttggtactgtttcttttgtcgatgctcaccctgttgtttg
gtgttacttctgca (SEQ ID NO:83)

The transformation experiments were conducted side-by-side using the same embryos from the same ears. Immature embryos of GS3 line were aseptically removed from each ear and divided into five portions. Each portion of the embryos was then infected with *A. tumefaciens* strain LBA4404 containing the expression cassettes from each of the five constructs, respectively. After 6 days co-cultivation, the embryos were transferred to fresh selection medium containing glyphosate. The transformed cells, which survived the glyphosate selection, proliferated and produced somatic embryogenic calli. After about two months subculture, the calli were then manipulated to regenerate whole transgenic plants with glyphosate presence and were transferred to the greenhouse. T0 plants were then subjected to glyphosate spray at 6X (156 oz/ac) Roundup Ready UltraMax™ at V3 or V4 stage in the greenhouse. Positive plants were sampled for quantitative PCR for copy number and western for expression. T0 plants were then crossed with inbred lines to obtain seeds for further evaluation. *Results*

Transformation efficiency was measured as the percentage of the infected embryos that produced resistant calli after selection. The average transformation efficiencies for PHP19288, PHP20118, PHP20120, PHP20122, and PHP20124 were 58%, 63%, 59%, 57%,

and 51%, respectively. The data indicated that all constructs had quite high and similar transformation efficiencies, although PHP20118 showed a slight increase (Fig 3).

T0 plant efficacy was defined as the percentage of the T0 events that were completely resistant to the 6x glyphosate spray. The efficacy of the non-35S construct (PHP19288) was 48.1%. In contrast, the efficacies of the 35S enhancer constructs (PHP20118, PHP20120, PHP20122, and PHP20124) were 96.6%, 93.5%, 89.1%, and 91.1%, respectively (Fig. 4). The data showed that all 35S enhancer constructs significantly increased the plant efficacy against glyphosate.

Another significant improvement of using 35S enhancer was in integration pattern of the transgene. The percentage of the tested events that were single copy for the non-35S enhancer construct was only 38%, but for the four 35S enhancer constructs (PHP20118, PHP20120, PHP20122, and PHP20124) single copy events represented 65%, 63%, 71%, and 88% of the events, respectively (Fig. 4).

A subset of events from all five constructs were sampled by Western analysis to look any comparative differences in GAT expression between non 35S and 35S events. This analysis showed that events from the non-35S enhancer construct had very low levels of GAT expression whereas the majority of the events from the 35S enhancer constructs showed very high levels of GAT expression (Fig. 5).

Example 9. Using 35S Enhancer GAT in Developing A Novel Callus-Based Gene/Construct Evaluation System.

Materials and Methods

This assay is being developed to improve the evaluation of expression of an insecticidal gene at a very early stage in the transformation process in order to identify potential problems with expression. The basis of this assay is the use of the glyphosate acetyl transferase (GAT) gene (SEQ ID NO:55) as a selectable marker. Both GAT and the insecticidal test gene will be driven by a strong constitutive promoter and linked in the same construct. The promoter employed comprised the ZmUBI PRO-5UTR-UBI INTRON1 with the 3X35S enhancer as described above in Example 7. As a result it is expected that selection on high levels of glyphosate will identify high insecticidal test gene expressors. The callus tissue from these putative high expressors will then be used in insect bioassays to determine whether the gene product is functional. Those constructs showing efficacy can be advanced into transformation. If the construct does not show efficacy then follow up biochemical and

molecular analyses can be conducted to identify the problem and the gene will be redesigned and retested in the system (Fig. 6).

Results

The assay is currently under development. Preliminary data has shown that the activity of an efficacious insect control gene can be detected at the callus stage. The correlation between the callus activity and the plant efficacy is currently being evaluated.

Example 10. GAT as a Selectable Marker

Materials and Method

Agrobacterium mediated transformation was used to introduce the *GAT* (SEQ ID NO:55) expression cassette into the corn genome. The *GAT* expression cassette comprises, promoter comprising ZmUBI PRO-5UTR-UBI INTRON1 with the 3X35S enhancer (as described above in Example 1) operably linked to the *gat* gene, and pinII terminator. *Agrobacterium tumefaciens*, strain LBA4404, was pathogenically disarmed by removing its native T-DNA. Instead, the T-DNA site on the Ti plasmid contained the *GAT* expression cassette.

Immature embryos of maize were aseptically removed from the developing caryopsis and treated with *A. tumefaciens* strain LBA4404 containing GAT expression cassettes. After a period of embryo and *Agrobacterium* co-cultivation on solid culture medium without glyphosate presence, the embryos were transferred to fresh selection medium that contained antibiotics and glyphosate. The antibiotics kill any remaining *Agrobacterium*. The selection medium is stimulatory to maize somatic embryogenesis and selective for those cells that contain an integrated *gat* gene. Therefore, callus that survives glyphosate to proliferate and produce embryogenic tissue is presumably genetically transformed. Callus samples were taken for molecular analysis to verify the presence of the transgene by PCR. The embryonic tissue is then manipulated to regenerate transgenic plants in the presence of glyphosate that are then transferred to the greenhouse. T0 plants are sprayed with glyphosate at different concentrations. Positive plants are sampled for molecular analysis for transgene copy number and crossed with inbred lines to obtain seeds from the initially transformed plants.

A glyphosate kill curve was established by testing non-transformed embryos response on media with different levels of glyphosate. GS3 embryos were isolated from an immature ear and placed onto media containing glyphosate at 0.0, 0.5, 1.0, and 2.0 mM. After about 40 days culture, the response of the embryos were observed and recorded. Similarly, infected GS3 embryos with the GAT construct were placed onto media containing glyphosate at 0.0,

0.5, 1.0, and 2.0 mM. After about 40 days culture, the response of the infected embryos were observed and recorded (Fig. 7).

A side-by-side experiment was conducted to compare the transformation efficiencies of GAT, bar and mopat. Immature embryos of GS3 line were aseptically removed from each ear and divided into three portions. Each portion of the embryos was then infected with *A. tumefaciens* strain LBA4404 containing the expression cassettes of GAT, bar, or mopat respectively. After co-cultivation, the embryos infected with GAT construct were selected on routine glyphosate medium and the embryos infected with bar or mopat constructs were selected on routine glufosinate medium. The subcultures were done every 2 weeks. At about 50 days selection the responses of the embryos were observed and recorded.

Results

From the glyphosate kill curve experiment, all embryos on medium with 0.0 mM glyphosate initiated healthy callus, while about half of the embryos on medium with 0.5 mM glyphosate showed callus initiation. There was very little callus growth with embryos on media containing 1.0 and 2.0 mm glyphosate. This indicated that 0.5 mM is not enough to inhibit all embryos growth, but 1 mM or 2 mM is strong enough to kill the non-transformed embryos. In the infected embryo experiment, more callus was grown on media with 0.0 and 0.5 mM glyphosate, but some embryos initiated resistant callus on media with 1.0 mM or 2.0 mM glyphosate. Western or PCR analysis has confirmed that these resistant calli were transformed. Currently, GAT has performed consistently as an effective selectable marker with excellent transformation efficiency in both GS3 and introEF09B genotypes (Fig. 10 and Table 45).

