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(54) Title: GLYPHOSATE RESISTANT PLANTS AND ASSOCIATED METHODS

[illegible]

FIG. 1

(57) Abstract: The present disclosure relates to certain polypeptides derived from prokaryotic DGT enzymes, and nucleic acids useful in encoding the same.

GLYPHOSATE RESISTANT PLANTS AND ASSOCIATED METHODS

PRIORITY CLAIM

This application claims the benefit of U.S. Provisional Patent Application
5 Serial No. 61/593,555 filed February 1, 2012, and also to U.S. Provisional Patent
Application Serial No. 61/625,222, filed April 17, 2012.

STATEMENT ACCORDING TO 37 C.F.R. § 1.821(c) or (e) - SEQUENCE LISTING SUBMITTED AS ASCII TEXT FILE

10 Pursuant to 37 C.F.R. § 1.821(c) or (e), a file containing an ASCII text version
of the Sequence Listing has been submitted concomitant with this application.

TECHNICAL FIELD

The present disclosure relates to plant biotechnology. Particular embodiments
15 relate to novel polypeptides involved in metabolism of N-(phosphonomethyl) glycine,
and nucleic acids encoding such polypeptides. Particular embodiments relate to plants,
plant plant parts, and plant cells that comprise a foregoing polypeptides and/or nucleic
acids.

20 BACKGROUND

Weed species have long been a problem in cultivated fields. Although weed
control can be a labor intensive operation, it has been made easier by the availability of
efficient weed killing chemical herbicides. The widespread use of herbicides, along
with improved crop varieties and fertilizers, has made a significant contribution to
25 the “green revolution” in agriculture. Particularly useful herbicides are those that
have a broad spectrum of herbicidal activity. Unfortunately, broad spectrum
herbicides typically have a deleterious effect on crop plants exposed to the herbicide.
One way to overcome this problem is to produce crop plants that are tolerant to the
broad spectrum herbicide.

30 One example of a broad spectrum herbicide is N-phosphonomethyl-glycine,
also known as glyphosate. Glyphosate has been used extensively by farmers
worldwide for controlling weeds prior to crop planting, for example, in no-till farming.
In addition, glyphosate is an efficient means to control weeds and volunteer plants

between production cycles or crop rotations. Glyphosate does not carry-over in soils after use, and it is widely considered to be one of the most environmentally safe and broadly effective chemical herbicides available for use in agriculture.

5 Glyphosate kills plants by inhibiting the shikimic acid pathway. This pathway leads to the biosynthesis of aromatic compounds, including amino acids, vitamins, and plant hormones. Glyphosate blocks the condensation of phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by binding to and inhibiting activity of the enzyme 5-enolpyruvylshikimate

10 -3-phosphate synthase, commonly referred to as "EPSP synthase," and "EPSPS." Unfortunately, no crop plants are known that are naturally tolerant to glyphosate, and, therefore, the utility of this herbicide for weed control in cultivated crops has been limited. One method to produce glyphosate-tolerant crop plants is to introduce a gene encoding a heterologous glyphosate-tolerant form of an EPSPS gene into the crop plant using the techniques of genetic engineering. Using

15 chemical mutagenesis, glyphosate tolerant forms of EPSPS have been produced in bacteria, and the heterologous genes were introduced into plants to produce glyphosate-tolerant plants. *See, e.g., Comai et al. (1983) Science 221:370-71.* The heterologous EPSPS genes may be overexpressed in the crop plants to obtain a desired level of tolerance.

20 The foregoing examples of the related art and limitations related therewith are intended to be illustrative and not exclusive. Other limitations of the related art will become apparent to those of skill in the art upon a reading of the specification.

DISCLOSURE OF THE INVENTION

25 Described herein are isolated polypeptides having at least 90% identity to SEQ ID NO:1, and nucleic acids encoding a polypeptide having at least 90% identity to SEQ ID NO:1 (*e.g.*, SEQ ID NOs:2-4). Also described are plants, plant parts, plant organs, plant seed, and plant cells comprising a heterologous polypeptide having at least 90% identity to SEQ ID NO:1.

30 Some embodiments include a plant, plant part, plant organ, plant seed, and/or plant cell comprising a heterologous nucleic acid encoding a polypeptide having at least 90% identity to SEQ ID NO:1. In particular examples, the heterologous nucleic acid comprises a nucleotide sequence having at least 80% sequence identity to SEQ ID

NO:2 or SEQ ID NO:3. Some embodiments include a plant, plant part, plant organ, plant seed, and/or plant cell comprising a heterologous nucleic acid that hybridizes to another nucleic acid having SEQ ID NO:2 or SEQ ID NO:3 under high-stringency conditions. In particular examples, the plant, plant part, plant organ, plant seed, and/or plant cell comprising a heterologous nucleic acid that hybridizes to another nucleic acid having SEQ ID NO:2 or SEQ ID NO:3 under high-stringency conditions comprises a polypeptide encoded by the heterologous nucleic acid that confers glyphosate tolerance (or increases glyphosate tolerance) to the plant, plant part, plant organ, plant seed, and/or plant cell.

10 In further embodiments, the disclosure relates to methods of generating a plant, plant part, plant organ, plant seed, and/or plant cell resistant to glyphosate comprising: transforming a plant, plant part, plant organ, plant seed, and/or plant cell with a nucleic acid encoding a polypeptide having at least 90% identity to SEQ ID NO:1; and expressing the nucleic acid so as to produce the polypeptide having at least 90% identity to SEQ ID NO:1.

