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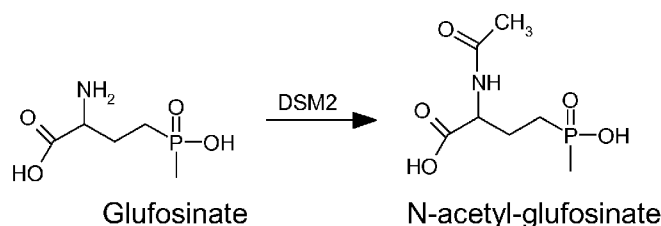
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(54) Title: CONSTRUCTS FOR EXPRESSING HERBICIDE TOLERANCE GENES, RELATED PLANTS, AND RELATED TRAIT COMBINATIONS

**FIGURE 1**

(57) **Abstract:** The subject invention relates in part to constructs for expressing herbicide tolerance genes, related plants, and related trait combinations. Such constructs and plants comprise a gene referred to herein as DSM-2. This gene was identified in *Streptomyces coelicolor* (A3). The DSM-2 protein is distantly related to PAT and BAR. DSM-2 can be used as a transgenic trait to impart tolerance in plant cells and plants to the herbicidal molecules glufosinate, phosphinothricin, bialaphos, and/or the like. Introduction of this gene into a variety of plants allows for excellent levels of tolerance and/or resistance to the herbicides glufosinate, bialaphos, and other herbicides. Preferred plants include canola and soybeans. The subject invention also relates to combination of the subject herbicide tolerant crop (HTC) traits along with other traits, including other HTC traits (including but not limited to glyphosate tolerance and 2,4-D tolerance), and/or insect resistance (IR) traits in some preferred embodiments. Some preferred stacks of DSM-2 with IR traits are in tobacco and corn. Thus, various uses of DSM-2 genes are included within the scope of the subject invention. Such uses include stacking of a DSM-2 gene with one or more other transgenic traits and introduction of a DSM-2 gene individually into preferred crops.



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## CONSTRUCTS FOR EXPRESSING HERBICIDE TOLERANCE GENES, RELATED PLANTS, AND RELATED TRAIT COMBINATIONS

### BACKGROUND OF THE INVENTION

5 A selectable marker is a detectable genetic trait or segment of DNA that can be identified and tracked. A marker gene typically serves as a flag for another gene, sometimes called the target gene. A marker gene is typically used with a target gene being used to transform target cells. Target cells that heritably receive the target gene can be identified by selecting for cells that also express the marker gene. The marker gene is near enough to the target gene so that the two  
10 genes (the marker gene and the target gene) are genetically linked and are usually inherited together. The current standard for selectable markers is the “pat” gene which encodes an enzyme called phosphinothricin acetyl transferase.

Glutamine synthetase (“GS”) in many plants is an essential enzyme for the development and life of plant cells. GS converts glutamate into glutamine. GS is also involved in ammonia  
15 assimilation and nitrogen metabolism. GS is involved in a pathway in most plants for the detoxification of ammonia released by nitrate reduction. Therefore, potent inhibitors of GS are very toxic to plant cells. Breakdown or modification of the herbicide inside the plant could lead to resistance.

A particular class of herbicides has been developed, based on the toxic effect due to  
20 inhibition of GS in plants. Bialaphos and phosphinothricin are two such inhibitors of the action of GS and possess excellent herbicidal properties. These two herbicides are non-selective; they inhibit growth of all the different species of plants present on the soil, accordingly causing their total destruction.

Bialaphos is also a broad spectrum herbicide. Bialaphos is composed of phosphinothricin  
25 (PPT or PTC; 2-amino-4-methylphosphinobutyric acid), an analogue of L-glutamic acid, and two L-alanine residues. Thus the structural difference between PPT and Bialaphos resides in the absence of two alanine amino acids in the case of PPT. Upon removal of the L-alanine residues of Bialaphos by intracellular peptidases, the PPT is released. PPT is a potent inhibitor of GS. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the  
30 plant cells.

Bialaphos was first disclosed as having antibiotic properties, which enabled it to be used as a pesticide or a fungicide. U.S. Patent No. 3,832,394 relates to cultivating *Streptomyces hygroscopicus*, and recovering Bialaphos from its culture media. However, other strains, such as *Streptomyces viridochromogenes*, also produce this compound. Other tripeptide antibiotics which contain a PPT moiety are also known to exist in nature, such as phosalacin. PPT is also obtained by chemical synthesis and is commercially distributed.

Bialaphos-producing *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes* are protected from PPT toxicity by an enzyme with phosphinothricin acetyl transferase activity. Plant Physiol, April 2001, Vol. 125, pp. 1585-1590 ("Expression of *bar* in the Plastid Genome Confers Herbicide Resistance," Lutz *et al.* ). The *Streptomyces* species that produce these antibiotics would themselves be destroyed if they did not have a self-defense mechanism against these antibiotics. This self-defense mechanism has been found in several instances to comprise an enzyme capable of inhibiting the antibiotic effect.

Phosphinothricin acetyl transferase is encoded by either the *bar* (bialaphos resistance; Thompson *et al.*, 1987) or *pat* (phosphinothricin acetyltransferase; Strauch *et al.*, 1988) genes, and detoxifies PPT by acetylation of the free amino group of PPT. The enzymes encoded by these two genes are functionally identical and show 85% identity at the amino acid level (Wohlleben *et al.*, 1988; Wehrmann *et al.*, 1996). PPT-resistant crops have been obtained by expressing chimeric *bar* or *pat* genes in the cytoplasm from nuclear genes. Herbicide-resistant lines have been obtained by direct selection for PPT resistance in tobacco (*Nicotiana tabacum* cv Petit Havana), potato, *Brassica napus*, *Brassica oleracea* (De Block *et al.*, 1987; De Block *et al.*, 1989), maize (Spencer *et al.*, 1990), and rice (Cao *et al.*, 1992).

A gene (*bar*) was identified adjacent to the *hrdD* sigma factor gene in *Streptomyces coelicolor* A3. The predicted *bar* product showed 32.2% and 30.4% identity to those of the *pat* and *bar* genes of the bialaphos producers *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus*, respectively. The *S. coelicolor bar* gene conferred resistance to bialaphos when cloned in *S. coelicolor* on a high-copy-number vector. Bedford *et al.*, Gene, 1991 Jul 31;104(1):39-45, "Characterization of a gene conferring bialaphos resistance in *Streptomyces coelicolor* A3(2)." Heterologous expression of this gene in other microbes, or transformation of this gene into plants, has not heretofore been reported.

The use of the herbicide resistance trait is referred to in DE 3642 829 A and U.S. Patent No. 5,879,903 (as well as 5,637,489; 5,276,268; and 5,273,894) wherein the *pat* gene is isolated from *Streptomyces viridochromogenes*. WO 87/05629 and U.S. Patent No. 5,648,477 (as well as 5,646,024 and 5,561,236) refer to the use of the *bar* gene from *S. hygroscopicus* for protecting plant cells and plants from glutamine synthetase inhibitors (such as PPT) and to the development of herbicide resistance in the plants. The gene encoding resistance to the herbicide BASTA (Hoechst phosphinothricin) or Herbiace (Meiji Seika bialaphos) was introduced by *Agrobacterium* infection into tobacco (*Nicotiana tabacum* cv *Petit Havan SR1*), potato (*Solanum tuberosum* cv *Benolima*), and tomato (*Lycopersicum esculentum*) plants, and conferred herbicide resistance.

#### BRIEF SUMMARY OF THE INVENTION

The subject invention relates in part to constructs for expressing herbicide tolerance genes, related plants, and related trait combinations. Such constructs and plants comprise a gene referred to herein as *DSM-2*. This gene was identified in *Streptomyces coelicolor* (A3). The *DSM-2* protein is distantly related to PAT and BAR. *DSM-2* can be used as a transgenic trait to impart tolerance in plant cells and plants to the herbicidal molecules glufosinate, phosphinothricin, bialaphos, and/or the like. Introduction of this gene into a variety of plants allows for excellent levels of tolerance and/or resistance to the herbicides glufosinate, bialaphos, and other herbicides. Preferred plants include canola and soybeans.

The subject invention also relates to combination of the subject herbicide tolerant crop (HTC) traits along with other traits, including other HTC traits (including but not limited to glyphosate tolerance and 2,4-D tolerance), and/or insect resistance (IR) traits in some preferred embodiments. Some preferred stacks of *DSM-2* with IR traits are in tobacco and corn.

Thus, various uses of *DSM-2* genes are included within the scope of the subject invention. Such uses include stacking of a *DSM-2* gene with one or more other transgenic traits and introduction of a *DSM-2* gene individually into preferred crops.

#### BRIEF DESCRIPTION OF THE FIGURES

**Figure 1:** shows deactivation of glufosinate by N-acetylation mediated by *DSM2*.

## BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the Native *DSM-2* sequence.

SEQ ID NO:2 is the Native Protein sequence.

SEQ ID NO:3 is the Hemicot *DSM-2* (v2) sequence.

5 SEQ ID NO:4 is the Rebuilt Protein sequence.

SEQ ID NO:5 is the *Pat* PTU primer (MAS123).

SEQ ID NO:6 is the *Pat* PTU primer (Per 5-4).

SEQ ID NO:7 is the *Pat* coding region primer

SEQ ID NO:8 is the *Pat* coding region primer

10 SEQ ID NO:9 is the *DSM-2* (v2) coding region primer

SEQ ID NO:10 is the *DSM-2* (v2) coding region primer

## DETAILED DESCRIPTION OF THE INVENTION

15 The subject invention relates in part to constructs for expressing herbicide tolerance genes, related plants, and related trait combinations. Such constructs and plants comprise a gene referred to herein as *DSM-2*. This gene was identified in *Streptomyces coelicolor* (A3). The *DSM-2* protein is distantly related to PAT and BAR. *DSM-2* can be used as a transgenic trait to impart tolerance in plant cells and plants to the herbicidal molecules glufosinate, phosphinothricin, bialaphos, and/or the like. Introduction of this gene into a variety of plants allows for excellent  
20 levels of tolerance and/or resistance to the herbicides glufosinate, bialaphos, and other herbicides. Preferred plants include canola and soybeans.

25 The subject invention also relates to combination of the subject herbicide tolerant crop (HTC) traits along with other traits, including other HTC traits (including but not limited to glyphosate tolerance and 2,4-D tolerance), and/or insect resistance (IR) traits in some preferred embodiments.

Thus, various uses of *DSM-2* genes are included within the scope of the subject invention. Such uses include stacking of a *DSM-2* gene with one or more other transgenic traits and introduction of a *DSM-2* gene individually into preferred crops.

30 PCT/US07/86813 (filed on December 7, 2007, by Dow AgroSciences LLC, Lira *et al.*) is herein incorporated by reference.

Experiments demonstrated that the *Escherichia coli* cell line BL21 – Star (DE3) (Invitrogen catalog #C6010-03) was inhibited on minimal media containing concentrations of 100 µg/ml of glufosinate ammonium (Basta). The expression of DSM-2 in the BL21 Star cell line complemented resistance on minimal media containing 400 µg/ml of glufosinate. These experiments indicate that the expression of DSM-2 can be used as a non-medicinal antibiotic selectable marker for cloning applications in bacteria that are inhibited by glufosinate.

Further experiments demonstrated that the plant promoters *Arabidopsis thaliana* PolyUbiquitin 10 (At Ubi10) and the viral promoter Cassava Vein Mosaic Virus (CsVMV) are functional in the *E. coli* strain BL21 – Star (DE3). Both promoters expressed adequate DSM-2 protein to provide resistance to minimal media containing 200 µg/ml of glufosinate. These plant promoters can be used to drive the expression of DSM-2 as a non-medicinal antibiotic selectable marker in *E. coli*. Functionality of a single plant promoter in both bacteria and plants eliminates the requirement of separate selectable markers for each species.

This gene can also be used as the basis for a novel, plant-transformation system in conjunction with a modified *Agrobacterium* strain. Novel strains of *Pseudomonas fluorescens*, or other microbial strains, for protein production using non-medicinal antibiotic resistance marker genes can also be produced according to the subject invention. Improvement in cloning and transformation processes and efficiency by elimination of fragment purification, away from medicinal antibiotic resistance elements can also be a benefit.

In addition to HTC traits, methods for controlling weeds using herbicides for which herbicide tolerance is created by the subject genes in transgenic crops is also within the scope of the subject invention. Combination of the subject HTC trait is also beneficial when combined with other HTC traits (including but not limited to glyphosate tolerance and 2,4-D tolerance), particularly for controlling species with newly acquired resistance or inherent tolerance to a herbicide (such as glyphosate). In addition, when rotating glyphosate tolerant crops (which are becoming increasingly prevalent worldwide) with other glyphosate tolerant crops, control of glyphosate resistant volunteers may be difficult. Thus, use of these transgenic traits stacked or transformed individually into crops may provide a tool for control of other HTC volunteer crops.

Additionally, DSM-2 alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (e.g., insect resistance, fungal resistance, or stress tolerance, *et al.*) or output (e.g., increased yield, improved oil profile, improved fiber quality, *et*

al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

*Proteins (and source isolates) of the subject invention.* The present invention provides functional proteins. By “functional activity” (or “active”) it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce “an effective amount” of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds. One cannot easily discuss the term “resistance” and not use the verb “tolerate” or the adjective “tolerant.” The industry has spent innumerable hours debating Herbicide Tolerant Crops (HTC) versus Herbicide Resistant Crops (HRC). HTC is a preferred term in the industry. However, the official Weed Science Society of America definition of resistance is “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis.” As used herein unless otherwise indicated, herbicide “resistance” is heritable and allows a plant to grow and reproduce in the presence of a typical herbicidally effective treatment by a herbicide for a given plant, as suggested by the current edition of *The Herbicide Handbook* as of the filing of the subject disclosure. As is recognized by those skilled in the art, a plant may still be considered

"resistant" even though some degree of plant injury from herbicidal exposure is apparent. As used herein, the term "tolerance" is broader than the term "resistance," and includes "resistance" as defined herein, as well an improved capacity of a particular plant to withstand the various degrees of herbicidally induced injury that typically result in wild-type plants of the same genotype at the same herbicidal dose.

Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

With the benefits of the subject disclosure, proteins and genes of the subject invention can be obtained from a variety of sources, including a variety of microorganisms such as recombinant and/or wild-type bacteria, for example.

Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

5 A protein “from” or “obtainable from” any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. “Derived from” also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a  
10 bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

Standard molecular biology techniques may be used to clone and sequence the proteins  
15 and genes described herein. Additional information may be found in Sambrook *et al.*, 1989, which is incorporated herein by reference.

*Polynucleotides and probes.* The subject invention further provides nucleic acid sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the  
20 desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

25 The polynucleotides of the subject invention can be used to form complete “genes” to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally,  
30 greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express

the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *DSM-2* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The “coding strand” is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the “anti-sense” strand of DNA. The “sense” or “coding” strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the

polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, *etc.*).

As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention

can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with <sup>32</sup>P-labeled gene-specific probes can be performed by standard methods (*see, e.g., Maniatis et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (T<sub>m</sub>) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

$$T_m = 81.5^\circ \text{C} + 16.6 \text{ Log}[\text{Na}^+] + 0.41(\% \text{G+C}) - 0.61(\% \text{formamide}) - 600/\text{length of duplex in base pairs}.$$

Washes can typically be carried out as follows:

- (1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).
- (2) Once at T<sub>m</sub>-20° C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (T<sub>m</sub>) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T<sub>m</sub> for oligonucleotide probes can be determined by the following formula:

$$T_m (^\circ \text{C}) = 2(\text{number T/A base pairs}) + 4(\text{number G/C base pairs})$$

(Suggs *et al.*, 1981).

Washes can typically be out as follows:

- (1) Twice at room temperature for 15 minutes 1x SSPE, 0.1% SDS (low stringency wash).
- (2) Once at the hybridization temperature for 15 minutes in 1x SSPE, 0.1% SDS (moderate stringency wash).

5 In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low: 1 or 2x SSPE, room temperature

Low: 1 or 2x SSPE, 42° C

Moderate: 0.2x or 1x SSPE, 65° C

10 High: 0.1x SSPE, 65° C.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

15 *PCR technology.* Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.*, 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*,

the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated  
5 between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

*Modification of genes and proteins.* The subject genes and proteins can be fused to other  
10 genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have  
15 substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein,  
20 reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid  
25 substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

30 In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation.

This can be referred to as gene “shuffling,” which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

“Shuffling” can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, “focused shuffling” can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using “gene shuffling” and other techniques, equivalent genes and proteins can be constructed that comprise certain segments having certain contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. Such techniques can be adjusted to obtain equivalent / functionally active proteins having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, and 170 contiguous amino acid residues corresponding to a segment (of the same size) in any of the exemplified or suggested sequences. Polynucleotides encoding such segments, particularly for regions of interest, are also included in the subject invention and can also be used as probes and/or primers, especially for conserved regions.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal31* or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By “truncated protein” it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, *B.t.* proteins can be used in a truncated (core protein) form (*see, e.g.,* Höfte *et al.* (1989), and Adang *et al.* (1985)). As used herein, the term “protein” can include functionally active truncations.