Table 22 GAT transformation efficiency in introEF09B

genotype	construct	selectable marker	# infected embryos	# events to GH	txn % based on # events to GH
EFWWBTX	GATHRA	GAT	1332	354	**27%**
EFWWCTX	GATHRA	GAT	136	47	**35%**
EFWWETX	GATHRA	GAT	1109	158	**14%**
EFWWZTX	GATHRA	GAT	1790	502	**28%**
			4367	**1061**	**24%**

In the side-by-side experiment to compare GAT, bar and mopat, GAT gave the best transformation efficiency at about 64%, bar at 34%, and mopat at 30%. Calli with GAT selection seem to grow faster that those selected on glufosinate (Fig. 8).

Example 11—Interaction Between Glyphosate, Metsulfuron-Methyl and Two Additives on the Control of Ryegrass

The example was conducted on non-glyphosate resistant ryegrass. As provided in Table 23, all treatments that included glyphosate [180 g a.i. L ha^{-1} (0.5 L glyphosate (SCAT®))] plus metsulfuron-methyl (BRUSH-OFF®) (5 and 10 g ha^{-1}) gave 100% control of the ryegrass. Metsulfuron-methyl (BRUSH-OFF®) on its own did nothing accept stunt the ryegrass plants slightly. Although 15 of the 16 plants (12%) in the 0.5 L ha^{-1} glyphosate (SCAT®) treatment eventually died, they took much longer to die. These results indicate that there was possible synergism between glyphosate (SCAT®) and metsulfuron-methyl (BRUSH-OFF®). The two additives, e.g., ADD-UP and VELOCITY, made no difference in the degree of control with any of the treatments.

Table 23. Interaction Between Glyphosate, metsulfuron-methyl and Two Additives on the Control of Ryegrass

Trial	Treatment/ha^{-1}	Control (%)
1	glyphosate (SCAT®) 0.5 L	94
2	Metsulfuron-methyl (BRUSH-OFF®) 5 g	0
3	Metsulfuron-methyl (BRUSH-OFF®) 10 g	0
4	glyphosate (SCAT®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 5 g	100
5	glyphosate (SCAT®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 10 g	100
6	glyphosate (SCAT®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 5 g + ammonium sulfate-based adjuvant (ADD-UP®) 1%	100

7	glyphosate (SCAT®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 10 g + ammonium sulfate-based adjuvant (ADD-UP®) 1%	100
8	glyphosate (SCAT®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 5 g + bispyribac-sodium (VELOCITY®) 1%	100
9	glyphosate (SCAT®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 10 g + bispyribac-sodium (VELOCITY®) 1%	100
10	Control	0

Biotype: non-glyphosate resistant ryegrass (70-04)

Population profile: Rup 0.25 control (%) 6.25

Rup 0.5 control (%) 81.25

Rup 1.0 control (%) 93.75

Growth Stage: 5-6 leaf stage

Conditions: hot and sunny

Volume rate: 200 L ha^{-1}

Example 12—Interaction Between Glyphosate, metsulfuron-methyl and Two Additives on the Control of Hairy Fleabane (*Conyza bonariensis*)

This example included the same treatments as used in Example 11, except the treatments were carried out on a glyphosate sensitive biotype of Kleinskraalhans (*Conyza bonariensis*). Glyphosate on its own gave poor control, killing only 1 out of 16 plants (12%). In this example, however, ammonium sulfate-based adjuvant (ADD-UP®) performed better than bispyribac-sodium (VELOCITY®) with the mixture.

Table 24. Interaction Between Glyphosate, metsulfuron-methyl and Two Additives on the Control of Hairy Fleabane (*Conyza bonariensis*)

Trial	Treatment/ha^{-1}	Control (%)
1	glyphosate (ROUNDUP®) 0.5 L	12
2	Metsulfuron-methyl (BRUSH-OFF®) 5 g	100
3	Metsulfuron-methyl (BRUSH-OFF®) 10 g	100
4	glyphosate (ROUNDUP®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 5g	100
5	glyphosate (ROUNDUP®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 10g	100
6	glyphosate (ROUNDUP®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 5g + ammonium sulfate-based adjuvant (ADD-UP®) 1%	100
7	glyphosate (ROUNDUP®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 10g + ammonium sulfate-based adjuvant (ADD-UP®) 1%	100
8	glyphosate (ROUNDUP®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 5g + bispyribac-sodium (VELOCITY®) 1%	75

9	glyphosate (ROUNDUP®) 0.5 L + metsulfuron-methyl (BRUSH-OFF®) 10g + bispyribac-sodium (VELOCITY®) 1%	100
10	Control	0

Biotype: Non-glyphosate resistant Conyza (Welgevallen-paraquat resistant)

Growth Stage: 10-15 leaf stage

Conditions: Cool and sunny

Volume rate: 200 L ha^{-1}

glyphosate (ROUNDUP): 360 g a.i. L^{-1}

Example 13—Interaction Between Glyphosate, metsulfuron-methyl and Two Additives on the Control of Glyphosate, Paraquat, and ACCase-Inhibitor Resistant Ryegrass

This example was conducted on one of the most resistant ryegrass types in the world, ryegrass resistant to non-selective herbicides, e.g., Fairview (Tulbagh), which is resistant to glyphosate, paraquat, and ACCase-inhibitors. In this example, the addition of metsulfuron-methyl (BRUSH-OFF®) at 5 and 10 g ha^{-1}, with 1% ammonium sulfate-based adjuvant (ADD-UP®) to 0.5 L Roundup improved control by 44% (e.g., 50% to 94%). Further, ammonium sulfate-based adjuvant (ADD-UP®) was superior to bispyribac-sodium (VELOCITY®) as an additive.

Table 25. Interaction Between Glyphosate, metsulfuron-methyl and Two Additives on the Control of Glyphosate, Paraquat, and ACCase-Inhibitor Resistant Ryegrass

Trial	Treatment/ha^{-1}	Control (%)
1	glyphosate (ROUNDUP®) 0.5L	50
2	glyphosate (ROUNDUP®) 0.5L + metsulfuron-methyl (BRUSH-OFF®) 5g	69
3	glyphosate (ROUNDUP®) 0.5L + metsulfuron-methyl (BRUSH-OFF®) 10g	88
4	glyphosate (ROUNDUP®) 0.5L + metsulfuron-methyl (BRUSH-OFF®) 5g + ammonium sulfate-based adjuvant (ADD-UP®) 1%	94
5	glyphosate (ROUNDUP®) 0.5L + metsulfuron-methyl (BRUSH-OFF®) 10g + ammonium sulfate-based adjuvant (ADD-UP®) 1%	94
6	glyphosate (ROUNDUP®) 0.5L + metsulfuron-methyl (BRUSH-OFF®) 5g + bispyribac-sodium (VELOCITY®) 1%	69
7	glyphosate (ROUNDUP®) 0.5L + metsulfuron-methyl (BRUSH-OFF®) 10g + bispyribac-sodium (VELOCITY®) 1%	75

Biotype: Fairview (Tulbagh) resistant to glyphosate, paraquat, and ACCase-inhibitors.

Growth Stage: 10-15 leaf stage

Conditions: Cool and sunny

Volume rate: 200 L ha^{-1}

glyphosate (ROUNDUP): 360 g ai L^{-1}

Example 14—Interaction Between Glyphosate and Representative SU's on the Control of Glyphosate, Paraquat, and ACCase-Inhibitor Resistant Ryegrass

In this example, four different SU's were applied together with glyphosate (SCAT®) on the resistant ryegrass. The results as to the best SU partner for glyphosate were inconclusive, but the average benefit of applying an SU with glyphosate on herbicide resistant ryegrass was 39% (e.g., 34% control with glyphosate (SCAT®) only and 57-83% control with glyphosate (SCAT®) plus metsulfuron-methyl (BRUSH-OFF®), chlorsulfuron (GLEAN®), or triasulfuron (LOGRAN®)).

Table 26. Interaction Between Glyphosate and Representative SU's on the Control of Glyphosate, Paraquat, and ACCase-Inhibitor Resistant Ryegrass

Trial	Treatment/ha^{-1}	Control (%)
1	Control	0
2	glyphosate (SCAT®) 6L	34
3	glyphosate (SCAT®) 6L + metsulfuron-methyl (BRUSH-OFF®) 10 g	67
4	glyphosate (SCAT®) 6L + chlorsulfuron (GLEAN®) 15 g	75
5	glyphosate (SCAT®) 6L + triasulfuron (LOGRAN®) 7 ½ g	83
6	glyphosate (SCAT®) 6L + triasulfuron (LOGRAN®) 15 g	67

Biotype: Fairview (Tulbagh) resistant to glyphosate, paraquat, and ACCase-inhibitors.

Growth Stage: 4 leaf stage

Conditions: Cool and sunny

Volume rate: 200 L ha^{-1}

All treatments sprayed with 1% ADD-UP

Example 15. GAT has no yield impact on soybean isolines

Abstract

Isolines from twelve selected SCP:GAT7::SAMS:ALS events were yield tested in 3 Iowa environments in 2004 and 6 midwest environments in 2005. When yield data for these environments is combined together, there is no significant yield difference between GAT7 positive lines and GAT7 negative lines across the construct, and within a specific event. For the three lead events (EAFS 3559.2.1, EAFS 3560.4.3, EAFS 3561.1.1), there were no

statistical yield differences detected when GAT7 positive lines were compared to GAT7 negative sister lines. Overall, the data for the lines tested indicate that presence of the GAT7 transgene does not appear to impact final yield.

Materials and Methods

2004 D test Materials and Methods:

The variety Jack was transformed with the constituative promoter (SCP1) driving expression of glyphosate acetyl transferase round 7 (GAT7), linked to the selectable marker insert SAMS:ALS. Forty initial events of SCP:GAT7::SAMS:ALS were advanced to the T2 generation. Zygosity of the advanced T2 lines was initially determined by screening 12 random plants per line for PCR amplification of the GAT insert. 454 lines were tentatively selected to be either homozygous positive or homozygous negative for GAT. Selected lines were blocked by event and grown in D level yield tests (1 replication of two 10 foot rows) at Cedar Falls (planted 6/5/04), Dallas Center (planted 6/4/04), and Johnston, IA (planted 6/9/04) during 2004. Twelve remnant T3 seed of each line was screened using either glyphosate spray at the V3 growth stage, or soaking remnant seed in sulfonylurea solution. Based upon the glyphosate treatment or SU seed soak, 342 SCP:GAT7::ALS lines from 30 events were confirmed to be homozygous positive or homozygous negative. Maturity scores were collected for all entries at the Dallas Center and Johnston locations. Yield data was collected and subject to multiple regression, ANOVA, and mean separation using SAS.

2005 C test Materials and Methods

Based upon yield performance and herbicide efficacy scores in 2004, 12 GAT7 events were advanced for C level yield testing in 2005. From these selected twelve events, 28 positive and 23 negative isolines were selected. 2005 C tests were designed as a randomized complete block (by event) and grown at Cedar Falls, IA; Johnston, IA; Stuart, IA; Monmouth, IL; Princeton, IL; and Napoleon, OH. Maturity scores and yield data were collected and subject to multiple regression, ANOVA and mean separation using PRISM and/or SAS.

Results and Discussion

When 2004 yield data for the selected lines tested was subject to ANOVA, the location, events, location*event, and GAT (positive or negative) variables were significantly different (data not shown). A mean of all positive lines was not significantly lower for yield

compared to a mean of all negative lines tested in 2004. In 2005, the location, event, location*event, GAT, and GAT*location variables were significantly different (data not shown). A mean of all positive lines tested was not significantly different from a mean of all negative lines tested in 2005 (data not shown). When the 2004 and 2005 data are combined, the year, location, event, year*event, location*event, year*GAT, and location*GAT variables are significantly different (data not shown). A mean of all GAT positive lines was not significantly different from a mean of all GAT negative lines tested across the 9 midwest environments (data not shown).

Based upon 2004 yield, herbicide efficacy, and molecular analyses, 3 lead GAT7 events were selected for potential regulatory and product development experiments. When the 2005 isoline yield data is combined with 2004 data, the locations were significantly different within EAFS 3559.2.1 (data not shown). Positive GAT lines within EAFS 3559.2.1 were not significantly different for yield compared to negative sister isolines (data not shown). Within EAFS 3560.4.3, the 9 locations and location*GAT interaction were significantly different. However, GAT positive isolines were not significantly different for yield when compared to GAT negative isolines within the same event (data not shown). Within event EAFS 3561.1.1, the locations were significantly different, while the GAT score and location*GAT interaction were not significantly different (data not shown). Within EAFS 3561.1.1, GAT positive lines were not significantly different for yield compared to GAT negative sister lines (data not shown).

Multiple regression of yield x maturity for the 6 environments tested in 2005 was performed on the 3 lead events to determine overall yield potential. In general, GAT positive and GAT negative lines within each of the 3 lead events appear to be random (data not shown). This suggests that yield does not appear to be significantly altered when the GAT7 transgene is present.

A modified t-test was completed to compare the GAT7 positive lines to a mean of the GAT7 negative lines within each specific event at each location tested (data not shown). Across the 9 locations tested, there is no apparent yield disadvantage for GAT7 positive lines compared to GAT7 negative sister lines within the same event. For the 3 lead events, GAT positive lines are within 2.6% of the negative mean, indicating overall yield parity exists in all environments tested (data not shown). ANOVA and LSD analyses performed on the individual lines indicate no distinct differences among lines tested in each event, except for EAFS 3560.3.2 (data not shown). Overall, there does not appear to be a yield difference between GAT7 positive lines and GAT7 negative lines.

Example 16 GAT soybeans have tolerance to glyphosate, and glyphosate + SU treatments

The objectives of this experiment was to evaluate the sulfonylurea (SU) herbicide tolerance of the lead GAT7 events in direct comparison to the tolerance of STS, and to determine if differences in tolerance could be detected among the lead GAT7 events across different glyphosate (Gly), Gly+SU and SU treatments. Across all the treatments, the four lead GAT7 events were rated with significantly less crop damage response compared to untransformed Jack and STS at 7 days after spraying, and again at 14 days after spraying. Among the 4 lead GAT7 events, there were some response differences detected, with EAFS 3560.4.3 performing the best over all the treatments, and EAFS 3560.3.2 showing the most herbicide response. In general, it appears there is a significantly better tolerance to several SU chemistries for the SAMS:ALS construct compared to STS. In addition, the GAT7 events tested had good tolerance to variable rates of glyphosate, sulfonylurea, and glyphosate plus sulfonylurea chemistry treatments.