In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.*, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 2** provides a listing of examples of amino acids belonging to each class.

| Table 2             |                                        |
|---------------------|----------------------------------------|
| Class of Amino Acid | Examples of Amino Acids                |
| Nonpolar            | Ala, Val, Leu, Ile, Pro, Met, Phe, Trp |
| Uncharged Polar     | Gly, Ser, Thr, Cys, Tyr, Asn, Gln      |
| Acidic              | Asp, Glu                               |
| Basic               | Lys, Arg, His                          |

In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

As used herein, reference to “isolated” polynucleotides and/or “purified” proteins refers to these molecules when they are in a state other than which they would be found in nature. Thus, reference to “isolated” and/or “purified” signifies the involvement of the “hand of man” as described herein. For example, a bacterial “gene” of the subject invention put into a plant for

expression is an “isolated polynucleotide.” Likewise, a protein derived from a bacterial protein and produced by a plant is an “isolated protein.”

Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled “Optimization of sequence for expression in plants.”

*Optimization of sequence for expression in plants.* To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

*Transgenic hosts.* The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf and pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

Thus, the subject invention can be adapted for use with vascular and nonvascular plants including monocots and dicots, conifers, bryophytes, algae, fungi, and bacteria. Animal cells and animal cell cultures are also a possibility.

In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when

cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid  
5 cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

10 *Insertion of genes to form transgenic hosts.* One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

A wide variety of methods are available for introducing a gene encoding a desired protein  
15 into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

Vectors comprising a *DSM-2* polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E.*  
20 *coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc.* Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then  
25 harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of  
30 inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border,

but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley *et al.* (1986); and An *et al.* (1985).

5           A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted  
10           has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred  
15           into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the  
20           T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a  
25           suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

          The transformed cells grow inside the plants in the usual manner. They can form germ  
30           cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the

normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said  
5 transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin,  
10 hygromycin, or chloramphenicol, *inter alia*. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate, (*PAT*), glyphosate (*EPSPS*), imazethyapyr (*AHAS*), and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell.  
15 Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include  
20 the introduction of genetic material coated onto microparticles directly into cells (U.S. Patent Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Patent Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S.  
25 Patent Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Patent No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Patent No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Patent Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Patent Nos.  
30 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. *See* U.S. Patent Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta.

Other direct DNA delivery transformation technology includes aerosol beam technology. *See* U.S. Patent No. 6,809,232. Electroporation technology has also been used to transform plants. *See* WO 87/06614 to Boyce Thompson Institute; U.S. Patent Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Patent No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Patent Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta *et al.* (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker

employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

In addition to a selectable marker, it may be desirable to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation

elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

*Selection agents.* In addition to glufosinate and bialaphos, selection agents that can be used according to the subject invention include all synthetic and natural analogs that may be inactivated by the acetyl transferase mechanism mediated by a *DSM-2* gene of the subject invention. See *e.g.* Figure 1.

Unless specifically indicated or implied, the terms “a”, “an”, and “the” signify “at least one” as used herein.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

#### EXAMPLE 1 – METHOD FOR IDENTIFYING GENES THAT IMPART RESISTANCE TO GLUFOSINATE *IN PLANTA*

As a way to identify genes which possess herbicide degrading activities *in planta*, or cell culture, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (*i.e.*,

phosphinothricin acetyltransferase). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only resulting sequences with  $\leq 50\%$  homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 30% amino acid conservation (relative to *pat* from *Streptomyces hygroscopicus*) can be used to select transformed plant cell cultures from untransformed.

*DSM-2* was identified from the NCBI database (*see* the [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov) website; accession #AAA26705) as a homologue with only 30% amino acid identity to *pat* and 28% to *bar*. Percent identity was determined by first translating the nucleotide sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

## EXAMPLE 2 – OPTIMIZATION OF SEQUENCE FOR EXPRESSION IN PLANTS AND BACTERIA

### 2.1 – Background.

To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression. See *e.g.* Kawabe *et al.* (2003), “Patterns of Codon Usage Bias in Three Dicot and Four Monocot Plant Species,” *Genes Genet. Syst.*, pp.343-352; and Ikemura *et al.* (1993), “Plant Molecular Biology Labfax”, Croy, ed., Bios Scientific Publishers Ltd., p. 3748), and all relevant references cited therein.

One reason for the reengineering of a bacterial protein for expression in maize, for example, is due to the non-optimal G+C content of the native gene. For example, the very low

G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.*, TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.*, polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

**Table 3** illustrates how high the G+C content is in maize. For the data in **Table 3**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (Accelrys, San Diego, California). Intron sequences were ignored in the calculations.

| <b>Table 3: Compilation of G + C contents of protein coding regions of maize genes</b> |               |                           |
|----------------------------------------------------------------------------------------|---------------|---------------------------|
| Protein Class <sup>a</sup>                                                             | Range % G + C | Mean % G + C <sup>b</sup> |
| Metabolic Enzymes (76)                                                                 | 44.4-75.3     | 59.0 (±.8.0)              |
| Structural Proteins (18)                                                               | 48.6-70.5     | 63.6 (±.6.7)              |
| Regulatory Proteins (5)                                                                | 57.2-68.8     | 62.0 (±.4.9)              |
| Uncharacterized Proteins (9)                                                           | 41.5-70.3     | 64.3 (±.7.2)              |
| All Proteins (108)                                                                     | 44.4-75.3     | 60.8 (±.5.2) <sup>c</sup> |

<sup>a</sup> Number of genes in class given in parentheses.

<sup>b</sup> Standard deviations given in parentheses.

<sup>c</sup> Combined groups mean ignored in mean calculation

Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This “codon bias” is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the

third position. It is thought that the presence of “minor” codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

In engineering genes encoding a bacterial protein for expression in maize (corn, or other plants, such as cotton, soybeans, wheat, *Brassica*/canola, rice; or more generally for oil crops, monocots, dicots, and hemicot/plants in general), the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 4**. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, *etc.* with the next preferred codon of choice.

**Table 4.** Preferred amino acid codons for proteins expressed in maize

| Amino Acid | Codon*  |
|------------|---------|
| Alanine    | GCC/GCG |
| Cysteine   | TGC/TGT |

|               |         |
|---------------|---------|
| Aspartic Acid | GAC/GAT |
| Glutamic Acid | GAG/GAA |
| Phenylalanine | TTC/TTT |
| Glycine       | GGC/GGG |
| Histidine     | CAC/CAT |
| Isoleucine    | ATC/ATT |
| Lysine        | AAG/AAA |
| Leucine       | CTG/CTC |
| Methionine    | ATG     |
| Asparagine    | AAC/AAT |
| Proline       | CCG/CCA |
| Glutamine     | CAG/CAA |
| Arginine      | AGG/CGC |
| Serine        | AGC/TCC |
| Threonine     | ACC/ACG |
| Valine        | GTG/GTC |
| Tryptophan    | TGG     |
| Tyrosine      | TAC/TAT |
| Stop          | TGA/TAG |

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It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

## 2.2 – DSM-2 Plant rebuild analysis.

Extensive analysis of the 513 base pairs (bp) of the DNA sequence of the native *DSM-2* coding region (SEQ ID NO:1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO:1 is presented as SEQ ID NO:2. To improve production of the recombinant protein in monocots as well as dicots, a “plant-optimized” DNA sequence *DSM-2 v2* (SEQ ID NO:3) was developed that encodes a protein which is identical to the native sequence disclosed as SEQ ID NO:2. In contrast, the native and plant-optimized DNA sequences of the coding regions are only 78.3% identical. **Table 5** shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences (Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

| <b>Table 5.</b> Codon composition comparisons of coding regions of Native <i>DSM-2</i> , Plant-Optimized version (v2) and a Theoretical Plant-Optimized version. |       |          |                |                     |            |       |          |                |                     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|----------|----------------|---------------------|------------|-------|----------|----------------|---------------------|
|                                                                                                                                                                  |       | A        | B              | C                   |            |       | D        | E              | F                   |
| Amino Acid                                                                                                                                                       | Codon | Native # | Plant Opt v2 # | Theor. Plant Opt. # | Amino Acid | Codon | Native # | Plant Opt v2 # | Theor. Plant Opt. # |
| ALA (A)                                                                                                                                                          | GCA   | 0        | 5              | 5                   | LEU (L)    | CTA   | 0        | 0              | 0                   |
|                                                                                                                                                                  | GCC   | 14       | 7              | 7                   |            | CTC   | 8        | 5              | 5                   |
|                                                                                                                                                                  | GCG   | 6        | 0              | 0                   |            | CTG   | 6        | 0              | 0                   |
|                                                                                                                                                                  | GCT   | 0        | 8              | 8                   |            | CTT   | 0        | 5              | 5                   |
| ARG (R)                                                                                                                                                          | AGA   | 2        | 4              | 4                   |            | TTA   | 0        | 0              | 0                   |
|                                                                                                                                                                  | AGG   | 1        | 5              | 5                   |            | TTG   | 0        | 4              | 4                   |
|                                                                                                                                                                  | CGA   | 1        | 0              | 0                   | LYS (K)    | AAA   | 0        | 1              | 1                   |
|                                                                                                                                                                  | CGC   | 8        | 3              | 3                   |            | AAG   | 3        | 2              | 2                   |
|                                                                                                                                                                  | CGG   | 3        | 0              | 0                   | MET (M)    | ATG   | 1        | 1              | 1                   |
|                                                                                                                                                                  | CGT   | 1        | 4              | 4                   | PHE (F)    | TTC   | 6        | 2              | 4                   |
| ASN (N)                                                                                                                                                          | AAC   | 2        | 1              | 1                   |            | TTT   | 0        | 4              | 2                   |
|                                                                                                                                                                  | AAT   | 0        | 1              | 1                   | PRO (P)    | CCA   | 1        | 6              | 6                   |
| ASP (D)                                                                                                                                                          | GAC   | 7        | 4              | 4                   |            | CCC   | 4        | 3              | 3                   |
|                                                                                                                                                                  | GAT   | 1        | 4              | 4                   |            | CCG   | 8        | 0              | 0                   |
| CYS (C)                                                                                                                                                          | TGC   | 0        | 0              | 0                   |            | CCT   | 1        | 5              | 5                   |
|                                                                                                                                                                  | TGT   | 0        | 0              | 0                   | SER (S)    | AGC   | 1        | 2              | 2                   |
| END                                                                                                                                                              | TAA   | 0        | 0              | 0                   |            | AGT   | 0        | 0              | 0                   |
|                                                                                                                                                                  | TAG   | 1        | 0              | 0                   |            | TCA   | 1        | 2              | 2                   |
|                                                                                                                                                                  | TGA   | 0        | 1              | 1                   |            | TCC   | 4        | 2              | 2                   |
| GLN (Q)                                                                                                                                                          | CAA   | 0        | 2              | 1                   |            | TCG   | 2        | 0              | 0                   |
|                                                                                                                                                                  | CAG   | 3        | 1              | 2                   |            | TCT   | 0        | 2              | 2                   |
| GLU (E)                                                                                                                                                          | GAA   | 1        | 6              | 6                   | THR (T)    | ACA   | 1        | 2              | 3                   |
|                                                                                                                                                                  | GAG   | 14       | 9              | 9                   |            | ACC   | 5        | 5              | 5                   |
| GLY (G)                                                                                                                                                          | GGA   | 2        | 4              | 4                   |            | ACG   | 4        | 0              | 0                   |
|                                                                                                                                                                  | GGC   | 9        | 4              | 4                   |            | ACT   | 2        | 5              | 4                   |
|                                                                                                                                                                  | GGG   | 2        | 2              | 2                   | TRP (W)    | TGG   | 3        | 3              | 3                   |
|                                                                                                                                                                  | GGT   | 1        | 4              | 4                   | TYR (Y)    | TAC   | 12       | 7              | 8                   |

| <b>Table 5.</b> Codon composition comparisons of coding regions of Native <i>DSM-2</i> , Plant-Optimized version (v2) and a Theoretical Plant-Optimized version. |               |          |                |                     |            |               |          |                |                     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|----------|----------------|---------------------|------------|---------------|----------|----------------|---------------------|
|                                                                                                                                                                  |               | A        | B              | C                   |            |               | D        | E              | F                   |
| Amino Acid                                                                                                                                                       | Codon         | Native # | Plant Opt v2 # | Theor. Plant Opt. # | Amino Acid | Codon         | Native # | Plant Opt v2 # | Theor. Plant Opt. # |
| HIS (H)                                                                                                                                                          | CAC           | 5        | 3              | 3                   | VAL (V)    | TAT           | 0        | 5              | 4                   |
|                                                                                                                                                                  | CAT           | 0        | 2              | 2                   |            | GTA           | 1        | 0              | 0                   |
| ILE (I)                                                                                                                                                          | ATA           | 0        | 1              | 1                   |            | GTC           | 5        | 3              | 3                   |
|                                                                                                                                                                  | ATC           | 4        | 2              | 2                   |            | GTG           | 4        | 4              | 4                   |
|                                                                                                                                                                  | ATT           | 0        | 1              | 1                   |            | GTT           | 1        | 4              | 4                   |
|                                                                                                                                                                  | <b>Totals</b> | 88       | 88             | 88                  |            | <b>Totals</b> | 84       | 84             | 84                  |

It is clear from examination of Table 5 that the native and plant-optimized coding regions, while encoding identical proteins, are substantially different from one another. The plant-optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the DSM-2 protein.

### EXAMPLE 3 – CLONING OF TRANSFORMATION VECTORS

#### 3.1 – Construction of Binary Plasmids Containing *DSM-2* (v2)

The *DSM-2* (v2) codon optimized gene coding sequence (DASPICO45) was cut with the restriction enzymes BbsI (New England Biolabs, Inc., Beverly MA, cat #R0539s) and SacI (New England Biolabs, Inc., cat #R0156s). The resulting fragment was ligated into pDAB773 at the corresponding restriction sites, NcoI (New England Biolabs, cat #R0193s) and SacI. Positive colonies were identified via restriction enzyme digestion. The resulting clones contained the Rb7 MAR v3 // At Ubi10 promoter v2 // gene of interest // Atu Orf 1 3'UTR v3. The plasmid that contained *DSM-2* (v2) as the gene of interest were labeled as pDAB3774.

The Rb7 MAR v3 // AtUbi10 promoter v2 // gene of interest // Atu Orf1 3'UTR v3 cassette was cloned into the binary vector pDAB3736 as an AgeI (New England Biolabs, Inc., cat #R0552s) restriction fragment. This cassette was cloned between the Left Hand and Right Hand Borders of the binary plasmid. Positive colonies were identified via restriction enzyme digestion and sequencing reactions. The constructs containing Rb7 MAR v3 // AtUbi10 promoter v2 // *DSM-2* v2 // Atu Orf1 3'UTR v3 were labeled as pDAB3778.

A control construct containing the Rb7 MAR v3 // AtUbi10 promoter v2 // *PAT* v3 // Atu Orf1 3' UTR v3 cassette was completed by removing the GateWay attR destination cassette from pDAB3736. pDAB3736 was digested with the PacI (New England Biolabs, Inc., cat #R0547s)

restriction enzyme. PacI flanks the GateWay attR destination cassette in pDAB3736. The PacI digested plasmid was self ligated and transformed into Escherichia coli Top10 cells (Invitrogen, Carlsbad CA, cat# C4040-10). Positive colonies were identified via restriction enzyme digestion and sequencing reactions. The resulting construct was labeled as pDAB3779.