MATERIALS AND METHODS

GAT round 7 (GAT7) transgenic events from construct PHP20163A were evaluated in 2004 for herbicide efficacy (JHM464TGATEFF) and yield potential (JHD4__GAT7 tests). Based upon these preliminary results and commercialization potential, four lead events were selected for additional efficacy testing in 2005 experiment JHM5G030. 92M90 was selected as a STS variety to compare directly with the SAMS:ALS construct in the GAT7 events. Variety Jack was utilized as an untransformed negative control.

Selected lines were grown in experiment JHM5G030 in two replications of paired twelve foot rows, with eleven different treatments applied. Lines were blocked by event and treatment to provide side-by-side comparison of the eleven different spray treatments, with a 2 row border between each treatment to catch any spray drift. Spray treatments (at V3 unless specified) were 0X (control), 35.03 g/ha ai Synchrony, 140.11 g/ha ai Synchrony, 8.75 g/ha ai tribenuron, 35.0 g/ha ai tribenuron, 8.75 g/ha ai rimsulfuron, 35.0 g/ha ai rimsulfuron, 3360.0 g/ha ai glyphosate, 3360.0 g/ha ai glyphosate at V3 followed by 3360.0 g/ha ai glyphosate at R1, 3360.0 g/ha ai glyphosate plus 35.0 g/ha ai tribenuron (tank mix) and 3360.0 g/ha ai glyphosate plus 70.0 g/ha ai rimsulfuron (tank mix). Lines were given a 100 (complete susceptibility, 100% damage) to 0 (complete tolerance, 0% damage) visual rating at 7 days after treatment and again at 14 days after treatment. Visual ratings were based upon overall degree of chlorosis, necrosis, and plant stunting (if evident) in the treated rows compared to

the respective unsprayed control rows. Rating data at 7 and 14 days after spraying were subject to ANOVA and mean separation using SAS.

RESULTS AND DISCUSSION

When all spray rating data for the different treatments at 7 and 14 days after application were subject to ANOVA, the events and treatments were significantly different, while the replication was not significantly different (data not shown). Jack had significantly higher spray response (damage scores) compared to STS and the GAT events at 7 and 14 days after spraying (DAS) (data not shown). The 4 GAT lines were scored with significantly less damage than Jack and STS for all the treatments at both 7 and 14 days after spray application (data not shown).

In examining individual treatments, the control plot appeared to have some spray drift evident, as both the Jack plot and STS plot were rated with significantly higher spray damage scores compared to the 4 GAT events at 7 DAS (data not shown) and 14 DAS (data not shown). Spray drift would potentially confound the results of the other plots, but was hopefully somewhat minimized by the use of a 2 row border between treatments.

Within the 8.75 g/ha ai rimsulfuron treatment, the 4 GAT events were scored with significantly less spray damage compared to Jack and STS at both 7 DAS (data not shown) and 14 DAS (data not shown). Among the 4 GAT events, there was no statistical difference noted at both rating times (data not shown). Examining the 35.0 g/ha ai rimsulfuron treatment, the 4 GAT events were scored with significantly less spray damage compared to Jack and STS at both 7 DAS (data not shown) and 14 DAS (data not shown). GAT7 Event EAFS 3560.4.3 was scored with less damage compared to GAT7 events EAFS 3559.2.1 and EAFS 3561.1.1 at both 7 DAS (data not shown) and 14 DAS (v).

After the 35.03 g/ha ai Synchrony treatment, the 4 GAT events were scored with significantly less spray damage compared to Jack and STS at both 7 DAS (data not shown) and 14 DAS (data not shown). Among the 4 GAT events, there was no statistical difference noted at both 7 DAS (data not shown) and 14 DAS (data not shown). In examining the 140.11 g/ha ai Synchrony treatment, the 4 GAT events were scored with significantly less spray damage compared to Jack and STS at both 7 DAS (data not shown) and 14 DAS (data not shown). GAT7 events EAFS 3560.4.3 and EAFS 3561.1.1 were scored with less damage compared to GAT7 events EAFS 3559.2.1 and EAFS 3560.3.2 at 7 DAS (data not shown). At 14 DAS the 140.11 g/ha ai Synchrony treatment, GAT7 events EAFS 3560.4.2, EAFS

3559.2.1, and EAFS 3561.1.1 were rated with no damage, while EAFS 3560.3.2 was scored similar to STS (data not shown).

In examining the 8.75 g/ha ai tribenuron treatment, the 4 GAT7 events had significantly less visual damage compared to STS and Jack at 7 DAS (data not shown) and 14 DAS (data not shown). The GAT7 events were not statistically different at 7 DAS (data not shown), but Evens EAFS 3560.4.3 and EAFS 3559.2.1 had significantly lower damage compared to EAFS 3560.3.2 at 14 DAS (data not shown). For the 35.0 g/ha ai tribenuron treatment, the 4 GAT7 events were rated with significantly less spray response compared to Jack and STS at 7 DAS (data not shown) and 14 DAS (data not shown). The 4 GAT events were not statistically different from each other at 7 DAS (data not shown), but the EAFS 3560.3.2 was rated with significantly more spray damage than the other 3 GAT7 events at 14 DAS (data not shown).

For the 3360.0 g/ha ai glyphosate and 3360.0 g/ha ai glyphosate at V3 followed by 3360.0 g/ha ai glyphosate at R1 treatments, the Jack and STS varieties were destroyed after 7 days, as expected (data not shown). The 4 GAT7 events did not have any observed spray damage for the 3360.0 g/ha ai glyphosate treatment at 7 DAS (data not shown) and 14 DAS (data not shown). Minimal spray damage was recorded for the 4 GAT7 events at 7 DAS for the treatment (data not shown), and events EAFS 3560.4.3 and EAFS 3559.2.1 were rated significantly better than EAFS 3560.3.2 at 14 DAS (data not shown).

Two tank-mix treatments of glyphosate plus a sulfonylurea herbicide provided similar results at 7 DAS and 14 DAS. For the 3360.0 g/ha ai glyphosate plus 70.0 g/ha ai rimsulfuron treatment, the 4 GAT events had a similar herbicide response at 7 DAS of about 40% damage (data not shown), and approximately 35% damage at 14 DAS (data not shown). Less of an overall crop response was observed for the 3360.0 g/ha ai glyphosate plus 35.0 g/ha ai tribenuron treatment, and the 4 GAT events were not statistically different at 7 DAS (data not shown), and 14 DAS (data not shown).

The mean of visual ratings at 7 DAS and 14 DAS were graphed for the four lead GAT events, Jack, and the STS line to allow visual interpretation of the data (data not shown). For the Rimsulfuron treatments, there was a crop response observed with the 4 lead GAT events, with EAFS 3560.4.3 having the most tolerance noted (data not shown). The response of the GAT7 events was significantly less than the STS and Jack controls for both Rimsulfuron treatments at 7 DAS and 14 DAS (data not shown).

In examining the mean response scores to the 1X and 140.11 g/ha ai Synchrony treatments, there was no apparent damage for GAT7 events EAFS 3560.4.3 and EAFS

3561.1.1, and minimal response of GAT 7 events EAFS 3559.2.1 and EAFS 3560.3.2 (data not shown). All GAT events had significantly less crop response compared to Jack and the STS line (data not shown).