5           3.2 – *Cloning of additional transformation constructs.*

All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular cloning methods (Maniatis *et al.*, 1982). **Table 6** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

| Table 6. Constructs used in transformation of various plant species. |        |                                  |                              |          |           |               |       |          |                             |                                  |                            |          |             |
|----------------------------------------------------------------------|--------|----------------------------------|------------------------------|----------|-----------|---------------|-------|----------|-----------------------------|----------------------------------|----------------------------|----------|-------------|
| pDAB #                                                               | pDAS # | Species *<br>Transformed<br>into | Gene of<br>interest<br>(GOI) | Promoter | Feature 1 | Feature 2     | GOI 2 | Promoter | Bacterial<br>Selection gene | Bacterial<br>Selection<br>gene 2 | Plant<br>Selection<br>gene | Promoter | Trxn Method |
| 3247                                                                 |        | A                                | DSM2 (v2)                    | CsVMV    | NtOsm     | RB7 Mar<br>v2 | -     | -        | Spectinomycin               | -                                | AAD12 (v1)                 | AtUbi10  | Agro binary |
| 3250                                                                 | 1941   | R, Cn                            | DSM2 (v2)                    | ZmUbi1   | NtOsm     | RB7 Mar<br>v2 | -     | -        | Spectinomycin               | -                                | -                          | -        | Whiskers    |
| 3251                                                                 | 1942   | R, Cn                            | PAT                          | ZmUbi1   | NtOsm     | RB7 Mar<br>v2 | -     | -        | Spectinomycin               | -                                | -                          | -        | Whiskers    |
| 3778                                                                 | 1861   | T                                | DSM2 (v2)                    | AtUbi10  | -         | RB7 Mar<br>v2 | -     | -        | Spectinomycin               | -                                | -                          | -        | Agro binary |
| 3779                                                                 | 1862   | T                                | PAT                          | AtUbi10  | -         | RB7 Mar<br>v2 | -     | -        | Spectinomycin               | -                                | -                          | -        | Agro binary |
| 9802                                                                 |        | S                                | DSM2 (v2)                    | CsVMV    |           | RB7 Mar<br>v2 |       |          | Spectinomycin               |                                  | AAD12 (v1)                 | CsVMV    | Agro binary |
| 9811                                                                 |        | S                                | DSM2 (v2)                    | CsVMV    |           | RB7 Mar<br>v2 |       |          | Spectinomycin               |                                  |                            |          | Agro binary |
| 9812                                                                 |        | S                                | DSM2 (v2)                    | CsVMV    |           |               |       |          | Spectinomycin               |                                  | AAD12 (v1)                 | AtUbi10  | Agro binary |
| 7602                                                                 |        | T                                | Cry gene                     | CsVMV    | NtOsm     | RB7 Mar<br>v2 | -     | -        | Spectinomycin               |                                  | DSM2 (v2)                  | AtUbi10  | Agro binary |
| 7604                                                                 |        | T                                | Cry gene                     | CsVMV    | NtOsm     | RB7 Mar<br>v2 | -     | -        | Spectinomycin               |                                  | DSM2 (v2)                  | AtUbi10  | Agro binary |
| 7606                                                                 |        | T                                | Cry gene                     | CsVMV    | NtOsm     | RB7 Mar<br>v2 | -     | -        | Spectinomycin               |                                  | DSM2 (v2)                  | AtUbi10  | Agro binary |
| 9104                                                                 |        | Cn                               | DSM2 (v2)                    | OsAct    |           |               | GUS   | ZmUbi1   | Spectinomycin               |                                  |                            |          | Agro binary |
| 9303                                                                 |        | Ca                               | DSM2 (v2)                    | CsVMV    |           |               | GUS   | AtUbi10  | Spectinomycin               |                                  |                            |          | Agro binary |

\*A = *Arabidopsis*

CsVMV = Cassava Vein Mosaic Virus Promoter

ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter

ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter

CsVMV = Cassava Vein Mosaic Virus Promoter

AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 PromoterRB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)NtOsm = *Nicotiana tabacum* Osmotin 5' and 3' Untranslated Regions

OsAct = Rice Actin Promoter

Cry gene = insect resistance gene

\* A = *Arabidopsis*

T = Tobacco

R = Rice

Cn = Corn

S = Soybean

Ca = Canola

EXAMPLE 4 – COMPLEMENTATION OF SENSITIVE E. COLI (BL-21) WITH *DSM-2 (V2)* USING PROKARYOTIC AND EUKARYOTIC PROMOTERS.

*4.1 – Construction of Escherichia coli Expression Plasmid Containing DSM-2 (v2)*

The *DSM-2 (v2)* codon optimized sequence was digested with the restriction enzymes BbsI and SacI. The resulting fragment was cloned into pDAB779 at the corresponding restriction sites of NcoI and SacI. pDAB779 is a pET28a(+) expression vector (Novagen, Madison WI, cat# 69864-3). Positive colonies containing the *DSM-2 (v2)* gene coding sequence were identified via restriction enzyme digestion. The *DSM-2* // pET28a(+) constructs were labeled as pDAB4412.

The expression plasmids pET (empty vector control), and pDAB4412 were transformed into the E. coli T7 expression strain BL21 – Star (DE3) (Invitrogen, Carlsbad CA, cat# C6010-03) using standard methods. Expression cultures were initiated with 10-200 freshly transformed colonies into 250 mL LB medium containing 50 µg/ml antibiotic and 75 µM IPTG (isopropyl- $\alpha$ -D-thiogalactopyranoside). The cultures were grown at 28 °C for 24 hours at 180-200 rpm. The cells were collected by centrifugation in 250 ml Nalgene bottles at 3,400 x g for 10 minutes at 4 C. The pellets were suspended in 4-4.5 mL Butterfield's Phosphate solution (Hardy Diagnostics, Santa Maria, CA; 0.3 mM potassium phosphate pH 7.2). The suspended cells were transferred to 50 mL polypropylene screw cap centrifuge tubes with 1 mL of 0.1 mm diameter glass beads (Biospec, Bartlesville, OK, catalog number 1107901). The cell-glass bead mixture was chilled on ice, then the cells were lysed by sonication with two 45 second bursts using a 2 mm probe with a Branson Sonifier 250 (Danbury CT) at an output of ~20, chilling completely between bursts. The lysates were transferred to 2 mL Eppendorf tubes and centrifuged 5 minutes at 16,000 x g. The supernatants were collected and the protein concentration measured. Bio-Rad Protein Dye Assay Reagent was diluted 1:5 with H<sub>2</sub>O and 1 mL was added to 10 µL of a 1:10 dilution of each sample and to bovine serum albumin (BSA) at concentrations of 5, 10, 15, 20 and 25 µg/mL. The samples were read on a spectrophotometer measuring the optical density at the wavelength of 595 nm in the Shimadzu UV160U spectrophotometer (Kyoto, JP). The amount of protein contained in each sample was calculated against the BSA standard curve and adjusted to between 3-6 mg/mL with phosphate buffer. Lysates were run on a SDS protein gel to visualize expressed protein.

#### 4.2 – Evaluation of common cloning strains for sensitivity to BASTA

Selected cell lines of *Escherichia coli* and *Agrobacterium tumefaciens* were inoculated on minimal media containing incrementally increasing concentrations of Glufosinate (BASTA). The cell lines; BL21 – Star (DE3), Top10, DH5 $\alpha$ , *Agrobacterium tumefaciens* C58, and *Agrobacterium tumefaciens* LBA4404s were initially grown up on complex media. The *Escherichia coli* strains were grown in LB and the *Agrobacterium tumefaciens* strains were grown in YEP. Five microliters of bacterial culture was inoculated and dispersed evenly onto minimal media plates containing various concentrations of glufosinate. The concentrations consisted of 0  $\mu\text{g/ml}$ , 250  $\mu\text{g/ml}$ , 500  $\mu\text{g/ml}$ , 1000  $\mu\text{g/ml}$ , 2000  $\mu\text{g/ml}$ , and 4000  $\mu\text{g/ml}$  of BASTA. In addition, the bacterial strains were inoculated onto a plate of complex media as a control – *Agrobacterium tumefaciens* strains were inoculated on YEP agar plates, and *Escherichia coli* strains were inoculated on LB agar plates. The plates containing the *Escherichia coli* strains were incubated at 37 °C for 24 hours. The plates containing the *Agrobacterium tumefaciens* strains were incubate at 25 °C for 48 hours. After the allotted incubation time the plates were observed for bacterial growth. **Table 7** illustrates the capability of the various strains to grow on minimal media containing glufosinate. Only one strain BL21 – Star (DE3) cell line was substantially inhibited by glufosinate.

| <b>Table 7: Variable bacterial strain response to glufosinate grown on minimal media</b> |                      |                   |                                                  |                                                  |                                                   |                                                   |                                                   |
|------------------------------------------------------------------------------------------|----------------------|-------------------|--------------------------------------------------|--------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
|                                                                                          | <b>Complex Cntrl</b> | <b>Min. Cntrl</b> | <b>Min. 250<math>\mu\text{g/ml}</math> BASTA</b> | <b>Min. 500<math>\mu\text{g/ml}</math> BASTA</b> | <b>Min. 1000<math>\mu\text{g/ml}</math> BASTA</b> | <b>Min. 2000<math>\mu\text{g/ml}</math> BASTA</b> | <b>Min. 4000<math>\mu\text{g/ml}</math> BASTA</b> |
| <b>E.coli –star BL21 (DE3)</b>                                                           | +++                  | +++               | --                                               | --                                               | --                                                | --                                                | --                                                |
| <b>E.coli DH5<math>\alpha</math></b>                                                     | +++                  | +++               | +++                                              | +++                                              | +++                                               | +++                                               | --                                                |
| <b>E.coli Top10</b>                                                                      | +++                  | +++               | +++                                              | +++                                              | +++                                               | +                                                 | --                                                |
| <b>Agrobacterium C58</b>                                                                 | +++                  | +++               | +++                                              | +++                                              | +++                                               | ++                                                | --                                                |
| <b>Agrobacterium LBA4404s</b>                                                            | +++                  | +++               | +++                                              | ++                                               | ++                                                | +                                                 | --                                                |

E.coli was grown for 24 hrs @ 37C. Agrobacterium was grown for 48hrs @ 25C. +++ = Heavy Lawn Growth // ++ = Light lawn growth // + = patchy lawn growth // -- = No Growth

#### 4.3 – Recombinant Expression of *DSM-2* (v2) to complement cell growth of *Escherichia coli* BL21 – Star (DE3)

A pET28a(+) expression plasmid containing *PAT* (v3) was constructed as a positive control. *PAT* (v3) was cloned as an NcoI – SacI fragment into corresponding restriction sites of pDAB779. Positive clones containing the *PAT* (v3) gene fragment were verified via restriction enzyme digestion. This construct was labeled as pDAB4434.

The plasmids, pDAB4434, pDAB4412, and an empty pET vector (control) were transformed into *Escherichia coli* BL21 – Star (DE3) bacterial cells. Expression cultures were initiated with 10-200 freshly transformed colonies into 250 mL LB medium containing 50 µg/ml antibiotic and 75 µM IPTG (isopropyl- $\alpha$ -D-thiogalactopyranoside). The cultures were grown at 28 °C for 24 hours at 180-200 rpm. Five microliters of the culture was inoculated onto a complex media control and minimal media containing incrementally increasing concentrations of glufosinate and 20 µM IPTG. The cultures were dispersed evenly over the plates and incubated at 28 °C for 24 hours. After the allotted incubation time the plates were observed for bacterial growth. These results are illustrated in **Table 8**.

| <b>Table 8:</b> |                      |                   |                            |                            |                             |                             |                             |
|-----------------|----------------------|-------------------|----------------------------|----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                 | <b>Complex Cntrl</b> | <b>Min. Cntrl</b> | <b>Min. 250µg/ml BASTA</b> | <b>Min. 500µg/ml BASTA</b> | <b>Min. 1000µg/ml BASTA</b> | <b>Min. 2000µg/ml BASTA</b> | <b>Min. 4000µg/ml BASTA</b> |
| <b>Cntrl</b>    | +++                  | +++               | --                         | --                         | --                          | --                          | --                          |
| <b>PAT</b>      | +++                  | +++               | +++                        | +++                        | +++                         | +++                         | +++                         |
| <b>DSM2</b>     | +++                  | +++               | +++                        | +++                        | +++                         | +++                         | +++                         |

*E.coli* was grown for 24 hrs @28C on media with 20uM IPTG. -- = No Growth // +++ = Distinct Colony Growth

#### 4.4 – Use of plant promoters to drive the recombinant expression of *DSM-2* in *Escherichia coli* BL21 – Star (DE3) Cells

Plasmid constructs in which the *DSM-2* (v2) and *PAT* gene coding sequences were expressed, under either the viral promoters CsVMV or the plant promoter AtUbi10, were assayed for complementation on minimal media containing glufosinate. The plasmids 3778 (Rb7 MARv3 // AtUbi10 promoter // *DSM-2* (v2) // Atu Orf 1 3'UTR), 3779 (Rb7 MARv3 // AtUbi10 promoter // *PAT* // Atu Orf 1 3'UTR), 3264 (CsVMV promoter // *DSM-2* // Atu Orf 24 3'UTR), 3037 (CsVMV promoter // *PAT* // Atu Orf 25/26 3'UTR), and 770 (control plasmid containing CsVMV promoter // GUS v3// Atu Orf 24 3'UTR) were transformed into *Escherichia coli*

BL21 – Star (DE3) and grown up in complex media. Five microliters of the cultures were plated on minimal media containing increasing concentrations of Glufosinate and incubated at 37 °C for 48 hours. The results are illustrated in **Table 9**. These data indicate that plant and viral promoters have moderate levels of functionality within bacterial cells and may be used to drive the expression of a selectable marker.

| <b>Table 9</b>              |                        |                       |                                    |                                    |                                     |                                     |                                     |
|-----------------------------|------------------------|-----------------------|------------------------------------|------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
|                             |                        | <b>Min.<br/>Cntrl</b> | <b>Min.<br/>250µg/ml<br/>BASTA</b> | <b>Min.<br/>500µg/ml<br/>BASTA</b> | <b>Min.<br/>1000µg/ml<br/>BASTA</b> | <b>Min.<br/>2000µg/ml<br/>BASTA</b> | <b>Min.<br/>4000µg/ml<br/>BASTA</b> |
| <b>AtUbi10<br/>Promoter</b> | <b>DSM-2<br/>-3778</b> | ++++                  | ++++                               | +++                                | +++                                 | ++                                  | ++                                  |
|                             | <b>PAT -<br/>3779</b>  | ++++                  | ++++                               | +++                                | +++                                 | +++                                 | +++                                 |
|                             | <b>AHAS<br/>- 4433</b> | ++++                  | (+)                                | --                                 | --                                  | --                                  | --                                  |
| <b>CsVMV<br/>Promoter</b>   | <b>DSM-2<br/>3264</b>  | ++++                  | ++                                 | +                                  | --                                  | --                                  | --                                  |
|                             | <b>PAT-<br/>3037</b>   | ++++                  | ++                                 | +                                  | --                                  | --                                  | --                                  |
|                             | <b>GUS-<br/>770</b>    | ++++                  | (+)                                | --                                 | --                                  | --                                  | --                                  |
|                             | <b>BL21<br/>Cntrl</b>  | ++++                  | (+)                                | --                                 | --                                  | --                                  | --                                  |

*E.coli* was grown for 48hrs @37C. +++++ = Heavy Lawn Growth; +++ = Lawn Growth; ++ = Lots of Distinct Colonies; + = Scattered Colony Growth.

#### EXAMPLE 5 – PURIFICATION OF DSM-2 FOR BIOCHEMICAL CHARACTERIZATION AND ANTIBODY PRODUCTION FOR WESTERN ANALYSES

##### 5.1 – Recombinant expression.

*E. coli* BL-21 (DE3) Star cells (purchased from Invitrogen, Carlsbad, CA) harboring codon-optimized *DSM-2* (v2) gene, in plasmid pDAB4412 was used to inoculate a 3 ml LB media supplemented with 50 µg/ml Kanamycin at 37 °C overnight for seed preparation. Approximately 2 ml of seed culture was transferred into a 1 L fresh LB containing Kanamycin (50 µg/ml) in a 2.8 L baffled Erlenmeyer flask. The cultures were incubated at 37 °C on a shaker (New Brunswick Scientific, Model Innova 44) at 250 RPM for approximately 6 hrs to obtain OD<sub>600</sub> close to 0.8-1.0. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to final 75 µM in the cultures and continued to incubate at 18 °C for overnight induction. Cells were harvested by centrifugation at

8,000 RPM at 4 °C for 15 min, and cell paste was stored at -80 °C or immediately processed for purification.

Approximately 5 g of wet weight *E. coli* cells from 1 L culture were thawed and resuspended in 300 ml of extraction buffer containing 20 mM Tris-HCl, pH 8.0 and 0.3 ml of  
5 Protease Inhibitor Cocktail (Sigma, cat# P8465), and disrupted on ice for 15 minutes by sonication. The lysate was centrifuged at 4 °C at 24,000 RPM for 20 min, and the supernatant was filtered through 0.8 µm and 0.45 µm membrane. All subsequent protein separations were performed using Pharmacia AKTA Explorer 100 and operated at 4 °C. The filtrate was applied at 10 ml/min to a QXL Sepharose Fast Flow column (Pharmacia HiPrep 16/10, 20 ml bed size)  
10 equilibrated with 20 mM Tris-HCl, pH 8.0 buffer. The column was washed with this buffer until the eluate OD<sub>280</sub> returned to baseline, proteins were eluted with 0.5 L of linear gradient from 0 to 0.4 M NaCl at a flow rate of 5 ml/min, while 5 ml fractions were collected. Fractions containing DSM-2 as determined by SDS-PAGE with apparent 20 kDa band (the predicted DSM-2 molecular weight is 19.3 kDa), also corresponding to the Glufosinate converting activity were  
15 pooled. The sample was diluted with 4 volumes of 20 mM Tris-HCl, pH 7.5 buffer contains 5 mM DTT, 0.5% Triton X-100, 5% glycerol, and re-applied to a Mono Q column (Pharmacia 10/100 GL, 8 ml bed size) at 4 ml/min. Proteins were eluted with 0.1-0.3 M NaCl gradient in the same buffer. A major peak containing DSM-2 was pooled, and solid ammonium sulfate was added to final 1.0 M, and applied to a Phenyl Fast Flow column (Pharmacia HiTrap, 5 ml bed size)  
20 equilibrated in 1.0 M ammonium sulfate in 20 mM Tris-HCl, pH 7.5. This column was washed with the equilibrating buffer at 4 ml/min until the OD<sub>280</sub> of the eluate returned to baseline, then proteins were eluted within 50 min (3 ml/min) by a linear gradient from 1.0 M to 0 Ammonium sulfate in 20 mM Tris-HCl, pH 7.5, and 3 ml fractions were collected. The main peak fractions contain DSM-2 eluted at 75 mS/cm was pooled, and concentrated to approximately 3 mg/ml using  
25 MWCO 10 kDa membrane centrifugal filter device (Millipore). The sample was then applied to a Superdex 75 gel filtration column (Pharmacia XK 16/60, 110 ml bed size) with PBS buffer at a flow rate of 1 ml/min. Peak fractions containing pure DSM-2 were pooled and stored at -80 °C. Protein concentration was determined by Bradford assay or total amino acid analysis using bovine serum albumin as standard. Activity of purified DSM-2 was measured based on a standard  
30 procedure for phosphinothricin acetyltransferase (PAT) assay. (Wehrmann *et al.* 1996)

### 5.2 – Antibody Production

Rabbit polyclonal antibody against DSM-2 was produced using the Rabbit Polyclonal Antibody - Standard Protocols provided by Invitrogen Antibody Services (South San Francisco, CA, Cat# M0300). E. coli-expressed and purified DSM-2 (see previous section) was supplied as immunogen. Briefly, two New Zealand rabbits were injected subcutaneously (SQ) with 1 mg of DSM-2 protein emulsified with 0.25 mg Keyhole Limpet Hemocyanin and Incomplete Freund's Adjuvant (IFA). The rabbits were rested for 2 weeks and boosted SQ three times with 0.5 mg of DSM-2 protein emulsified in IFA with three weeks of rest period in between. Two weeks after the final boost, sera were collected from each rabbit and tested on direct ELISA for titer (data not shown). Two additional boosts and terminal bleed (Invitrogen Cat# M0311 and M0313) were conducted on rabbit number 2, which gave better titer on specific antibodies.

### 5.3 – Western Blotting Analysis

Approximately 100 mg of tobacco calli tissue was put into a 2 mL microfuge tube containing 3 stainless steel BB beads. 250  $\mu$ L of extraction buffer (phosphate buffered saline containing 0.1% Triton X-100, 10 mM DTT and 5  $\mu$ L per mL protease inhibitors cocktail) was added and the tubes were secured in the Geno/Grinder (Model 2000-115, Certiprep, Metuchen, NJ) and shaken for 6 min with setting at 1x of 500 rpm. Tubes were centrifuged at 10,000x g for 10 min and supernatant containing the soluble proteins was pipetted into separate tubes and stored in ice. The pellet was extracted a second time as described above and the supernatant was pooled with previous fraction and assayed.

Extracted proteins from plant samples were denatured in Laemmli Buffer and incubated at 95°C for 10 min. Denatured proteins were separated on Novex 8-16% Tris-Glycine pre-cast gels (Invitrogen Cat# EC60452BOX) according to manufacturer's protocol, followed by transferring onto nitrocellulose membrane using standard protocol.

All Western blotting incubation steps were conducted at room temperature for one hour. The blot was first blocked in PBS containing 4% milk (PBSM) and then incubated in DSM-2-specific rabbit polyclonal antibody (see previous paragraph) diluted 5000-fold in PBSM. After three 5-min washes in PBS containing 0.05% Tween-20 (PBST), goat anti-rabbit antibody/horseradish peroxidase conjugate was incubated on the blot. Detected proteins were visualized using chemiluminescent substrate (Pierce Biotechnology, Rockford, IL Cat# 32106) and exposure to X-ray film.

EXAMPLE 6 – TRANSFORMATION INTO *ARABIDOPSIS* AND SELECTION6.1 – *Arabidopsis thaliana* growth conditions.

Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150  $\mu\text{mol}/\text{m}^2\text{sec}$  under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2 – *Agrobacterium* transformation.

An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5 $\alpha$  colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50  $\mu\text{l}$  of competent Agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant agitation for 4

hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28 °C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed *Agrobacterium* cells, suspended in 10 µl of water, at 100 °C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C for 3 minutes, 2) 94° C for 45 seconds, 3) 55° C for 30 seconds, 4) 72° C for 1 minute, for 29 cycles then 1 cycle of 72° C for 10 minutes. The reaction was maintained at 4° C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

### 6.3 – *Arabidopsis* transformation.

*Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog salts/Gamborg's B5 vitamins,

10% (w/v) sucrose, 0.044 $\mu$ M benzylamino purine (10  $\mu$ l/liter of 1 mg/ml stock in DMSO) and 300  $\mu$ l/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

#### 6.4 – Selection of transformed plants.

Freshly harvested T<sub>1</sub> seed [*DSM-2 (v2)* gene] was allowed to dry for 7 days at room temperature. T<sub>1</sub> seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T<sub>1</sub> seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using 2,4-D postemergence spray (selecting for the co-transformed *AAD-12* gene; see USSN 60/731,044).

Seven days after planting (DAP) and again 11 DAP, T<sub>1</sub> plants (cotyledon and 2-4-leaf stage, respectively) were sprayed with a 0.016% solution of 2,4-D herbicide (456 g ae/L 2,4-D Amine 4, Dow AgroSciences LLC, Indianapolis, IN) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 50 g ae/ha 2,4-D DMA per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22 $\pm$ 5° C, 50 $\pm$ 30% RH, 14 h light;10 dark, minimum 500  $\mu$ E/m<sup>2</sup>s<sup>-1</sup> natural + supplemental light) at least 1 day prior to testing for the ability of *DSM-2 (v2)* to provide glufosinate herbicide resistance.

T<sub>1</sub> plants were then randomly assigned to various rates of glufosinate. For *Arabidopsis* 140 g ai/ha glufosinate is an effective dose to distinguish sensitive plants from ones with

meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (280, 560, or 1120g ai/ha). Table 10 shows comparisons drawn to an aryloxyalkanoate herbicide resistance gene (*AAD-12 v1*); see USSN 60/731,044.

All glufosinate herbicide applications were applied by track sprayer in a 187 L/ha spray volume. The commercial Liberty<sup>TM</sup> formulation (200 g ai/L, Bayer Crop Science, Research Triangle Park, NC). T<sub>1</sub> plants that exhibited tolerance to glufosinate were further accessed in the T<sub>2</sub> generation.

#### 6.5 – Results of selection of transformed plants.

The first *Arabidopsis* transformations were conducted using *DSM-2 (v2)* (plant optimized gene). T<sub>1</sub> transformants were first selected from the background of untransformed seed using a 2,4-D DMA selection scheme. Over 100,000 T<sub>1</sub> seed were screened and 260 2,4-D resistant plants (*AAD-12* gene) were identified, equating to a transformation/selection frequency of 0.26% which is slightly higher than the normal range of selection frequency of constructs where *AAD-12* + 2,4-D are used for selection. T<sub>1</sub> plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial glufosinate herbicide. **Table 10** compares the response of *DSM-2 (v2)* and control genes to impart glufosinate resistance to *Arabidopsis* T<sub>1</sub> transformants. Response is presented in terms of % visual injury 2 WAT. Data were presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T<sub>1</sub> is an independent transformation event, one can expect significant variation of individual T<sub>1</sub> responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. Untransformed-wildtype *Arabidopsis* served as a glufosinate sensitive control. The *DSM-2 (v2)* gene imparted herbicide resistance to individual T<sub>1</sub> *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at above 140 g ai/ha glufosinate, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 10** simply to demonstrate the significant difference between the plants transformed with *DSM-2 (v2)* versus the wildtype or *AAD-12* + *PAT*-transformed controls. Many *DSM-2 (v2)* individuals survived 1,120 g ai/ha glufosinate with little or no injury.

**Table 10.** T<sub>1</sub> *DSM-2 v2* (plant optimized)-transformed T<sub>1</sub> *Arabidopsis* response to a range of glufosinate rates applied postemergence compared to Wildtype and T<sub>1</sub> *AAD-12 + PAT* plants.

| <b>DSM-2 (v2) gene + AAD-12</b> |      |        |      | <b>% Injury</b> |         |
|---------------------------------|------|--------|------|-----------------|---------|
| Averages                        | <20% | 20-40% | >40% | Ave             | Std dev |
| 0 g ai/ha glufosinate           | 20   | 0      | 0    | 0.0             | 0.0     |
| 140 g ai/ha glufosinate         | 20   | 0      | 0    | 0.0             | 0.0     |
| 280 g ai/ha glufosinate         | 19   | 1      | 0    | 3.0             | 5.0     |
| 560 g ai/ha glufosinate         | 19   | 0      | 1    | 6.0             | 22.0    |
| 1120 g ai/ha glufosinate        | 17   | 1      | 2    | 13.0            | 19.0    |
| <b>Wildtype control</b>         |      |        |      | <b>% Injury</b> |         |
| Averages                        | <20% | 20-40% | >40% | Ave             | Std dev |
| 0 g ai/ha glufosinate           | 10   | 0      | 0    | 0.0             | 0.0     |
| 140 g ai/ha glufosinate         | 0    | 0      | 10   | 98.0            | 5.0     |
| 280 g ai/ha glufosinate         | 0    | 0      | 10   | 100.0           | 0.0     |
| 560 g ai/ha glufosinate         | 0    | 0      | 10   | 100.0           | 0.0     |
| 1120 g ai/ha glufosinate        | 0    | 0      | 10   | 100.0           | 0.0     |
| <b>PAT gene + AAD-12</b>        |      |        |      | <b>% Injury</b> |         |
| Averages                        | <20% | 20-40% | >40% | Ave             | Std dev |
| 0 g ai/ha glufosinate           | 10   | 0      | 0    | 0.0             | 0.0     |
| 140 g ai/ha glufosinate         | 10   | 0      | 0    | 0.0             | 0.0     |
| 280 g ai/ha glufosinate         | 10   | 0      | 0    | 0.0             | 0.0     |
| 560 g ai/ha glufosinate         | 10   | 0      | 0    | 0.0             | 0.0     |
| 1120 g ai/ha glufosinate        | 10   | 0      | 0    | 3.0             | 3.0     |

#### 6.6 – *DSM-2 (v2)* as a selectable marker.

The ability to use *DSM-2 (v2)* as a selectable marker using glufosinate as the selection agent was analyzed with *Arabidopsis* transformed as described above. Approximately 100 T<sub>1</sub> generation *Arabidopsis* seed (100-150 seeds) containing for *DSM-2 (v2)* or 2mg homozygous T<sub>5</sub> plants containing *PAT* were spiked into approximately 10,000 wildtype (sensitive) seed. Each tray of plants received two application timings of 280 g ai/ha glufosinate at the following treatment times: 7 DAP and 11 DAP. Treatments were applied with a DeVilbiss spray tip as previously described. Another 2mg T<sub>1</sub> generation *Arabidopsis* seed from each was sown and not sprayed as a comparison count. Plants were identified as Resistant or Sensitive 17 DAP. Counts between treated and untreated were shown to be similar in **Table 11**. These results indicate *DSM-2 (v2)* can effectively be used as an alternative selectable marker for a population.

**Table 11.** Mass of seed sown and count of the number of plants that survived following a treatment of 280 g ai/ha glufosinate.

| Trt | Mass WT<br>Planted | Mass <i>PAT</i><br>Planted | Mass <i>DSM-2</i> ( <i>v2</i> )<br>Planted | Number of Surviving<br>Plants Following<br>glufosinate Spray |
|-----|--------------------|----------------------------|--------------------------------------------|--------------------------------------------------------------|
| 1   | 200 mg             | 0 mg                       | 0 mg                                       | 0                                                            |
| 2   | 0 mg               | 2 mg                       | 0 mg                                       | 154                                                          |
| 3   | 200 mg             | 2 mg                       | 0 mg                                       | 121                                                          |
| 4   | 0 mg               | 0 mg                       | 2 mg                                       | 117                                                          |
| 5   | 200 mg             | 0 mg                       | 2 mg                                       | 121                                                          |

### 6.7 – Molecular Analysis:

#### 6.7.1 – Tissue harvesting DNA isolation and quantification.

Fresh tissue is placed into tubes and lyophilized at 4° C for 2 days. After the tissue is fully dried, a tungsten bead (Heavy Shot) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (Wavelength 485/530-BioTek) with known standards to obtain the concentration in ng/μl.

#### 6.7.2 – Invader assay analysis.

The DNA samples are diluted to 0.7 ng/μl then denatured by incubation in a thermocycler at 95° C for 10 minutes. The Invader assay reaction mix is then prepared by following the 96 well format procedure published by Third Wave Technologies. 7.5 μl of the prepared reaction mix is dispersed into each well of the a 96 well plate followed by an aliquot of 7.5 μl of controls and 0.7 ng/μl diluted, denatured unknown samples. Each well is overlaid with 15 μl of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe (FAM dye wavelength 560/620) divided by the % signal over background internal control probe (RED dye wavelength 485/530) will calculate the ratio. The ratio was then used to determine the event's zygosity.

### 6.8 – Heritability.

A variety of T<sub>1</sub> events were self-pollinated to produce T<sub>2</sub> seed. These seed were progeny tested by applying glufosinate (200 g ai/ha) to 100 random T<sub>2</sub> siblings. Each individual T<sub>2</sub> plant was transplanted to 3-inch square pots prior to spray application (track sprayer at 187 L/ha applications rate). Sixty-three percent of the T<sub>1</sub> families (T<sub>2</sub> plants) segregated in the anticipated 3

Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ( $P > 0.05$ ).

Invader for zygosity was performed on 16 randomly selected plants from each of the lines that segregated as a single locus. Seed were collected from homozygous invader determined  $T_2$  individuals ( $T_3$  seed). Twenty-five  $T_3$  siblings from each of 4 homozygous invader determined  $T_2$  families were progeny tested as previously described. All of the  $T_2$  families that were anticipated to be homozygous (non-segregating populations) were non-segregating. These data show *DSM-2* (*v2*) is stably integrated and inherited in a Mendelian fashion to at least three generations.

## EXAMPLE 7 – WHISKERS-MEDIATED TRANSFORMATION OF CORN USING HERBIACE

### 7.1 – Cloning of *DSM-2* (*v2*).

The *DSM-2* (*v2*) gene was cut out of the DASPICO45 vector as a Bbs1/Sac1 fragment. This was ligated directionally into the similarly cut pDAB3812 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5 $\alpha$  cells. Minipreps were performed on the resulting colonies using Qiagen's QIASpin mini prep kit, and the colonies were digested to check for orientation. The final construct was designated pDAB3250, which contains ZmUbi1/*DSM-2* (*v2*) / ZmPer5 3'UTR. An identical control vector containing the *PAT* gene was built as above. This construct was designated pDAB3251.

### 7.2 – Callus/suspension initiation.

To obtain immature embryos for callus culture initiation,  $F_1$  crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.*, 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L AgNO<sub>3</sub>, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was selectively transferred at bi-weekly

intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L  $\alpha$ -naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L *myo*-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

### 7.3 – Cryopreservation and thawing of suspensions.

Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C/minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until “boiling” subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium,

2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters  
5 PCV was subcultured into fresh H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on  
10 a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L *myo*-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and  
15 during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was  
20 drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml  
25 fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

Approximately 6 mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were  
30 cultured for 1 week at 28° C in a dark box.

After 1 week, 20 of the filter papers were transferred to 60x20 mm plates of GN6 (1 Herbiace), 20 of the filter papers were transferred to 60x20 mm plates of GN6 (2 Herbiace) and 20 of the filter papers were transferred to 60x20 mm plates of GN6 (4 Herbiace) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 1, 2, or 4 mg/L bialaphos (from Herbiace) and, 2.5 g/L Gelrite, pH 5.8) Plates were placed in boxes and cultured for an additional week.

After an additional week, all of the filter papers were transferred to the same concentrations of GN6 + Herbiace medium (1H, 2H and/or 4H) again. The plates were placed in boxes and cultured for an additional week.

Three weeks post-transformation, the tissue was embedded by scraping  $\frac{1}{2}$  of the cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C) containing either 1, 2, or 4 mg/L bialaphos from Herbiace. The tissue was broken up and the 3 mL of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (1H, 2H or 4H), depending on the concentration that the cells were originally cultured on. This was repeated with the other  $\frac{1}{2}$  of the cells on each plate. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for about 4 weeks at 28° C in dark boxes.

#### *7.4 – Protocol for Plant Regeneration.*

Putatively transformed isolates are typically first visible 5-8 weeks post-transformation. Any potential isolates are removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained growth is evident after approximately 2 weeks, an event is deemed to be resistant. A subset of the resistant events are then submitted for molecular analysis.