The four GAT7 events had significantly less crop response to 1X and 35.0 g/ha ai tribenuron applications at 7 DAS and 14 DAS when compared to Jack and STS (data not shown). Among the GAT7 events, EAFS 3560.4.3 performed the best, while EAFS 3560.3.2 appeared to show the most response overall (data not shown).

For the 3360.0 g/ha ai glyphosate application, there was no apparent crop response for all 4 GAT7 events at 7 DAS and 14 DAS (data not shown). For the 3360.0 g/ha ai glyphosate at V3 followed by 3360.0 g/ha ai glyphosate at R1, there were no statistical differences noted among the four GAT7 events at 7 DAS, but EAFS 3560.4.3 and EAFS 3559.2.1 appeared to have less response compared to EAFS 3561.1.1 and EAFS 3560.3.2 at 14 DAS (data not shown).

Of the two tank mix treatments, the 70.0 g/ha ai rimsulfuron plus 3360.0 g/ha ai glyphosate caused a higher level of crop damage response compared to the 35.0 g/ha ai tribenuron plus 3360.0 g/ha ai glyphosate treatment (data not shown). Among the four lead GAT7 events, there were no statistical differences observed for the visual ratings at 7 DAS and 14 DAS (data not shown).

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A method for controlling weeds in an area of cultivation comprising

a) planting the area with crop seeds or plants which comprise

i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in said plant; and,

ii) a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide operably linked to a promoter active in said plant;

b) applying to any crop, crop part, weed or area of cultivation thereof a combination of herbicides comprising at least an effective amount of a first herbicide and an effective amount of a second herbicide, wherein said combination of herbicides does not include glyphosate and wherein said effective amount of said first and said second herbicide is tolerated by the crop and controls weeds.

2. The method of claim 1, wherein said first herbicide comprises an ALS inhibitor.

3. The method of claim 2, wherein said second herbicide comprises a second ALS inhibitor.

4. The method of claim 2 or 3, wherein said effective amount of the first ALS inhibitor comprises about 0.1 to about 5000 g ai/hectare.

5. The method of claim 4, wherein said effective amount of the first ALS inhibitor comprises about 0.5 to about 350 g ai/hectare or about 1 to about 150 g ai/hectare.

6. The method of claim 3, wherein said effective amount of the second ALS inhibitor comprises about 0.1 to about 5000 g ai/hectare, about 0.5 to about 350 g ai/hectare or about 1 to about 150 g ai/hectare.

7. The method of any one of claims 1-6, wherein said first polynucleotide encodes a glyphosate-N-acetyltransferase.

8. The method of any one of claims 1-6, wherein said first polynucleotide encodes a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase or a glyphosate-tolerant glyphosate oxido-reductase.

9. The method of any one of claims 1-6, wherein said ALS inhibitor-tolerant polypeptide comprises a mutated acetolactate synthase polypeptide.

10. The method of claim 9, wherein said mutated acetolactate synthase polypeptide comprises HRA.

11. The method of any one of claims 1-10, wherein said combination of herbicides comprises an ALS inhibitor selected from the group consisting of a sulfonylurea, a triazolopyrimidine, a pyrimidinyloxy(thio)benzoate, an imidazolinone, and a sulfonylaminocarbonyltriazolinone.

12. The method of claim 11, wherein said ALS inhibitor is selected from the group consisting of:

a) Azimsulfuron;
b) Chlorimuron-ethyl;
c) Metsulfuron-methyl;
d) Nicosulfuron;
e) Rimsulfuron;
f) Sulfometuron-methyl;
g) Thifensulfuron-methyl;
h) Tribenuron-methyl;
i) Amidosulfuron;
j) Bensulfuron-methyl;
k) Chlorsulfuron;
l) Cinosulfuron;
m) Cyclosulfamuron;
n) Ethametsulfuron-methyl;
o) Ethoxysulfuron;
p) Flazasulfuron;
q) Flupyrsulfuron-methyl;

r) Foramsulfuron;

s) Imazosulfuron;

t) Iodosulfuron-methyl;

u) Mesosulfuron-methyl;

v) Oxasulfuron;

w) Primisulfuron-methyl;

x) Prosulfuron;

y) Pyrazosulfuron-ethyl;

z) Sulfosulfuron;

aa) Triasulfuron;

bb) Trifloxysulfuron;

cc) Triflusulfuron-methyl;

dd) Tritosulfuron;

ee) Halosulfuron-methyl;

ff) Flucarbazone;

gg) Procarbazone;

hh) Cloransulam-methyl;

ii) Flumetsulam;

jj) Diclosulam;

kk) Florasulam;

ll) Imazamox;

mm) Bispyribac;

nn) Pyriftalid;

oo) Pyribenzoxim;

pp) Pyrithiobac;

qq) Pyriminobac-methyl;

rr) Imazapyr;

ss) Imazethapyr;

tt) Imazaquin;

uu) Imazapic;

vv) Imazamethabenz-methyl;

ww) Imazamox;

xx) Flucetosulfuron;

yy) Metosulam;

zz) Penoxsulam; and,

aaa) Pyroxsulam.

13. The method of any one of claims 1-12, wherein said combination of herbicides comprises at least a triazine or a phosphinic acid.

14. The method of any one of claims 1, 2, 4 or 7-12, wherein said second herbicide and said first herbicide have different mechanisms of action.

15. The method of any one of claims 1-13, wherein said second herbicide and said first herbicide have the same mechanism of action.

16. The method of any one of claims 1-15, wherein said combination of herbicides comprises at least an effective amount of a third herbicide.

17. The method of any one of claims 1-16, wherein

said first herbicide is applied pre-emergence, post-emergence or pre- and post-emergence to the weeds or the crop; and,

said second herbicide is applied pre-emergence, post-emergence or pre- or post-emergence to the weed or the crop.

18. The method of any one of claims 1-17, wherein said first and said second herbicides are applied together or are applied separately.

19. The method of any one of claims 1-17, wherein at least said first and said second herbicides comprise a homogeneous granule blend.

20. The method of any one of claims 1-19, wherein said method further comprises applying an agricultural chemical selected from the group consisting of a fungicide, a nematicide, a growth regulator, a safener, an adjuvant, an insecticide, and any combination thereof.

21. The method of claim 20, wherein said agricultural chemical is applied pre-emergence, post-emergence or pre- and post-emergence to the weed or the crop.

22. The method of any one of claims 1-21, wherein said agricultural chemical is applied together or separately with said first herbicide, said second herbicide, or said first and said second herbicide.

23. The method of any one of claims 1-22, wherein the first herbicide comprises a sulfonylurea and the second herbicide comprises an ALS inhibitor.

24. The method of any one of claims 1-22, wherein the first herbicide comprises a sulfonylurea and the second herbicide comprises an imidazolinone.

25. The method of claim 23 or 24, wherein the effective amount of said first or said second herbicide provides a safening effect.

26. The method of any one of claims 1-25, wherein at least one of said first or said second polynucleotides is operably linked to at least one copy of the enhancer sequence set forth in SEQ ID NO: 72.

27. The method of any one of claims 1-26, wherein said crop is a dicot.

28. The method of claim 27, wherein said dicot is soybean, canola, sunflower, cotton, alfalfa, an ornamental, a fruit, a vegetable, a sugar beet, or an *Arabidopsis*.