Regeneration is initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (1H), containing, (MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 1 mg/L bialaphos, 2.5 g/L Gelrite; pH 5.7,) Cells are allowed to grow in low light ( $13 \mu\text{Em}^{-2}\text{s}^{-1}$ ) for one week, then higher light ( $40 \mu\text{Em}^{-2}\text{s}^{-1}$ ) for another week, before being transferred to regeneration medium, 36 (1H), which is identical to 28 (1H) except that it lacks plant growth regulators. Small (3-5 cm) plantlets are removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972;

1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they are transplanted to soil in the greenhouse.

### *7.5 – Molecular Analysis: Maize Materials and Methods.*

#### *7.5.1 – Tissue harvesting DNA isolation and quantification.*

5            Fresh tissue is placed into tubes and lyophilized at 4° C for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to  
10           obtain the concentration in ng/μl.

#### *7.5.2 – PAT Invader assay analysis.*

              The DNA samples are diluted to 20 ng/μl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and MgCl<sub>2</sub> (Third Wave Technologies). An aliquot of 7.5 μl is placed in each well of the Invader  
15           assay plate followed by an aliquot of 7.5 μl of controls, standards, and 20 ng/μl diluted unknown samples. Each well is overlaid with 15 μl of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis  
20           is used to identify the estimated copy of the unknown events.

#### *7.5.3 – Polymerase chain reaction for PAT.*

              A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *PAT* PTU are (Forward MAS123 – GAACAGTTAGACATGGTCTAAAGG) (SEQ ID NO:5) and (Reverse  
25           Per5-4 – GCTGCAACACTGATAAATGCCAACTGG) (SEQ ID NO:6). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 62° C for 30 seconds, and 72° C for 3 minute and 15 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR *PAT* are (Forward – ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:7) and (Reverse – CGGGC  
30           AGGCCTAACTCCACCAA) (SEQ ID NO:8). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes

and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR for *DSM-2* are (Forward –ATGCCTGGAAGTCTGAGGTC) (SEQ ID NO:9) and (Reverse –TGAGCGATGCCAGCATAAGCT) (SEQ ID NO:10). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 45 seconds followed by 72° C for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

#### 7.5.4 – Southern blot analysis.

Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 5 µg of total genomic DNA is subjected to an overnight digestion with NcoI and SwaI to obtain integration data. A digestion of 5 µg with restriction enzyme SspI was used to obtain the PTU data. After analyzing the SspI digestion data, restriction enzyme MfeI was used to digest all of the remaining samples because it appeared to be a better choice in enzyme. After the overnight digestion an aliquot of ~100 ngs is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85 % agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 120,000 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment is prepared using coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization step at 60°C for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling reaction (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Hybridization buffer are used to hybridize the Southern blots overnight. After the overnight hybridization the blots are then

subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

### 7.6 – Results

Three whisker-mediated transformations of maize were performed with each of the 2 constructs (*PAT* and *DSM-2*) described earlier. From those collective experiments, 230 isolates were recovered. On media containing 1 and 2 mg/L bialaphos (from Herbiace), event recovery between *PAT* and *DSM-2* was very similar, however, event recovery on media containing 4 mg/L bialaphos was higher for *PAT* than for *DSM-2*.

**Table Ex. 7.6-1**

| Construct | Gene of Interest | Overall Events Recovered Per Bottle |            |            |
|-----------|------------------|-------------------------------------|------------|------------|
|           |                  | 1 Herbiace                          | 2 Herbiace | 4 Herbiace |
| pDAB3250  | DSM2             | 46                                  | 41         | 17         |
| pDAB3251  | PAT              | 44                                  | 37         | 45         |

Forty-eight of the *DSM-2* events selected upon 1 or 2 Herbiace were submitted for copy number analysis and presence of an intact plant transcription unit (PTU) via Southern blot. All 48 events contained at least one copy of the *DSM-2* gene. A subset of results from the lower-copy events (3 or less) are presented below.

**Table Ex. 7.6-2**

| Sample #    | Integration        | PTU                  |
|-------------|--------------------|----------------------|
|             | DSM2 Southern Blot | DSM2 Southern Blot   |
| 1941[1]-005 | 2                  | larger than expected |
| 1941[1]-006 | 3                  | yes                  |
| 1941[1]-008 | 1                  | yes                  |
| 1941[1]-009 | 2                  | yes                  |
| 1941[1]-021 | 2                  | yes                  |
| 1941[1]-023 | 2                  | yes                  |
| 1941[2]-012 | 2                  | yes                  |
| 1941[2]-014 | 2                  | yes                  |
| 1941[2]-016 | 3                  | yes                  |
| 1941[2]-017 | 2                  | yes                  |
| 1941[2]-029 | 2                  | yes                  |
| 1941[4]-038 | 1                  | yes                  |
| 1941[5]-044 | 2                  | yes                  |
| 1941[5]-045 | 2                  | yes                  |
| 1941[5]-047 | 3                  | yes                  |

Forty-eight of the *PAT* events selected upon 1, 2, or 4 Herbiace were submitted for copy number estimate and presence of an intact plant transcription unit (PTU) via Invader Assay and

PCR, respectively. All 48 events contained at least one copy of the PAT gene. A subset of results from the lower-copy events (3 or less) are presented below.

**Table Ex. 7.6-3**

| Plant ID    | Copy # | PTU PCR |
|-------------|--------|---------|
| 1942[1]-001 | 1      | +       |
| 1942[1]-007 | 3      | +       |
| 1942[2]-017 | 2      | +       |
| 1942[3]-018 | 1      | +       |
| 1942[3]-021 | 1      | +       |
| 1942[3]-022 | 1      | +       |
| 1942[3]-024 | 3      | +       |

5 Approximately 6-10 T<sub>0</sub> plants were regenerated from each of 15 DSM-2-containing events and 7-8 plants were regenerated from each of 7 PAT-containing events listed earlier in order to assess tolerance to Liberty.

10 Callus tissue samples from 31 different events together with an untransformed callus (negative control) were analyzed with Western Blotting experiment. All samples except the negative control had one band observed with relative molecular weight of 20 kDa to the marker. It agreed with the predicted size of the protein of 19.7 kDa. In addition, the band also has the same size as the standard, i.e. purified DSM-2 protein purified from *E. coli*. With 0.7 µg/mL of the standard on the gel and given an arbitrary score of 5 (+++++), the bands from different events were relatively graded and listed in the table below.

15

**Table Ex. 7.6-4**

| Count | Event #     | Total Grams | Western Detection |
|-------|-------------|-------------|-------------------|
| 1     | 1941[1]-018 | 0.090       | ++                |
| 2     | 1941[1]-019 | 0.070       | ++++              |
| 3     | 1941[1]-020 | 0.080       | ++                |
| 4     | 1941[1]-021 | 0.060       | ++                |
| 5     | 1941[1]-022 | 0.070       | ++++              |
| 6     | 1941[1]-023 | 0.050       | +                 |
| 7     | 1941[1]-024 | 0.080       | ++                |
| 8     | 1941[1]-025 | 0.050       | ++                |
| 9     | 1941[1]-026 | 0.070       | +                 |
| 10    | 1941[1]-027 | 0.060       | ++                |
| 11    | 1941[2]-028 | 0.080       | ++++              |
| 12    | 1941[2]-029 | 0.090       | +++               |
| 13    | 1941[2]-030 | 0.110       | ++                |
| 14    | 1941[2]-031 | 0.140       | +++               |
| 15    | 1941[2]-032 | 0.130       | +++               |

| Count | Event #     | Total Grams | Western Detection |
|-------|-------------|-------------|-------------------|
| 16    | 1941[2]-033 | 0.120       | +++               |
| 17    | 1941[2]-034 | 0.070       | +++               |
| 18    | 1941[2]-035 | 0.140       | +                 |
| 19    | 1941[4]-036 | 0.160       | +                 |
| 20    | 1941[4]-037 | 0.140       | ++                |
| 21    | 1941[4]-038 | 0.140       | +/-               |
| 22    | 1941[4]-039 | 0.130       | +                 |
| 23    | 1941[4]-040 | 0.100       | +/-               |
| 24    | 1941[4]-041 | 0.130       | ++                |
| 25    | 1941[4]-042 | 0.090       | +/-               |
| 26    | 1941[4]-043 | 0.110       | +                 |
| 27    | 1941[5]-044 | 0.090       | +++               |
| 28    | 1941[5]-045 | 0.090       | ++                |
| 29    | 1941[5]-046 | 0.100       | +++               |
| 30    | 1941[5]-047 | 0.090       | +++               |
| 31    | 1941[5]-048 | 0.110       | +                 |

#### 7.6.1 – Leaf Paint Direct Comparison in $T_0$ Corn.

$T_0$  *DSM-2* (*v2*) plants were painted with a rundown of glufosinate herbicide. Four siblings from each of 15  $T_0$  events were tested, and 4 leaves on each individual plant received a rundown of glufosinate at approximately V8 stage. Rundown treatments were randomized for each rate allowing for variation of treatment location on individual leaves. For corn, 0.25% v/v glufosinate is the minimum effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (0.5%, 1.0%, and 2.0% v/v). Glufosinate treatments were applied using cotton tipped applicators to a treatment area of approximately 2.5 cm in diameter.

**Table 11** compares the response of *DSM-2* (*v2*) and control genes to impart glufosinate resistance to corn  $T_0$  transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each  $T_0$  is an independent transformation event, one can expect significant variation of individual  $T_0$  responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. Untransformed wildtype corn served as a glufosinate sensitive control. The *DSM-2* (*v2*) gene imparted herbicide resistance to individual  $T_0$  corn plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at up to 2% v/v glufosinate, *DSM-2* (*v2*) performs better overall than *PAT*-

transformed plants. An overall population injury average by rate is presented in **Table 12** simply to demonstrate the significant difference between the plants transformed with *DSM-2 (v2)* versus the wildtype or *PAT*-transformed controls.

**Table 12.** *T<sub>0</sub> DSM-2 (v2)* (plant optimized)-transformed Corn plants response to a range of glufosinate rates applied as leaf paints to plants postemergence compared to Wildtype and *T<sub>0</sub> PAT-transformed* plants.

|           | Wildtype | PAT      | DSM-2(v2) |
|-----------|----------|----------|-----------|
| Treatment | % Injury | % Injury | % Injury  |
| 2% v/v    | 90       | 9        | 4         |
| 1% v/v    | 84       | 6        | 2         |
| 0.5% v/v  | 50       | 3        | 1         |
| 0.25% v/v | 33       | 1        | 0         |

#### 7.6.2 – Verification of high glufosinate tolerance in *T<sub>2</sub> corn*.

The seed from the cross of *T<sub>1</sub> DSM-2 (v2)* x 5XH751 were planted into 4-inch pots containing Metro Mix media and at 2 leaf stage were sprayed in the track sprayer set to 187 L/ha at 560 g ai/ha glufosinate to remove nulls. At 7 DAT nulls were removed and resistant plants were sprayed in the track sprayer as above at the following rates: 0, 560, 1120, 2240, and 4480 g ai/ha glufosinate. Plants were graded at 3 and 14 DAT and compared to 5XH751 x Hi II control plants. **Table Ex. 7.6.2-1** below shows that there are individual *DSM-2 (v2)* plants that provided up to 2240 g ai/ha glufosinate with less than 20% injury. *DSM-2 (v2)* also provided similar tolerance to 4480 g ai/ha glufosinate at the *PAT* transformed controls.

**Table Ex. 7.6.2-1.** *T<sub>2</sub> Corn* response to a range of glufosinate rates applied postemergence (14DAT).

| <i>DSM-2 v2 (pDAS1941)</i> | % Injury |        |      | % Injury |         |
|----------------------------|----------|--------|------|----------|---------|
|                            | <20%     | 20-40% | >40% | Ave      | Std dev |
| Averages                   |          |        |      |          |         |
| Untreated control          | 4        | 0      | 0    | 3        | 3       |
| 560 g ai/ha glufosinate    | 4        | 0      | 0    | 6        | 3       |
| 1120 g ai/ha glufosinate   | 2        | 2      | 0    | 18       | 6       |
| 2240 g ai/ha glufosinate   | 1        | 3      | 0    | 21       | 5       |
| 4480 g ai/ha glufosinate   | 0        | 4      | 0    | 29       | 5       |

| <b>PAT (pDAS1942)</b>    |      |        |      | % Injury |         |
|--------------------------|------|--------|------|----------|---------|
| Averages                 | <20% | 20-40% | >40% | Ave      | Std dev |
| Untreated control        | 4    | 0      | 0    | 0        | 0       |
| 560 g ai/ha glufosinate  | 4    | 0      | 0    | 6        | 5       |
| 1120 g ai/ha glufosinate | 0    | 1      | 3    | 25       | 10      |
| 2240 g ai/ha glufosinate | 2    | 1      | 1    | 25       | 12      |
| 4480 g ai/ha glufosinate | 0    | 3      | 1    | 33       | 5       |
| <b>WT</b>                |      |        |      | % Injury |         |
| Averages                 | <20% | 20-40% | >40% | Ave      | Std dev |
| Untreated control        | 4    | 0      | 0    | 3        | 3       |
| 560 g ai/ha glufosinate  | 0    | 0      | 4    | 100      | 0       |
| 1120 g ai/ha glufosinate | 0    | 0      | 4    | 100      | 0       |
| 2240 g ai/ha glufosinate | 0    | 0      | 4    | 100      | 0       |
| 4480 g ai/ha glufosinate | 0    | 0      | 4    | 100      | 0       |

### 7.6.3 – *DSM-2 (v2)* heritability in corn.

The seed from the cross of T<sub>1</sub> *DSM-2 (v2)* x 5XH751 were planted into 3-inch pots containing Metro Mix media and at 2 leaf stage were sprayed in the track sprayer set to 187 L/ha at 0, 280, 560, 1120, 2240, and 4480 g ai/ha glufosinate. Plants were graded at 3 and 14 DAT and compared to 5XH751 x Hi II control plants. Plants were graded as before with overall visual injury from 0-100%. To determine segregation of each population the rate of 1120 g ai/ha and higher was chosen. Resistant and sensitive plants were counted and it was determined that all of the T<sub>1</sub> families segregated as a single locus, dominant Mendelian trait (1R:1S) as determined by Chi square analysis. Surviving plants were selfed to produce the T<sub>2</sub> generation. *DSM-2 (v2)* is heritable as a robust glufosinate resistance gene in multiple species when reciprocally crossed to a commercial hybrid.

A progeny test was also conducted on five *DSM-2 (v2)* T<sub>2</sub> families. The seeds were planted in three-inch pots as described above. At the 3 leaf stage all plants were sprayed with 560 g ai/ha glufosinate in the track sprayer as previously described. After 7 DAT, resistant and sensitive plants were counted. Four out of the five lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis.

### 7.6.4 – *Stacking of DSM-2(v2) to increase herbicide spectrum*

The cross of T<sub>1</sub> plants with BE1146RR have been made. *DSM-2(v2)*-transformed plants can be conventionally bred to other corn lines containing additional traits of interest. An inbred (BE1146RR) containing the glyphosate tolerance trait *CP4* was crossed with T<sub>1</sub> plants containing

*DSM-2(v2)*. Plants of the subsequent generation can be tested for efficacy of both herbicide tolerance traits by application of glufosinate and glyphosate in sequence or in tankmix at rates equal to or exceeding normally lethal herbicide rates (e.g the plants could be sprayed with 280, 560, 1120 g ae/ha, or more, of both herbicides). This would identify the ability to use both herbicides in combination or sequential applications for herbicide resistance management.

#### *7.6.4 – Stacking of DSM-2(v2) with an Insect Resistance trait*

*DSM-2 (v2)* will be used to select corn that has been successfully transformed with an insect resistance trait including but not limited to those listed in Example 9. Plants containing both the *DSM-2 (v2)* gene and an insect resistance gene will be evaluated for levels of resistance in appropriate bioassay's as described in Example 9.

### EXAMPLE 8 – PROTEIN DETECTION FROM TRANSFORMED PLANTS VIA ANTIBODY

#### *8.1 – Polyclonal Antibody Production.*

Five milligrams purified DSM-2 (see previous section) was delivered to Invitrogen Custom Antibody Services (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 4 injections in the period of 12 weeks with each injection contained 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both direct-ELISA and Western blotting experiments to confirm specificity and affinity.

#### *8.2 – Extracting DSM-2 from callus tissue.*

Four maize (Hi-II) leaf discs using a single-hole puncher were put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., Cat# 145462-000) and 500 µL plant extraction buffer (PBS containing 0.1 % Triton X-100 and 5 µL per mL protease inhibitors cocktail (Sigma Cat # P9599). The tubes were secured in the Geno/Grinder (Model 2000-115, Certiprep, Metuchen, NJ) and shook for 6 min with setting at 1x of 500 rpm. Tubes were centrifuged at 5000x g for 10 min and the supernatant containing the soluble proteins was assayed in Western Blotting experiments to detect the presence of DSM-2 .