29. The method of any one of claims 1-26, wherein said plant is a monocot.

30. The method of claim 29, wherein said monocot is maize, wheat, rice, barley, sorghum, sugar cane, Switchgrass, or rye.

31. The method of claim 1, wherein said crop is maize and said first herbicide is applied at the V7 stage or later.

32. The method of claim 1, wherein step (b) is performed at least once prior to step (a).

33. A method for controlling weeds in an area of cultivation comprising:

a) evaluating environmental conditions in an area of cultivation;

b) selecting an effective amount of a combination of herbicides comprising at least an effective amount of a first herbicide and an effective amount of a second herbicide, wherein said combination of herbicides does not include glyphosate and wherein said effective amount of said first and said second herbicide is tolerated by a crop and controls weeds; and,

c) applying the combination of herbicides to a crop, crop part, seed or an area of cultivation of said crop, wherein the crop comprises a plant having

i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in said plant; and,

ii) a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide operably linked to a promoter active in said plant.

34. The method of claim 33, wherein said first herbicide comprises an ALS inhibitor.

35. The method of claim 34, wherein said second herbicide comprises a second ALS inhibitor.

36. The method of claim 34 or 35, wherein said effective amount of the first or said second ALS inhibitor comprises about 0.1 to about 5000 g ai /hectare.

37. The method of claim 34 or 35, wherein said effective amount of the first or the second ALS inhibitor comprises about 1 to about 150 g ai/hectare.

38. The method of claim 34 or 35, wherein said effective amount of the first or the second ALS inhibitor comprises about 0.5 to about 350 g ai/hectare.

39. The method of any one of claims 33-38, wherein said first polynucleotide encodes a glyphosate-N-acetyltransferase.

40. The method of any one of claims 33-38, wherein said ALS inhibitor-tolerant polypeptide comprises a mutated acetolactate synthase polypeptide.

41. The method of claim 40, wherein said mutated acetolactate synthase polypeptide comprises HRA.

42. The method of any one of claim 33-41, wherein said herbicide combination comprises an ALS inhibitor selected from the group consisting of a sulfonylurea, a triazolopyrimidine, a pyrimidinyloxy(thio)benzoate, an imidazolinone, and a sulfonylaminocarbonyltriazolinone.

43. The method of claim 42, wherein said ALS inhibitor is selected from the group consisting of:

a) Azimsulfuron;
b) Chlorimuron-ethyl;
c) Metsulfuron-methyl;
d) Nicosulfuron;
e) Rimsulfuron;
f) Sulfometuron-methyl;
g) Thifensulfuron-methyl;
h) Tribenuron-methyl;
i) Amidosulfuron;
j) Bensulfuron-methyl;
k) Chlorsulfuron;
l) Cinosulfuron;
m) Cyclosulfamuron;
n) Ethametsulfuron-methyl;
o) Ethoxysulfuron;
p) Flazasulfuron;
q) Flupyrsulfuron-methyl;
r) Foramsulfuron;
s) Imazosulfuron;
t) Iodosulfuron-methyl;
u) Mesosulfuron-methyl;
v) Oxasulfuron;
w) Primisulfuron-methyl;

x) Prosulfuron;
y) Pyrazosulfuron-ethyl;
z) Sulfosulfuron;
aa) Triasulfuron;
bb) Trifloxysulfuron;
cc) Triflusulfuron-methyl;
dd) Tritosulfuron;
ee) Halosulfuron-methyl;
ff) Flucarbazone;
gg) Procarbazone;
hh) Cloransulam-methyl;
ii) Flumetsulam;
jj) Diclosulam;
kk) Florasulam;
ll) Imazamox;
mm) Bispyribac;
nn) Pyriftalid;
oo) Pyribenzoxim;
pp) Pyrithiobac;
qq) Pyriminobac-methyl;
rr) Imazapyr;
ss) Imazethapyr;
tt) Imazaquin;
uu) Imazapic;
vv) Imazamethabenz-methyl;
ww) Imazamox.
xx) Flucetosulfuron;
yy) Metosulam;
zz) Penoxsulam; and,
aaa) Pyroxsulam.

44. The method of any one of claims 33-43, wherein said second herbicide and said first herbicide have the same or a different mechanism of action.

45. The method of any one of claims 33-44, wherein

said first herbicide is applied pre-emergence, post-emergence or pre- and post-emergence to the weeds or the crop; and,

said second herbicide is applied pre-emergence, post-emergence or pre- and post-emergence to the weed or the crop.

46. The method of any one of claims 33-44, wherein said first and said second herbicides are applied together or are applied separately.

47. The method of any one of claims 33-44, wherein at least said first and said second herbicides comprise a homogeneous granule blend.

48. The method of any one of claims 33-47, wherein said method further comprises applying an agricultural chemical selected from the group consisting of a fungicide, a nematicide, a growth regulator, a safener, an adjuvant, an insecticide, or any combination thereof.

49. The method of any one of claims 33-48, wherein at least one of said first or said second polynucleotides is operably linked to at least one copy of the enhancer sequence set forth in SEQ ID NO: 72.

50. The method of any one of claims 33-49, wherein said crop is a dicot.

51. The method of claim 50, wherein said dicot is soybean, canola, sunflower, cotton, alfalfa, an ornamental, a fruit, a vegetable, a sugar beet, or an *Arabidopsis*.

52. The method of any one of claims 33-39, wherein said plant is a monocot.

53. The method of claim 52, wherein said monocot is maize, wheat, rice, barley, sorghum, sugar cane, Switchgrass, or rye.

54. A method for controlling weeds in an area of cultivation comprising

a) planting the area with crop seeds or plants which comprise

i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in a plant; and,

ii) a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide operably linked to a promoter active in a plant;

b) applying to the crop, crop part, weed or area of cultivation thereof a combination of herbicides comprising at least an effective amount of a first and a second ALS inhibitor, wherein

i) the effective amount of the combination of herbicides controls weeds;

ii) the effective amount of the first ALS inhibitor is not tolerated by the crop when applied alone when compared to a control crop that has not been exposed to the first ALS inhibitor; and,

iii) the effective amount of the second ALS inhibitor is sufficient to produce a safening effect, wherein said safening effect provides an increase in crop tolerance upon the application of the first and the second ALS inhibitor when compared to the crop tolerance when the first ALS inhibitor is applied alone.

55. The method of claim 54, wherein at least said first or said second ALS inhibitor comprises a sulfonylurea.

56. The method of claim 54, wherein said second ALS inhibitor comprises a sulfonylurea and the first ALS inhibitor comprises an imidazolinone.

57. The method of claim 54, wherein said combination of herbicides further comprises glyphosate.

58. A method for controlling weeds in an area of cultivation comprising:

a) evaluating environmental conditions in an area of cultivation;

b) selecting a combination of herbicides comprising at least an effective amount of a first and a second ALS inhibitor, wherein said effective amount of the combination controls weeds, and the effective amount of the first ALS inhibitor is not tolerated by the crop when applied alone when compared to a control crop that has been applied with the first ALS inhibitor, and the effective amount of the second ALS inhibitor is sufficient to produce a safening effect, wherein said safening effect provides an increase in

crop tolerance upon the application of the first and the second ALS inhibitor when compared to the crop tolerance when the first ALS inhibitor is applied alone; and,

c) applying the combination of herbicides to a crop, crop part, seed or an area of cultivation of said crop, wherein the crop comprises a plant having

i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in said plant; and,

ii) a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide operably linked to a promoter active in said plant.

59. The method of claim 58, wherein at least said first or said second ALS inhibitor comprises a sulfonylurea.

60. The method of claim 58, wherein said second ALS inhibitor comprises a sulfonylurea and the first ALS inhibitor comprises an imidazolinone.

61. A method for controlling weeds in an area of cultivation comprising

a) planting the area with crop seeds or plants which comprise

i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in a plant; and,

ii) a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide operably linked to a promoter active in a plant;

b) applying to any crop, crop part, weed or area of cultivation thereof an effective amount of a combination of herbicides selected from the group consisting of:

i) the combination of herbicides comprising glyphosate, imazapyr, chlorimuron-ethyl, quizalofop, and fomesafen, wherein said effective amount is tolerated by the crop and controls weeds;

ii) the combination of herbicides comprising glyphosate, imazapyr, chlorimuron-ethyl, quizalofop, and fomesafen, wherein said effective amount is tolerated by the crop and controls weeds; or,

iii) the combination of herbicides comprising glyphosate, chlorimuron-ethyl, and thifensulfuron-methyl, wherein said effective amount is tolerated by the crop and controls weeds.

62. The method of claim 61, wherein said crop is soybean, corn or cotton.

63. The method of claim 59, wherein the effective amount of the combination of herbicides of part (b)(i) comprises glyphosate comprising about 1110 to about 1130 g ai/hectare; the effective amount of imazapyr comprises about 7.5 to about 27.5 g ai/hectare; the effective amount of chlorimuron-ethyl comprises about 7.5 to about 27.5 g ai/hectare; the effective amount of quizalofop comprises about 50 to about 70 g ai/hectare; and, the effective amount of fomesafen comprises about 240 to about 260 g ai/hectare.

64. A plant comprising a polynucleotide encoding a polypeptide which confers tolerance to glyphosate or a polynucleotide that encodes and ALS inhibitor that encodes an ALS inhibitor-tolerant polypeptide, wherein said polynucleotide is operably linked to

(a) a promoter that drives expression in the plant; and,

(b) at least one copy of the enhancer sequence set forth in SEQ ID NO: 1, 72, 85, 88, or 89 or an enhancer sequence having at least 90% sequence identity to SEQ ID NO: 1, 72, 85, 88, or 89, wherein said enhancer sequence modulates the level of transcription

wherein said plant is tolerant to glyphosate or an ALS inhibitor applied at a level effective to inhibit the growth of a control plant which does not comprise said polynucleotide operably linked to said enhancer sequence.

65. The plant of claim 64, wherein said polynucleotide which confers tolerance to glyphosate encodes a glyphosate-N-acetyltransferase.

66. The plant of claim 64, wherein said polynucleotide which confers tolerance to glyphosate encodes a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase or a glyphosate-tolerant glyphosate oxido-reductase.

67. The transgenic plant of claim 64, wherein said ALS inhibitor-tolerant polypeptide comprises HRA.

68. The transgenic plant of any one of claim 64-67, wherein said polynucleotide is operably linked to at least three copies of the enhancer sequence set forth in SEQ ID NO: 72.

69. The transgenic plant of any one of claims 64-67, wherein said promoter is a ubiquitin promoter.

70. The transgenic plant of any one of claims 64-68, wherein said enhancer is heterologous to said promoter.

71. The transgenic plant of any one of claims 64-70, wherein said promoter is a maize ubiquitin promoter or an Arabidopsis ubiquitin promoter.

72. The plant of any one of claims 64-71, wherein said crop is a dicot.

73. The plant of claim 72, wherein said dicot is is soybean, canola, sunflower, cotton, alfalfa, an ornamental, a fruit, a vegetable, a sugar beet, or an *Arabidopsis*.

74. The plant of any one of claims 64-70, wherein said plant is a monocot.

75. The plant of claim 74, wherein said monocot is maize, wheat, rice, barley, sorghum, sugar cane, Switchgrass, or rye.

76. An expression cassette comprising a first polynucleotide that encodes a polypeptide which confers tolerance to glyphosate or a polynucleotide that encodes an ALS inhibitor-tolerant polypeptide, wherein said polynucleotide is operably linked to:

(a) a promoter that drives expression in the plant; and

(b) at least one copy of the enhancer sequence set forth in SEQ ID NO: 1, 72, 85, 88, or 89, or an enhancer sequence having at least 90% sequence identity to SEQ ID NO: 1, 72, 85, 88, or 89, wherein said enhancer sequence modulates transcription and said enhancer sequence is heterologous to said promoter;

wherein said expression cassette confers glyphosate tolerance or ALS inhibitor tolerance on a plant containing it, wherein said plant is tolerant to glyphosate or the ALS inhibitor applied at a level effective to inhibit the growth of a control plant which does not comprise said expression cassette.

77. The expression cassette of claim 76, wherein said polynucleotide which confers tolerance to glyphosate encodes a glyphosate-N-acetyltransferase.

78. The expression cassette of claim 76, wherein said polynucleotide which confers tolerance to glyphosate encodes a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase or a glyphosate-tolerant glyphosate oxido-reductase.

79. The expression cassette of any one of claims 76-78, wherein said polynucleotide is operably linked to at least three copies of the enhancer sequence set forth in SEQ ID NO: 72.

80. The expression cassette of any one of claims 76-79, wherein said promoter is a ubiquitin promoter.

81. The expression cassette of any one of claims 76-79, wherein said enhancer is heterologous to said promoter.

82. The expression cassette of claim 81, wherein said promoter is an Arabidopsis ubiquitin promoter or a maize ubiquitin promoter.

83. The expression cassette of any one of claims 76-83, wherein said polypeptide comprises the sequence set forth in SEQ ID NO:45.

84. The expression cassette of any one of claims 76-83, wherein said expression cassette comprises the polynucleotide which confers tolerance to glyphosate and the polynucleotide that encodes the ALS inhibitor-tolerant polypeptide.

86. The expression cassette of claim 76 or 84, wherein said ALS inhibitor-tolerant polypeptide comprises HRA.

87. A method of selecting cotton which has been transformed with a first polynucleotide encoding a first polypeptide which confers glyphosate tolerance and a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide, said method comprising the step of growing transformed cotton callus while exposing it to glyphosate and/or an ALS inhibitor herbicide.

88. The method of claim 87, wherein said polypeptide which confers glyphosate tolerance comprise GAT and said ALS inhibitor-tolerant polypeptide comprises HRA.

89. The method of claim 88, wherein the callus is grown while exposing it to a medium containing chlorsulfuron at a concentration of 50-200 micrograms per liter.

90. The method of claim 88, wherein the callus is grown while exposing it to a medium containing glyphosate at a concentration of up to 450 μM.

91. A method for controlling weeds in an area of cultivation comprising

a) planting the field with crop seeds or plants which comprise

i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in a plant; and,

ii) a second polynucleotide encoding an ALS inhibitor resistance polypeptide operably linked to a promoter active in a plant;

b) applying to said crop, crop part, seed of said crop or the area of cultivation thereof an effective amount of an herbicide, wherein said effective amount is tolerated by the crop and controls weeds, wherein said herbicide is not glyphosate, chlorimuron-ethyl, rimsulfuron, tribenuron-methyl or thifensufuron-methyl.