#### *8.3 – Western Blotting Analysis.*

The leaf extract was spiked with various concentrations of purified DSM-2 and incubated with Laemmli sample buffer at 95 °C for 10 min followed by electrophoretic separation in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, DSM-2 protein was detected by

anti-DSM-2 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham Cat.# RPN 21058).

#### 8.4 – Results.

5 Polyclonal antibodies for DSM-2 were generated using protein expressed in and purified from *E. coli* cells. After four injections, the antisera had high titer of anti-DSM-2 antibodies as observed in direct-ELISA. At 100,000-fold dilution, the serum still provided signal six times above background.

10 In Western blotting analysis (of DSM-2 in wild type maize (Hi-II) leaf matrix), the serum could detect a major band of approximately 22 kDa, which is comparable to the predicted molecular weight based on the *DSM-2 (v2)* gene. The same band could still be detected when the extract was spiked at 5 ng/mL, the lowest concentration tested. Minor bands were also observed at high DSM-2 concentrations, which are believed to be aggregate of the target protein as these were not observed at lower concentrations. A single major band with molecular weight  
15 comparable to the predicted one was observed. (The lanes run on this gel were a molecular weight marker, and leaf extracts containing DSM-2 protein at concentration 0.005, 0.05, 0.5 and 5 µg/mL, respectively.) In addition, the polyclonal antibody did not cross react to any maize leaf proteins as little background signal was observed.

20 Expression of DSM-2 from transformed Tobacco callus tissue was determined using Western blotting analysis. Detected band with comparative size as the standard (approx. 22 kDa) was observed in different events indicating that DSM-2 was expressed in transgenic tobacco tissue.

#### EXAMPLE 9 – TOBACCO, CHILI, AND CELL CULTURE TRANSFORMATION

25 Four days prior to transformation, a 1 week old NT-1 tobacco suspension, which was being subcultured every 7 days, was subcultured to fresh medium by adding 2 ml of the NT-1 culture or 1 ml of packed cells into 40 ml NT-1 B media. The subcultured suspension was maintained in the dark at  $25 \pm 1^{\circ}\text{C}$  on a shaker at 125 rpm.

**Table Ex. 9-1**

NT-1 B medium recipe

| <i>Reagent</i>                  | <i>Per liter</i> |
|---------------------------------|------------------|
| MS salts (10X)                  | 100 ml           |
| MES                             | 0.5 g            |
| Thiamine-HCl (1 mg/ml)          | 1 ml             |
| <i>myo</i> -inositol            | 100 mg           |
| K <sub>2</sub> HPO <sub>4</sub> | 137.4 mg         |
| 2,4-D (10 mg/ml)                | 222 $\mu$ l      |
| Sucrose                         | 30 g             |
| pH to 5.7 $\pm$ 0.03            |                  |

A 50% glycerol stock of *Agrobacterium tumefaciens* [strain LBA4404] harboring a binary vector of interest was used to initiate a liquid overnight culture by adding 20, 100, or 500  $\mu$ l to 30 ml YEP liquid (10 g/L yeast extract, 10 g/L Peptone, 5 g/L NaCl, 0-10 g/L Sucrose) containing 50-100 mg/L spectinomycin. The bacterial culture was incubated in the dark at 28°C in an incubator shaker at 150-250 rpm until the OD<sub>600</sub> was 1.5  $\pm$  0.2. This took approximately 18-20 hrs.

For each vector tested, 20-70 mL of 4-day old suspension cells were transferred into a sterile vessel, to which 500 – 1750  $\mu$ l of *Agrobacterium* suspension at the proper OD was added. To ensure a uniform mixture was achieved, the cells were pipetted up and down 5 times with a 10 ml wide-bore pipet. The uniform suspension was then drawn up into a 25 ml barrel of a repeat pipetter and 250  $\mu$ l of the suspension was dispensed per well into 24-well plates, continuing until the suspension was exhausted. The well plates were wrapped in parafilm and cultured in the dark at 25  $\pm$  1°C without shaking for a 3 day co-cultivation.

Following the co-cultivation, all excess liquid was removed from the individual wells with a 1 mL pipet tip, and remaining cells were resuspended in 1 ml NTC liquid (NT-1 B medium containing 500 mg/L carbenicillin, added after autoclaving). The contents of an individual well were dispersed across the entire surface of 100 x 25 mm selection plates using disposable transfer pipets. Selection media consisted of NTC media solidified with 8g/l TC agar supplemented with

7.5 to 15 mg/L bialaphos or technical grade glufosinate ammonium, added after autoclaving. All selection plates, left unwrapped, were maintained in the dark at 28°C.

Putative transformants appeared as small clusters of callus on a background of dead, non-transformed cells. Calli were isolated approximately 2-6 weeks post-transformation. Each callus isolate was transferred to its own 60x20 mm plate containing the same selection medium and allowed to grow for approximately 2 weeks before being submitted for analysis.

### 9.1 – Results

A side-by-side experiment comparing *DSM-2 (v2)* with *PAT* was completed. In the study, 100% of the *PAT* selection plates produced at least one PCR positive isolate on 10 mg/L bialaphos media, whereas 79% of the *DSM-2 (v2)* selection plates produced at least one PCR positive isolate. All events were assayed for the presence of the *DSM-2 (v2)* or *PAT* gene via coding region PCR, and were found to be positive.

**Table Ex. 9-2**

| Construct | Gene of Interest | Transformation Frequency on 10 mg/L Bialaphos following PCR Verification |
|-----------|------------------|--------------------------------------------------------------------------|
| pDAB3778  | DSM-2            | 79%                                                                      |
| pDAB3779  | PAT              | 100%                                                                     |

Western blots were performed on a small subset of the *DSM-2 (v2)*-selected events, and three positive events were identified as seen in the data below. In a second experiment, *DSM-2 (v2)*-treated tobacco cells were selected upon 7.5, 10, 12.5, or 15 mg/L bialaphos or technical grade glufosinate ammonium. Transformation frequencies (% of selection plates producing at least one callus) following verification by coding region PCR are listed in the table below.

**Table Ex. 9-3**

| Concentration in mg/L | Glufosinate | Bialaphos |
|-----------------------|-------------|-----------|
| 7.5                   | 55.6%       | 38.9%     |
| 10                    | 50.0%       | 44.4%     |
| 12.5                  | 44.4%       | 38.9%     |
| 15                    | 38.9%       | 22.2%     |

### 9.2 – Tobacco Transformation

To create tobacco plants that can resist harmful insects, the *DSM-2 (v2)* was used to select plants that were successfully transformed via *Agrobacterium*. The *DSM-2 (v2)* gene was

molecularly stacked independently with each of the following insect resistance traits: Cry5B, Cry6A, Cry12A, Cry14A, and Cry21A. The *DSM-2 (v2)* gene can also be molecularly stacked with at least one of the following insect resistance traits: Cry1Aa1, Cry1Ac1, Cry1Bb1, Cry1Fa1, Cry1Ja1, Cry2Ac7, Cry4Ba4, Cry8Ga2, Cry19Aa1, Cry32Ca1, Cry43Aa2, Cyt2Ba3, Cry1Aa2, Cry1Ac2, Cry1Bc1, Cry1Fa2, Cry1Jb1, Cry2Ac8, Cry4Ba5, Cry8Ha1, Cry19Ba1, Cry32Da1, Cry43Ba1, Cyt2Ba4, Cry1Aa3, Cry1Ac3, Cry1Bd1, Cry1Fb1, Cry1Jc1, Cry2Ac9, Cry4Ca1, Cry8Ia1, Cry20Aa1, Cry33Aa1, Cry44Aa, Cyt2Ba5, Cry1Aa4, Cry1Ac4, Cry1Bd2, Cry1Fb2, Cry1Jc2, Cry2Ac10, Cry5Aa1, Cry9Aa1, Cry21Aa1, Cry34Aa1, Cry45Aa, Cyt2Ba6, Cry1Aa5, Cry1Ac5, Cry1Be1, Cry1Fb3, Cry1Jd1, Cry2Ac11, Cry5Ab1, Cry9Aa2, Cry21Aa2, Cry34Aa2, Cry46Aa, Cyt2Ba7, Cry1Aa6, Cry1Ac6, Cry1Be2, Cry1Fb4, Cry1Ka1, Cry2Ac12, Cry5Ac1, Cry9Ba1, Cry21Ba1, Cry34Aa3, Cry46Ab, Cyt2Ba8, Cry1Aa7, Cry1Ac7, Cry1Bf1, Cry1Fb5, Cry1La1, Cry2Ad1, Cry5Ad1, Cry9Bb1, Cry22Aa1, Cry34Aa4, Cry47Aa, Cyt2Ba9, Cry1Aa8, Cry1Ac8, Cry1Bf2, Cry1Fb6, Cry2Aa1, Cry2Ad2, Cry5Ba1, Cry9Ca1, Cry22Aa2, Cry34Ab1, Cry48Aa, Cyt2Bb1, Cry1Aa9, Cry1Ac9, Cry1Bg1, Cry1Fb7, Cry2Aa2, Cry2Ad3, Cry5Ba2, Cry9Ca2, Cry22Aa3, Cry34Ac1, Cry48Aa2, Cyt2Bc1, Cry1Aa10, Cry1Ac10, Cry1Ca1, Cry1Ga1, Cry2Aa3, Cry2Ad4, Cry6Aa1, Cry9Da1, Cry22Ab1, Cry34Ac2, Cry48Aa3, Cyt2Ca1, Cry1Aa11, Cry1Ac11, Cry1Ca2, Cry1Ga2, Cry2Aa4, Cry2Ad5, Cry6Aa2, Cry9Da2, Cry22Ab2, Cry34Ac3, Cry48Ab, Cry1Aa12, Cry1Ac12, Cry1Ca3, Cry1Gb1, Cry2Aa5, Cry2Ae1, Cry6Aa3, Cry9Db1, Cry22Ba1, Cry34Ba1, Cry48Ab2, Cry1Aa13, Cry1Ac13, Cry1Ca4, Cry1Gb2, Cry2Aa6, Cry2Af1, Cry6Ba1, Cry9Ea1, Cry23Aa1, Cry34Ba2, Cry49Aa, Cry1Aa14, Cry1Ac14, Cry1Ca5, Cry1Gc, Cry2Aa7, Cry3Aa1, Cry7Aa1, Cry9Ea2, Cry24Aa1, Cry34Ba3, Cry49Aa2, Cry1Aa15, Cry1Ac15, Cry1Ca6, Cry1Ha1, Cry2Aa8, Cry3Aa2, Cry7Ab1, Cry9Ea3, Cry24Ba1, Cry35Aa1, Cry49Aa3, Cry1Ab1, Cry1Ac16, Cry1Ca7, Cry1Hb1, Cry2Aa9, Cry3Aa3, Cry7Ab2, Cry9Ea4, Cry24Ca1, Cry35Aa2, Cry49Aa4, Cry1Ab2, Cry1Ac17, Cry1Ca8, Cry1Ia1, Cry2Aa10, Cry3Aa4, Cry7Ab3, Cry9Ea5, Cry25Aa1, Cry35Aa3, Cry49Ab1, Cry1Ab3, Cry1Ac18, Cry1Ca9, Cry1Ia2, Cry2Aa11, Cry3Aa5, Cry7Ab4, Cry9Eb1, Cry26Aa1, Cry35Aa4, Cry50Aa1, Cry1Ab4, Cry1Ac19, Cry1Ca10, Cry1Ia3, Cry2Aa12, Cry3Aa6, Cry7Ab5, Cry9Ec1, Cry27Aa1, Cry35Ab1, Cry51Aa1, Cry1Ab5, Cry1Ac20, Cry1Ca11, Cry1Ia4, Cry2Ab1, Cry3Aa7, Cry7Ba1, Cry9Ed1, Cry28Aa1, Cry35Ab2, Cry52Aa1, Cry1Ab6, Cry1Ac21, Cry1Cb1, Cry1Ia5, Cry2Ab2, Cry3Aa8, Cry7Ca1, Cry10Aa1, Cry28Aa2, Cry35Ab3, Cry53Aa1, Cry1Ab7, Cry1Ac22, Cry1Cb2, Cry1Ia6, Cry2Ab3, Cry3Aa9, Cry8Aa1, Cry10Aa2, Cry29Aa1, Cry35Ac1, Cry54Aa1, Cry1Ab8,

Cry1Ac23, Cry1Cb3, Cry1Ia7, Cry2Ab4, Cry3Aa10, Cry8Ab1, Cry10Aa3, Cry30Aa1, Cry35Ba1,  
 Cry55Aa1, Cry1Ab9, Cry1Ad1, Cry1Da1, Cry1Ia8, Cry2Ab5, Cry3Aa11, Cry8Ba1, Cry11Aa1,  
 Cry30Ba1, Cry35Ba2, Cry55Aa2, Cry1Ab10, Cry1Ad2, Cry1Da2, Cry1Ia9, Cry2Ab6, Cry3Aa12,  
 Cry8Bb1, Cry11Aa2, Cry30Ca1, Cry35Ba3, Cyt1Aa1, Cry1Ab11, Cry1Ae1, Cry1Db1, Cry1Ia10,  
 5 Cry2Ab7, Cry3Ba1, Cry8Bc1, Cry11Aa3, Cry30Da1, Cry36Aa1, Cyt1Aa2, Cry1Ab12, Cry1Af1,  
 Cry1Db2, Cry1Ia11, Cry2Ab8, Cry3Ba2, Cry8Ca1, Cry11Ba1, Cry30Ea1, Cry37Aa1, Cyt1Aa3,  
 Cry1Ab13, Cry1Ag1, Cry1Dc1, Cry1Ia12, Cry2Ab9, Cry3Bb1, Cry8Ca2, Cry11Bb1, Cry31Aa1,  
 Cry38Aa1, Cyt1Aa4, Cry1Ab14, Cry1Ah1, Cry1Ea1, Cry1Ia13, Cry2Ab10, Cry3Bb2, Cry8Ca3,  
 Cry12Aa1, Cry31Aa2, Cry39Aa1, Cyt1Aa5, Cry1Ab15, Cry1Ah2, Cry1Ea2, Cry1Ib1, Cry2Ab11,  
 10 Cry3Bb3, Cry8Da1, Cry13Aa1, Cry31Aa3, Cry40Aa1, Cyt1Aa6, Cry1Ab16, Cry1Ai1, Cry1Ea3,  
 Cry1Ib2, Cry2Ab12, Cry3Ca1, Cry8Da2, Cry14Aa1, Cry31Aa4, Cry40Ba1, Cyt1Ab1, Cry1Ab17,  
 Cry1Ba1, Cry1Ea4, Cry1Ib3, Cry2Ac1, Cry4Aa1, Cry8Da3, Cry15Aa1, Cry31Aa5, Cry40Ca1,  
 Cyt1Ba1, Cry1Ab18, Cry1Ba2, Cry1Ea5, Cry1Ic1, Cry2Ac2, Cry4Aa2, Cry8Db1, Cry16Aa1,  
 Cry31Ab1, Cry40Da1, Cyt1Ca1, Cry1Ab19, Cry1Ba3, Cry1Ea6, Cry1Ic2, Cry2Ac3, Cry4Aa3,  
 15 Cry8Ea1, Cry17Aa1, Cry31Ab2, Cry41Aa1, Cyt2Aa1, Cry1Ab20, Cry1Ba4, Cry1Ea7, Cry1Id1,  
 Cry2Ac4, Cry4Ba1, Cry8Ea2, Cry18Aa1, Cry31Ac1, Cry41Ab1, Cyt2Aa2, Cry1Ab21, Cry1Ba5,  
 Cry1Ea8, Cry1Ie1, Cry2Ac5, Cry4Ba2, Cry8Fa1, Cry18Ba1, Cry32Aa1, Cry42Aa1, Cyt2Ba1,  
 Cry1Ab22, Cry1Ba6, Cry1Eb1, Cry1If1, Cry2Ac6, Cry4Ba3, Cry8Ga1, Cry18Ca1, Cry32Ba1,  
 Cry43Aa1, Cyt2Ba2, Cry1A.105, Cry3Bb1.11098, Cry2Ab, Cry1Ab-Bt11, mCry3A, and Vip3A,  
 20 for example.

Leaf disks were inoculated (for 5-10 min) in bacterial culture (final OD<sub>600</sub> 0.5) that had  
 been resuspended 1/2x MS liquid medium. Explants were blotted dry on filter paper and transferred  
 to filter paper on top of the agar-solidified MS medium with 1 mg/l BAP and 0.1 mg/l IAA  
 without antibiotics for 2-3 days at 27°C. Then, leaf disks were collected and washed in sterile  
 25 water, blotted on filter paper and transferred to MS medium with 1 mg/l BAP and 0.1 mg/l IAA  
 with cefotaxime (claforan, ~500 mg/l) and PPT (5 mg/l). Explants were transferred ~ every 2  
 weeks to a fresh MS medium as above. Shoots appeared in 1-3 months and have been rooted  
 either on the same medium or on 1/2 MS with 2.5 mg/l of PPT and 125-400 mg/l cefotaxime.  
 Positive tobacco transformants were confirmed via PCR amplification of the Cry and *DSM-2* (v2)  
 30 transgene insertion region.

### 9.3 – PPT Selection

Initial experiments were done with PPT at 10 mg/L. This selection was extremely severe on non-transformed controls, so 5 mg/L was used for most experiments. In early experiments, plants were rooted on medium containing 2.5 mg/L PPT after selection of shoots on medium with 5 mg/L PPT. Shoot selections have been done on medium with 2.5 mg/L PPT. This level seems totally sufficient for selections; it is still a very strong selection against non-transformed controls, and an escape at this level has not been detected.

Based on PCR testing, 95% of transformed shoots were recovered with this protocol. Below is data, **Table 13**, from some of the original transformations we performed showing 1 PCR negative out of 31 plants tested with the 5 mg/L shoot selection and 2.5 mg/L rooting regimens.

**Table 13; Transformation efficiency based on PCR positive plants.**

| N | Construct    | Date     | Inoculated Explants | Resistant shoots | PCR (+)                | Western (+) |
|---|--------------|----------|---------------------|------------------|------------------------|-------------|
| 1 | pDAB7602     | 01/20/07 | 39                  | 9                | 7                      | 2 of 5      |
| 2 | pDAB7602     | 02/08/07 | 152                 | 13               | 8/8 tested             | 0 of 17     |
|   | <b>total</b> |          | 191                 | ?                | <b>15 of 16 tested</b> | 2 of 22     |
| 3 | pDAB7604     | 01/20/07 | 29                  | 6                | 6                      | 3 of 9      |
| 4 | pDAB7604     | 02/12/07 | 73                  | 5                | Not tested             | 4 of 4      |
|   | <b>total</b> |          | 156                 | ?                | <b>6 of 6 tested</b>   | 7 of 13     |
| 5 | pDAB7606     | 01/20/07 | 49                  | 11               | 9 of 9 tested          | 4 of 10     |
|   | <b>total</b> |          | 49                  | ?                | <b>9 of 9 tested</b>   | 4 of 10     |

#### 9.3.1 PPT Selection of Chili Plants with Hairy Roots

PPT is being titrated for use in selecting chimeric chili plants with hairy roots, and we have found it is a very potent selection against wild type chili shoots at 1 mg/L. The selection is visually apparent after only a few days and shoots are dead within a week at this level. It is being assessed at 0.1, 0.25, and 0.5 mg/L for these selections.

Prior to propagation, T<sub>1</sub> plants will be sampled for DNA analysis to determine the insert copy number. Fresh tissue will be placed into tubes and lyophilized at 4° C for 2 days. After the

tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure will then be followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ $\mu$ l.

The DNA samples will be diluted to 9 ng/ $\mu$ l and then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and MgCl<sub>2</sub> (Third Wave Technologies). An aliquot of 7.5  $\mu$ l is placed in each well of the Invader assay plate followed by an aliquot of 7.5  $\mu$ l of controls, standards, and 20 ng/ $\mu$ l diluted unknown samples. Each well is overlaid with 15  $\mu$ l of mineral oil (Sigma). The plates are then incubated at 63° C for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

All events will be assayed for the presence of the *DSM-2 (v2)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA is used as template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit. The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Clonal lineages from each PCR positive events with 1-3 copies of *DSM-2 (v2)* gene (and presumably a Cry gene of interest, since these genes are physically linked) will be regenerated and moved to the greenhouse.

#### 9.4 – Postemergence herbicide tolerance in *DSM-2 (v2)* transformed *T<sub>0</sub>* tobacco

T<sub>1</sub> plants from each positive events were challenged with a wide range of glufosinate, sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. Glufosinate will be applied at s560 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT.

#### 9.5 – Evaluation of insect resistance

Protocol for bioassay is as follows:

32-well trays are filled with 2% agar solution (CD International, Pitman, NJ). Leaf pieces (roughly 1" square) are taken from transformed plants. There are 2 leaf/plant pieces per pest tested (including but not limited to insect targets from the orders Thysanoptera, Hemiptera, Homoptera, Lepidoptera, Coleoptera, Diperta, and parasitic worms from the phylum Nematoda). At least 5 neonate insects (OR an egg mass, if available) are placed in each well. Wells are covered with perforated sticky lids. Trays are incubated at 28C (40% RH, 16:8 light:dark) for 3 days. Grading is on a % damage basis; each leaf piece is given a % damage score and recorded.

#### EXAMPLE 10 – *AGROBACTERIUM* TRANSFORMATION OF OTHER CROPS

In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated transformation of rye, see, e.g., Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, see, e.g., Hinchey *et al.*, 1988. For *Agrobacterium*-mediated transformation of sorghum, see, e.g., Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, see, e.g., Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, see, e.g., Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, see, e.g., Hiei *et al.*, 1997.

The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform *DSM-2 (v1)*, for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta*, and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Canola/rapeseed (*Cruciferae Brassica* spp), Sugar cane (*Arenga pinnata*), Tomato (*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Compositae Lactuca sativa*, *perennis*, and *pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), Sorghum (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans

(*Leguminosae Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (*Leguminosae Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Compositae Helianthus annuus*), Squash (*Dicotyledoneae Cucurbita* spp.), Cucumber (*Dicotyledoneae genera*), Tobacco (*Solanaceae Nicotiana* spp.), *Arabidopsis* (*Cruciferae Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, and other families), and Clover (*Leguminosae*). Such plants, with *DSM-2 (v2)* genes, for example, are included in the subject invention. Vegetation control in plants endowed with glufosinate or bialaphos resistance as a result of transformation with *DSM-2(v2)* can be improved by selectively applying glufosinate.

*DSM-2 (v2)* has the potential to increase the applicability of herbicides, that can be inactivated by *DSM-2* (e.g., glufosinate, bialaphos, and/or phosphinothricin), for in-season use in many deciduous and evergreen timber cropping systems. Glufosinate or bialaphos-resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited to: alder, ash, aspen, beech, birch, cherry, eucalyptus, hickory, maple, oak, pine, and poplar. Use of glufosinate or bialaphos resistance for the selective control in ornamental species is also within the scope of this invention. Examples could include, but not be limited to, roses, Euonymus, petunia, begonia, and marigolds.

#### EXAMPLE 11 – *DSM-2 (V2)* STACKED WITH GLYPHOSATE TOLERANCE TRAIT IN ANY CROP

The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; [isb.vt.edu/cfdocs/fieldtests1.cfm](http://isb.vt.edu/cfdocs/fieldtests1.cfm), 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *DSM-2 (v2)* with a GT trait,

either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where *DSM-2 (v2)* and a GT trait are stacked in any monocot or dicot crop species:

- 5           a)       Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (e.g., *Commelina* spp, *Ipomoea* spp, etc), 280-2240 g ae/ha (preferably 350-1700
- 10           g ae/ha) glufosinate can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.
- b)       Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *DSM-2 (v2)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). Glufosinate (at 280-2240 g ae/ha, more preferably 350-1700 g ae/ha) could then
- 15           be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control.

One skilled in the art will recognize that other herbicides, (e.g. bialaphos) can be enabled by

20           transformation of plants with *DSM-2(v2)*. Specific rates can be determined by the herbicides labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2003). Each alternative herbicide enabled for use in HTC's by *DSM-2 (v2)*, whether used alone, tank mixed, or

25           sequentially, is considered within the scope of this invention.

#### EXAMPLE 12 – *DSM-2 (V2)* STACKED WITH *AHAS* TRAIT IN ANY CROP

Imidazolinone herbicide tolerance (*AHAS*, *et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional

30           imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox,

imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS* *et al.* Commercial imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agrilience, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking *DSM-2 (v2)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where *DSM-2 (v2)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

- a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.
  - i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis*, *Ambrosia trifida*, *Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 350-1700 g ae/ha glufosinate.
  - ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea spp.* can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 350-1700 g ae/ha glufosinate.

One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, and glufosinate based herbicides, alone or in multiple combinations, is enabled by *DSM-2 (v2)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the

Crop Protection Guide from Agrilience (2003). Each alternative herbicide enabled for use in HTCs by *DSM-2 (v2)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

5      EXAMPLE 13 – *DSM-2 (V2)* IN RICE

          13.1 – *Media description.*

          The culture media was adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytigel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml  
10      liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26 °C (Zhang *et al.* 1996).

          Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion  
15      of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Herbicide-resistant callus was selected on NB medium supplemented with 8 mg/l Bialaphos for 9 weeks with subculturing every 3 weeks.

### 13.2 – Tissue culture development.

Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described by Zhang et al. 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3–5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH8 herbiace selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 3 weeks. Newly formed callus was subcultured to fresh NBH8 medium twice every 3 weeks.

### 13.3 – Microprojectile bombardment.

All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA, 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

### 13.4 – Microprojectile bombardment.

Sixteen to twenty h after bombardment, tissues were transferred from NBO medium onto NBH8 herbiace selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 3 weeks. Newly formed callus was subcultured to fresh NBH8 medium twice every 3 weeks.

EXAMPLE 14 – *DSM-2 (V2)* STACKED WITH *AAD-1 (V3)* IN ANY CROP

Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agrilience, 2003).

By stacking *AAD-1* (v3) (see USSN 11/587,893; WO 2005/107437) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1* (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1* (v3) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

- a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.
  - i) Inherently tolerant grass weed species (e.g., *Echinochloa* spp or *Sorghum* spp) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
  - ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.
- b) A three-way combination of glufosinate (200-500 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum

provides an additional mechanism for the management or delay of herbicide resistant weeds.

EXAMPLE 15 – *DSM-2 (V2)* STACKED WITH *AAD-12 (V2)* IN ANY CROP

5           Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agrilience, 2005).

10           By stacking *AAD-12 (v1)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

15           a)     Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

20           i)     Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis*, *Apocynum cannabinum*, and *Conyza canadensis*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species. Triclopyr and fluroxypyr would be acceptable components to consider in the weed control regimen. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

- b) A multiple combination of glufosinate (200-500 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

#### EXAMPLE 16 – ADDITIONAL GENE STACKING COMBINATIONS

The subject invention also includes plants that produce one or more enzymes of the subject invention “stacked” together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, ALS- (imidazolinone, sulfonylurea), aryloxyalkanoate-, HPPD-, PPO-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

In some embodiments, the invention provides monocot and dicot plants tolerant to bialaphos, phosphinothricin, or glufosinate and one or more commercially available herbicides (e.g., glyphosate, glufosinate, paraquat, ALS-inhibitors (e.g., sulfonylureas, imidazolinones, triazolopyrimidine sulfonanilides, et al), HPPD inhibitors (e.g, mesotrione, isoxaflutole, *et al.*), 2,4-D, fluroxypyr, triclopyr, dicamba, bromoxynil, aryloxyphenoxypropionates, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as glufosinate).

The subject DSM-2 genes can be stacked with one or more *pat/bar* genes, for an additional mechanism for glufosinate tolerance, which are well known in the art.

For use of a gene, in plants, that encodes an HPPD (hydroxyl-phenyl pyruvate dioxygenases), *see e.g.* U.S. Patent Nos. 6,268,549 and 7,297,541. Such “stacked” plants can be combined with other gene(s) for glufosinate resistance, and such stacked plants (and various other plants and stacked plants of the subject invention) can be used to prevent the development of glyphosate resistance.

Genes encoding enzymes with glyphosate N-acetyltransferase (GAT) activity can also be used (stacked) with DSM-2 gene(s) of the subject invention. *See e.g.* Castle *et al.* (2004), “Discovery of Directed Evolution of a Glyphosate Tolerance Gene,” *Science* Vol. 34, pp. 1151-1154; and WO 2002/36782.

The subject DSM-2 genes can also be stacked with the AAD-1 and AAD-12, and AAD-13 genes of WO 2005/107437, WO 2007/053482, and USSN 60/928,303, respectively, and can be used for combating glyphosate resistance in some preferred embodiments, as disclosed therein.

The subject DSM-2 genes can also be stacked with other insect resistance traits in any crop, such as those expressing RNA interference genes (RNAi) Baum *et al* (2007), Gordon *et al* (2007)

#### EXAMPLE 17 – DSM-2 IN CANOLA

##### 17.1 – Canola transformation.

The DSM-2 (*v2*) selectable marker gene was used to transform *Brassica napus* var. Nexera\* 710 with *Agrobacterium*-mediated transformation along with GUS and plasmid pDAB9303. The construct contained GUS reporter gene and DSM-2 (*v2*) gene both driven by CsVMV promoter.

Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 23° C, and a photoperiod of 16 hrs light/8 hrs dark.

Hypocotyl segments (3 mm) were excised from 5 day old seedlings and placed on callus induction medium MSK1D1 (MS medium with 1 mg/L kinetin and 1 mg/L 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a 100 x 25 petri plate containing 20 mL of M liquid for a 1 hour pretreatment and were then treated with *Agrobacterium* Z707S strain containing pDAB9303. The *Agrobacterium* was grown for 16 hours overnight at 23° C in the

dark on an enclosed shaker at 200 rpm, centrifuged at 6,000 rpm for 15 minutes and subsequently re-suspended in the culture medium to a final density of klett 50 with a red filter.

After 30 min treatment of the hypocotyl segments with *Agrobacterium*, they were placed back on the callus induction medium for 3 days. Following co-cultivation at 23°C with 16 hours indirect light/8 hours dark, the segments were placed directly on selection medium MSK1D1H0.1 or H1 (above medium with 1 mg/L Herbiace or 0.1 mg/L Herbiace). Carbenicillin and timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace, which contains 20% bialaphos as the active ingredient (a.i.), inhibited the growth of the non-transformed cells and the growth of transformed cells.

Callused hypocotyl segments were then placed on MSB3Z1H0.25 to H3 (MS medium, 3 mg/L benzylaminopurine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 0.25 to 3 mg/L Herbiace, carbenicillin and timentin) shoot regeneration medium. After 2 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to MSB3Z1H0.5 to H5 medium (same media as before except with higher Herbiace level to 5 mg/L) for another 2 weeks.

Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH0.75 to H10 (MS, 0.5 gm/L MES, 0.75 to 10 mg/L Herbiace, carbenicillin, timentin) for two 2 week passes. The elongated shoots are cultured for root induction on ½ MS + IBA + timentin (MS with 0.1 mg/L Indolebutyric acid, 1 mg/ml IBA, and 50 mg/ml timentin). Once the plants had a well established root system, they were transplanted into 5 1/4" pots containing metro mix soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

For tissue harvesting DNA isolation and quantification, fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. The results of the transformation are described in **Table 14**.

**Table 14; Expression of DSM-2 (v2) in canola plants**

| Herbiace® concentration | pDAB9303 | Putative Events | Confirmed Transgenic | Notes:                                       |
|-------------------------|----------|-----------------|----------------------|----------------------------------------------|
| H0                      | 300      | 0               | 0                    | All contaminated (no Carb/timentin in media) |
| H.1                     | 300      | 121             | 6                    | 2% transformation                            |
| H1                      | 300      | 18              | 9                    | 3% transformation                            |
| H2                      | 300      | 0               | 0                    | all died on selection                        |
| H10                     | 300      | 0               | 0                    | all died on selection                        |
| TOTALS                  | 1500     | 139             | 15                   | 1% overall transformation                    |

## EXAMPLE 18 – SOYBEAN TRANSFORMATION

Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgett *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of apical meristems (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (four target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee *et al.*, 1989), apical meristems (McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue.

## 18.1 – Soybean transformation constructs

Constructs containing the following plant expression cassettes were labeled as: pDAB9812 (RB7 MARv3 // AtUbi10 promoter // *AAD-12* (v1) // AtuORF23 3'UTR // CsVMV promoter // *DSM-2* (v2) // AtuORF1 3'UTR); pDAB9811 (RB7 MARv3 // CsVMV promoter // *DSM-2* (v2) // AtuORF1 3'UTR) (see Table 6). These constructs were confirmed via restriction enzyme digestion and sequencing. Transformation with these constructs demonstrated the use of *DSM-2* (v2) as an *in vitro* selectable marker and as an effective glufosinate tolerance trait for new, selective use of glufosinate in transgenic soybean. Combination of two herbicide tolerant genes

AAD-12 (v1) with DSM-2 (v2) will allow flexible combinations of 2,4-D, triclopyr, or fluroxypyr with glufosinate for weed control in soybeans.

18.2 – *Transformation Method 1: cotyledonary node transformation of soybean mediated by Agrobacterium tumefaciens.*

The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Though this method was described as early as 1988 (Hinchee *et al.*, 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

18.3 – *Plant transformation production of DSM-2 (v2) tolerant phenotypes.* Seed derived explants of “Maverick” and the *Agrobacterium* mediated cot-node transformation protocol was used to produce DSM-2 (v2) transgenic plants.