92. The method of claim 91, wherein said herbicide is selected from the group consisting of

(i) a sulfonylaminocarbonyltriazolinone;

(ii) a triazolopyrimidine;

(iii) a pyrimidinyl(thio)benzoate;

(iv) an imidazolinone;

(v) a triazine; and

(vi) a phosphinic acid.

93. A method for controlling weeds in a field containing a crop comprising

a) planting the field with crop seeds or plants which comprise

i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate; and,

ii) a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide;

b) applying to any crop and weeds in the field an effective amount of a first herbicide comprising an ALS inhibitor and an effective amount of at least one additional agricultural agent selected from the group consisting of a fungicide, a nematicide, an insecticide, a safener, or an adjuvant,

wherein said effective amount of said ALS inhibitor herbicide is tolerated by the crop, and,

wherein said effective amount of said combination is not tolerated by a control plant which does not express both of said first and said second polypeptide.

94. The method of claim 93, wherein step (b) is performed at least once prior to step (a).

95. A method for improving transformation efficiency, increasing a single copy integration event, or increasing transformation efficacy of a plant comprising:

a) introducing in the plant a cassette comprising a polynucleotide encoding a polypeptide that confers tolerance to glyphosate or a polynucleotide encoding an ALS inhibitor-tolerant polypeptide, wherein said polynucleotide is operably linked to a promoter active in said plant and is operably linked to at least one copy of the enhancer sequence set forth in SEQ ID NO:1, 72, 85, 88, or 89 or an enhancer sequence having at least 90% sequence identity to SEQ ID NO: 1, 72, 85, 88, or 89, wherein said sequence modulates transcription;

b) contacting said plant with an effective concentration of an appropriate herbicide; and,

c) selecting the plant expressing said polynucleotide.

96. The method of claim 95, wherein said cassette further comprises a polynucleotide of interest.

97. The method of claim 95 or 96, wherein said polypeptide conferring tolerance to glyphosate comprises a GAT polypeptide or said ALS inhibitor-tolerant polypeptide comprises HRA.

98. A method for controlling weeds comprising

a) planting the area with crop seeds or plants which express

i) a first nucleotide sequence encoding a polypeptide that can confer tolerance to glyphosate; and,

ii) a second nucleotide sequence encoding an ALS inhibitor-tolerant polypeptide;

b) applying to said crop, crop part, seed of said crop or the area under cultivation, an effective amount of a herbicides, wherein said effective amount comprises

i) an amount that is not tolerated by a first control crop when applied to the first control crop, crop part, seed or the area of cultivation, wherein said first control crop expresses the first polynucleotide that confers tolerance to glyphosate and does not express the second polynucleotide that encodes an ALS inhibitor-tolerant polypeptide;

ii) an amount that is not tolerated by a second control crop when applied to the second crop, crop part, seed or the area of cultivation, wherein said second control crop expresses the second polynucleotide and does not express the first polynucleotide; and,

iii) an amount that is tolerated when applied to the crop, crop part, seed, or the area of cultivation thereof of step (b).

99. A method for controlling weeds in an area of cultivation comprising:

a) evaluating environmental conditions in an area of cultivation;

b) selecting an effective amount of a herbicide wherein the effective amount of the herbicide comprises

i) an amount that is not tolerated by a first control crop when applied to the first control crop, crop part, seed or the area of cultivation, wherein said first control crop expresses a first polynucleotide that confers tolerance to glyphosate and does not express a second polynucleotide that encodes an ALS inhibitor-tolerant polypeptide;

ii) an amount that is not tolerated by a second control crop when applied to the second crop, crop part, seed or the area of cultivation, wherein said second control crop expresses the second polynucleotide and does not express the first polynucleotide; and,

iii) an amount that is tolerated when applied to a crop of interest, a crop part or interest, a seed of the crop of interest, or the area of cultivation thereof, wherein said crop of interest expresses the first nucleotide sequence encoding the polypeptide that can

confer tolerance to glyphosate; and, the second nucleotide sequence encoding the ALS inhibitor-tolerant polypeptide.

c) applying to the crop of interest, crop part of interest, weed or area of cultivation thereof, the effective amount of said herbicide.

100. A method for controlling weeds in an area of cultivation, the method comprising:

a) planting the area with crop seeds or crop plants, wherein the crop seeds or crop plants comprise:

i) a first polynucleotide encoding a polypeptide that can confer tolerance to glyphosate operably linked to a promoter active in the crop seeds or crop plants; and

ii) a second polynucleotide encoding an ALS inhibitor-tolerant polypeptide operable linked to a promoter active in the crop seeds or crop plants; and

b) applying to any crop, crop part, weed, area of cultivation, or a combination thereof, an effective amount of a herbicide composition comprising at least one of a synergistically effective amount of glyphosate and a synergistically effective amount of a sulfonylurea herbicide, or agriculturally suitable salts thereof, wherein at least one of:

i) the synergistically effective amount of the glyphosate is lower than an amount of glyphosate required to control the weeds in the absence of the sulfonylurea herbicide;

ii) the synergistically effective amount of the sulfonylurea herbicide is lower than an amount of the sulfonylurea required to control the weeds in the absence of glyphosate; and

iii) combinations thereof; and

wherein the effective amount of the herbicide composition is tolerated by the crop seeds or crop plants and controls the weeds in the area of cultivation.

101. The method of claim 100, wherein the first polynucleotide encodes a glyphosate-N-acetyltransferase.

102. The method of claim 100 or 101, wherein the ALS inhibitor-tolerant polypeptide comprises a mutated acetolactate synthase polypeptide comprising HRA.

103. The method of claim 100, 101, or 102, wherein the sulfonylurea herbicide is selected from the group consisting of metsulfuron-methyl, chlorsulfuron, and triasulfuron.

104. The method of claim 100, 101, 102, or 103, wherein

the glyphosate is applied pre-emergence, post-emergence or pre- and post-emergence to the weeds or the crop plants; and

the sulfonylurea herbicide is applied pre-emergence, post-emergence or pre- and post-emergence to the weeds or the crop plants.

105. The method of any one of claims 100-104, wherein the glyphosate and the sulfonylurea herbicide are applied together or are applied separately.

106. The method of claim 100, wherein step (b) is performed at least once prior to step (a).

107. The method of any one of claim 100-106, wherein the herbicide composition comprises an ammonium sulfate-based adjuvant.

108. The method of claim 107, wherein the herbicide composition comprises an effective amount of a pyrimidinyloxy(thio)benzoate herbicide.

109. The method of claim 108, wherein the pyrimidinyloxy(thio)benzoate herbicide comprises bispyribac, or an agriculturally suitable salt thereof.

110. The method of claim 54, 58, 61, 91, 93, 98, or 100, wherein the crop plant is a dicot.

111. The method of claim 110, wherein the dicot is soybean, canola, sunflower, cotton, alfalfa, an ornamental, a fruit, a vegetable, a sugar beet, or an *Arabidopsis*.

112. The method of claim 54, 58, 61, 91, 93, 98, or 100, wherein the crop plant is a monocot.

113. The method of claim 112, wherein the monocot is maize, wheat, rice, barley, sorghum, sugar cane, Switchgrass, or rye.