18.3.1 – *Agrobacterium preparation and inoculation*

*Agrobacterium* strain EHA101 (Hood et al. 1986), carrying either pDAB9811 or pDAB9812 (Table 6) was used to initiate transformation. Each binary vector contains the *DSM-2* (*v2*) gene as the plant-selectable gene and depending on the construct used includes *AAD-12* (*v1*) as a second gene of interest within the T-DNA region. Each plasmid was mobilized into the EHA101 strain of *Agrobacterium* by electroporation. The selected colonies were analyzed for the integration of genes before the *Agrobacterium* treatment of the soybean explants. Maverick seeds were used in all transformation experiments and the seeds were obtained from University of Missouri, Columbia, MO.

*Agrobacterium*-mediated transformation of soybean (*Glycine max*) using the *DSM-2* (*v2*) gene as a selectable marker coupled with the herbicide glufosinate as a selective agent was carried out using a modified procedure of Zeng et al. (2004). Sterilized seeds were germinated on B5 basal medium (Gamborg et al. 1968) solidified with 3 g/L Phytigel (Sigma-Aldrich, St. Louis, Mo.). Cotyledonary node explants were prepared from 5-6 days old seedlings and infected with *Agrobacterium* as described by Zhang et al., 1999. Cocultivation was carried out for 5 days on the co-cultivation medium containing 400 mg/L L-cysteine (Olhoft and Somers 2001). Shoot initiation, shoot elongation, and rooting media were supplemented with 50 mg/L cefotaxime, 50 mg/L timentin, 50 mg/L vancomycin, and solidified with 3 g/L Phytigel. Selected shoots were then transferred to the rooting medium. The optimal selection scheme used glufosinate at 3 to 10 mg/L at the second shoot initiation stage in the medium and 1-5 mg/L during shoot elongation in the medium.

Prior to transferring elongated shoots (3-5 cm) to rooting medium, the excised end of the internodes were dipped in 1 mg/L indole 3-butyric acid for 1-3 min to promote rooting (Khan et al. 1994). The shoots that have generated roots in 25×100 mm glass culture tubes containing rooting medium were transferred to soil mix (Sogemix by Premium Horticulture Inc., Quakertown, PA) in open Magenta boxes in Conviron for acclimatization of plantlets. Glufosinate, the active ingredient of Liberty herbicide (Bayer Crop Science), was used for selection during shoot initiation and elongation. The rooted plantlets were acclimated in open Magenta boxes for several weeks before they were screened and transferred to the greenhouse for further acclimation and establishment.

18.3.2 –Assay of putatively transformed plantlets, and analyses established  $T_0$  plants in the greenhouse.

The terminal leaflets of selected leaves of these plantlets were leaf painted with 50-100 mg/L of glufosinate twice with a week interval to observe the results to screen for putative transformants. The screened plantlets were transferred to the greenhouse and after acclimation the leaves were painted with glufosinate again to confirm the tolerance status of these plantlets in the GH and deemed to be putative transformants. The screened plants were sampled and molecular analyses for the confirmation of genomic integration of the *DSM-2 (v2)* and *AAD12 (v1)* genes.

*DSM-2 (v2)* transformed  $T_0$  cotton leaf tissue was collected and gDNA was isolated. PCR reactions of the plant transcription units (PTU) were completed of the *DSM-2 (v2)* (pDAB9811) or *DSM-2 (v2)* and *AAD12 (v1)* (pDAB9812). The presence of the expected band size indicated that the plants contained an integrated copy of the transgene within the genome. The results of these PCR screens is provided below in **Table 15**.

**Table 15; Soybean Transgenic Production using DSM2 as a Selectable Marker**

| Construct | Genes      | # of Explants | # of $T_0$ Plants Produced* | # of $T_0$ Plants Analyzed | PTU PCR-Positive | % PTU PCR-Positive |
|-----------|------------|---------------|-----------------------------|----------------------------|------------------|--------------------|
| pDAB9811  | DSM2 only  | 500           | 28                          | 25                         | 16               | 64%                |
| pDAB9812  | DSM2+AAD12 | 500           | 24                          | 22                         | 17               | 77%                |

#### EXAMPLE 19 – COTTON TRANSFORMATION

##### 19.1 –Cotton plant preparation.

Cotton seeds (Co310 genotype) were surface-sterilized in 2% available chlorine plus Tween 20 for one hour, the mixture was placed on a rotary wheel to allow washing of all surfaces.

Seeds were then rinsed a minimum of three times with sterile water. Four seeds were placed in a sundae cup (Solo, SD5) with tall lids (Solo, TN20) for germination on cotton seed media (CSM) (Table 16) and maintained under dark conditions at 28°C for 10 days by which time the seedling growth had reached the top of the container.

#### 19.2 –*Agrobacterium* preparation.

A frozen single use glycerol stock of *Agrobacterium* containing the binary plasmid, pDAB9811 or pDAB9812, was removed from the -80°C freezer and allowed to thaw. 20µl of *Agrobacterium* stock was pipetted onto the surface of a Y-media (Table 16) plate containing streptomycin and spectinomycin. A four quadrant streak of the plate, exchanging loops between each quadrant to dilute the *Agrobacterium* in each area to produce single colonies was performed. These plates were incubated in the dark at 28°C, unwrapped and placed upside down for 48 hours to recover single colonies. After two days, the plates were removed and mini-*Agrobacterium* cultures were initiated by placing 5 ml of Y-media (Table 16) in a Falcon tube (Falcon, item#1309) with 50µg/ml of spectinomycin and 125mg/ml of streptomycin and a single colony from the plate streaked. This culture was then placed in the incubator at 28°C in the dark overnight. The tubes were placed on a rotary drum in the shaker to allow for aeration and mixing. The next day, 35ml of Y media was placed in a 125 ml tri-baffled flask for the start of the over night cultures. Each flask had 100mg/L of spectinomycin and 250mg/L of streptomycin, with 1000µL of mini-*Agrobacterium* culture. These were placed on a shaker at 200rpm in the dark at 28°C overnight. The next day, the *Agrobacterium* solution was poured into a sterile Oakridge tube (Nalge-Nunc, 3139-0050), and centrifuged in the Beckman J2-21 at 8,000 rpm for 5 minutes. The supernatant was poured off and the pellet resuspended in 25 ml of GH1 (Table 16) and vortexed. An aliquot was placed into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). The new suspension was diluted using M liquid media to a Klett-meter reading of  $10^8$  colony forming units per ml with a total volume of 40 ml.

#### 19.3 –*Cotton transformation protocol.*

Hypocotyls were removed from the etiolated seedlings and cut into 1.5-2 cm sections in a sterile Petri dish (Nunc, item #0874728) containing GH3 liquid media (Table 16). The GH3 liquid media was removed and cut segments were treated with an *Agrobacterium* solution for 3 minutes and then transferred to semi-solid GH3 media (Table 16) to undergo co-cultivation for 3

days. Following co-cultivation, segments were transferred to GH1 media (**Table 16**). Carbenicillin was added to kill the *Agrobacterium* and the selection agent, glufosinate-ammonium, was used to select for growth of only those cotton cells that contain the transferred gene.

After four to six weeks, the hypocotyl segments and callus were subcultured to GH2 media (**Table 16**). Every four to six weeks the callus was transferred to this media utilizing a step down selection of 1.0mg/L of glufosinate-ammonia (GLA) and the subsequent transfers to 0.5mg/L of GLA. After 16 weeks, embryogenic callus began to form from the hypocotyl segments and callus. The embryogenic callus could be distinguished from non-embryogenic callus by its yellowish-white color and granular appearance. Callus tissue was collected and gDNA was isolated. PCR reactions of the plant transcription units (PTU) were completed of the *DSM-2* (*v2*) (pDAB9811) or *DSM-2* (*v2*) and *AAD12* (*v1*) (pDAB9812) to identify positive transformants. The presence of the expected band size indicated that the plants contained an integrated copy of the transgene within the genome. The results of these PCR screens is provided below in **Table 17**.

**Table 17: Transgenic Cotton Production Using DSM2 as a Selectable Marker**

| Construct | Construct Details                         | # of Explants | PTU PCR-Positive | Protocol Selection  |
|-----------|-------------------------------------------|---------------|------------------|---------------------|
| pDAB9811  | CsVMV-DSM2-AtORF1                         | 250           | 0                | 1mg/L to .5mg/L GLA |
| pDAB9812  | AtuUbi10-AAD12-AtORF23::CsVMV DSM2-AtORF1 | 250           | 5                | 1mg/L to .5mg/L GLA |
| No Agro   |                                           | 50            | 0                | 1mg/L to .5mg/L GLA |
| No Agro   |                                           | 50            | 0                | 0 GLA               |

As embryos started to form, they were distinguishable from the non-embryogenic callus as they were a distinct green color. One of the methods used to speed up the process of the regeneration of cotton embryos is to stress the callus tissue. Desiccation is a common technique used to accomplish the differentiation of the embryos from the callus tissue. Desiccation was accomplished by changing the microenvironment of the tissue and plate, by using less culture media and/or adopting various modes of plate enclosure (taping versus parafilm) was done as needed to the cultures.

Larger, well-developed embryos were isolated and transferred to GHE1 media (Table 16) for embryo development. After 4-6 weeks (or when the embryos have developed), germinated embryos were transferred to fresh media for shoot and root development. Plantlets were maintained to allow for shoot and root formation, they were placed in individual cups to allow space for growth. Plantlets were observed on a regular basis, and any well-developed plants were transferred into soil and grown to maturity. Once plants matured in the greenhouse leaf paints were done to confirm resistance.

**Table 16.** Media for Cotton Transformation

| Ingredients in 1 liter          | CSM      | GH3 liquid | GH3        | GH1        | GH2        | GHE1     | Y        |
|---------------------------------|----------|------------|------------|------------|------------|----------|----------|
| MS Salts (10X) (Phytotech M524) | 50 ml    | 100 ml     | 100 ml     | 100 ml     | 100 ml     |          |          |
| Glucose                         | 15 grams | 30 grams   | 30 grams   | 30 grams   | 30 grams   | 20 grams |          |
| Gamborg B5 vitamins             | 0.5 ml   | 1 ml       | 1 ml       | 1 ml       | 1 ml       | 1 ml     |          |
| Magnesium chloride hexahydrate  |          | 0.90 grams | 0.90 grams | 0.90 grams | 0.90 grams |          |          |
| 2,4-D (1mg/ml)                  |          |            |            | 100 µl     |            |          |          |
| Gelrite                         | 2 grams  |            | 2 grams    | 2 grams    | 2 grams    | 3 grams  |          |
| Potassium Nitrate               |          | 1.9 grams  | 1.9 grams  | 1.9 grams  | 1.9 grams  |          |          |
| MS Salts (10X) (Phytotech M499) |          |            |            |            |            | 100 ml   |          |
| Sucrose 3%                      |          |            |            |            |            |          | 10 grams |
| NAA                             |          |            |            |            |            |          |          |
| Carbenicillin (250 mg/ml)       |          |            |            | 2 ml       | 2 ml       |          |          |
| GLA (1 mg/ml)                   |          |            |            | 1 ml       | 0.5 ml     |          |          |
| Peptone                         |          |            |            |            |            |          | 10 grams |
| Yeast Extract                   |          |            |            |            |            |          | 10 grams |
| NaCl                            |          |            |            |            |            |          | 5 grams  |

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CLAIMS

5           1.       A transgenic plant cell comprising a polynucleotide that encodes a protein that has phosphinothricin acetyltransferase activity, wherein said polynucleotide hybridizes under conditions of 6XSSC at 65° C with the full complement of a nucleic acid probe that encodes SEQ ID NO:4, and wherein said plant is selected from the group consisting of canola, soybean, cotton, chili, and rice.

10           2.       A transgenic plant comprising a polynucleotide that encodes a protein that has phosphinothricin acetyltransferase activity, wherein said protein is at least 95% identical to SEQ ID NO:4, and wherein said plant is selected from the group consisting of canola, soybean, cotton, chili, and rice.

15           3.       The plant cell of claim 1 wherein said plant cell further comprises an AAD-12 gene.

20           4.       The plant of claim 2 wherein said plant further comprises an AAD-12 gene.

25           5.       A plant comprising a plurality of plant cells of claim 1

30           6.       A method of using a *DSM-2* gene as a selectable marker, said method comprising the steps of

          providing a vector to a plurality of plant cells for expression,  
          culturing the cells in a medium,  
          exposing the cells to phosphinothricin, and  
          determining whether cells are resistant to the phosphinothricin due to expression of a polynucleotide in said vector,

35           wherein said plant cells are selected from the selected from the group consisting of canola, soybean, cotton, tobacco, chili, and rice cells,

said vector comprising a promoter operable in said plant cell, and said polynucleotide operably linked to said promoter, wherein said polynucleotide encodes a protein that has phosphinothricin acetyltransferase activity, and wherein said polynucleotide hybridizes under conditions of 6XSSC at 65° C with the full complement of a nucleic acid probe that encodes an amino acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

7. The method of claim 6 wherein said protein is at least 95% identical to SEQ ID NO:4.

8. The method of claim 6 wherein said vector further comprises a second polynucleotide encoding a second protein of interest.

9. The method of claim 8 wherein said second protein of interest is an insecticidal protein.

10. The method of claim 9 wherein said insecticidal protein is an insecticidal Cry protein.

11. The method of claim 6 wherein said method comprising selecting for a plant cell comprising said vector, growing said plurality of cells in a concentration of a herbicide that permits cells that express said polynucleotide to grow while killing or inhibiting the growth of cells that do not comprise said vector, and wherein said herbicide comprises a phosphinothricin molecule.

12. The method of claim 11 wherein said herbicide is selected from the group consisting of bialaphos and glufosinate.

13. The method of claim 11 wherein said method comprises identifying, selecting, and regenerating plant cells that comprise said vector.

14. A seed of a plant of claim 2.

15. A plant cell of claim 1 wherein said cell further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis*, *Photorhabdus*, and *Xenorhabdus*.

5 16. A plant cell of claim 1 wherein said cell further comprises a second herbicide-tolerance gene.

17. A plant of claim 2 wherein said plant is tolerant to a second herbicide.

10 18. A plant of claim 2 wherein said plant comprises a second herbicide-tolerance gene.

19. A process for generating phosphinothricin-tolerant plant cells, plants, and their propagates wherein said method comprises regenerating plant cells of claim 1 to plants, and producing propagates from said plants.

15 20. A process for producing a plant that is tolerant to the herbicidal activity of a glutamine synthetase inhibitor including phosphinothricin or a compound with a phosphinothricin moiety, wherein said process comprises the steps of a) producing a plant cell of claim 1, and b) regenerating a plant from said cell, said plant comprising said polynucleotide in its nuclear genome.

20 21. A process for increasing yield of a group of cultivated plants in a field, wherein said method comprises destroying weeds in said field, wherein said plants are according to claim 2, and wherein said weeds are destroyed by application of a herbicide comprising a glutamine synthetase inhibitor as an active ingredient.

25 22. A process for producing a pure culture of plant cells of claim 1 that have a foreign DNA incorporated into their nuclear genome, said method comprising the steps of:

30 i) transforming starting plant cells in a plant cell culture with a foreign DNA, said foreign DNA comprising:

a) a promoter recognized by a polymerase of said starting plant cell, and

- b) a coding region comprising said polynucleotide; and
- ii) selecting the transformed plant cells by applying to the plant cell culture a glutamine synthetase inhibitor, including phosphinothricin or a compound with a phosphinothricin moiety, at a sufficient concentration to kill the untransformed plant cells.

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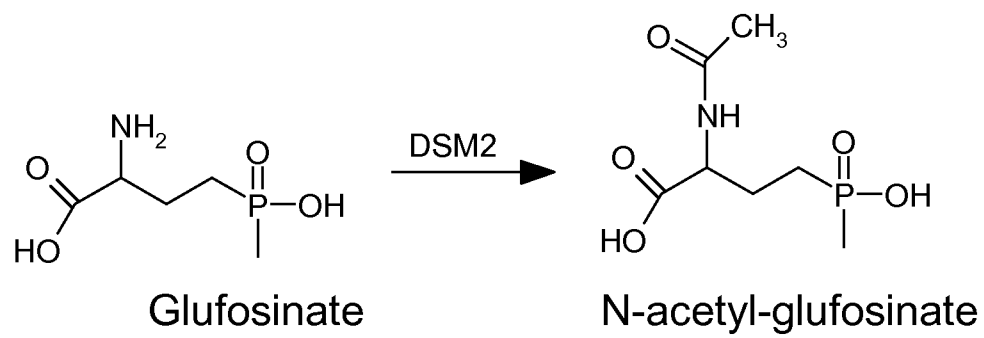
23. The method of claim 6 wherein said method comprises selecting for a plant cell comprising said vector, wherein said method comprises

- a) providing said vector to a plurality of plant cells; and
- b) growing said plurality of cells in a concentration of a herbicide that permits

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cells that express said polynucleotide to grow while killing or inhibiting growth of cells that lack said vector, wherein said herbicide comprises phosphinothricin.

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**FIGURE 1**