15 Other embodiments include vectors comprising a nucleic acid encoding a polypeptide having at least 90% identity to SEQ ID NO:1. Particular examples include vectors comprising a nucleic acid encoding a polypeptide having at least 95% identity to SEQ ID NO:1. For example, a vector may comprise a nucleic acid sequence having at least 90% identity to SEQ ID NO:2 or SEQ ID NO:3.

20 Particular embodiments include glyphosate tolerant plants and plant cells expressing a heterologous polypeptide having at least 90% identity to SEQ ID NO:1.

25 Additional embodiments include methods for controlling weeds in a field or area under cultivation containing glyphosate-resistant plants, wherein such a method may comprise: planting a plant or a plant seed comprising a nucleic acid encoding a heterologous polypeptide having at least 90% identity to SEQ ID NO:1 in the field or area under cultivation; and applying to the field or area under cultivation a sufficient amount of glyphosate to control weeds in the field without significantly affecting the plant.

30 In some embodiments, the disclosure relates to regenerable cells for use in tissue culture of plants resistant to glyphosate. Such a tissue culture may be capable of regenerating plants having the physiological and morphological characteristics of the foregoing glyphosate-resistant plants, and also of regenerating plants having

substantially the same genotype as the glyphosate-resistant plants. Regenerable cells in such tissue cultures may be, for example, embryos, protoplasts, meristematic cells, callus, pollen, leaves, anthers, roots, root tips, flowers, seeds, pods, and stems. Particular embodiments relate to plants regenerated from a tissue culture according to the foregoing.

In some embodiments, the disclosure relates to cells that are not regenerable to produce plants, for example for use in producing plant cell lines resistant to glyphosate. In other embodiments, the disclosure relates to plants comprising in part such cells.

In certain embodiments, the present disclosure relates to the application of multiple herbicides to crops planted in an area under cultivation. An over the top application of glyphosate in addition to multiple herbicides takes advantage of the different herbicide properties, so that weed control is provided with an improved combination of flexibility and economy. For example, individual herbicides may have different longevities in the area under cultivation; *i.e.*, some herbicides may persist and be effective for a relatively long time after they are applied to the area, while other herbicides may be quickly broken down into other and/or non-active compounds. An improved herbicide application system according to particular embodiments allows the use of glyphosate and multiple herbicides so that a grower can tailor the selection of particular herbicides for use in a particular situation.

In other embodiments, the present disclosure relates to methods and compositions for making and using a plant that is tolerant to more than one herbicide or class or subclass of herbicide, as described below. In particular embodiments, a plant is provided that is tolerant to both glyphosate and at least one other herbicide (or class or subclass of herbicide) or chemical (or class or subclass of chemical) (*e.g.*, fungicides, insecticides, plant growth regulators and the like). Such plants may find use, for example, in methods comprising treatment of crop plants with multiple herbicides. Thus, the disclosure provides herbicide-resistant plants which tolerate treatment with an herbicide or combination of herbicides (including a combination of herbicides that each act through a different mode of herbicidal activity) or which tolerate treatment with a combination of at least one herbicide and at least one other chemical. In this manner, the disclosure describes improved methods of growing crop plants in which weeds are selectively controlled.

An herbicide-resistant plant according to some embodiments may comprise a nucleic acid molecule that encodes a heterologous polypeptide that confers tolerance to glyphosate and a nucleic acid molecule encoding a polypeptide that confers tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D). According to the foregoing paragraphs, plants are provided that comprise at least a third nucleic acid molecule encoding a polypeptide imparting to the plant a trait selected from the group consisting of an herbicide tolerance trait; an insect resistance trait; an agronomic trait; a disease resistance trait; a modified fatty acid trait; and a reduced phytate trait.

In some examples, an herbicide-resistant plant comprises a heterologous nucleic acid molecule encoding a polypeptide that confers tolerance to glyphosate and a nucleic acid molecule encoding a polypeptide that confers tolerance to glufosinate. Some examples include an herbicide-resistant plant comprising a nucleic acid molecule encoding a polypeptide imparting to the plant a trait selected from the group consisting of an herbicide tolerance trait; an insect resistance trait; an agronomic trait; a disease resistance trait; a modified fatty acid trait; and a reduced phytate trait.

In particular examples, a herbicide-resistant plant comprises a heterologous nucleic acid molecule encoding a polypeptide that confers tolerance to glyphosate and a nucleic acid molecule encoding a polypeptide that confers tolerance to a herbicide that inhibits acetolactate synthase (ALS) (Lee *et al.* (1988) EMBOJ. 7:1241), also known as acetohydroxyacid synthase (AHAS) enzyme (Miki *et al.* (1990) Theor. Appl. Genet. 80:449). Some examples include an herbicide-resistant plant comprising a nucleic acid molecule encoding a polypeptide imparting to the plant a trait selected from the group consisting of an herbicide tolerance trait; an insect resistance trait; an agronomic trait; a disease resistance trait; a modified fatty acid trait; and a reduced phytate trait.

In some embodiments, a nucleic acid may be combined (or “stacked”) in a plant with any other nucleic acid molecule, for example and without limitation, to provide additional resistance or tolerance to glyphosate or another herbicide, to provide resistance to select insects or diseases, to provide nutritional enhancements, to provide improved agronomic characteristics, and to provide a protein or other product useful in feed, food, industrial uses, pharmaceutical uses, and/or other uses. Examples include the stacking of two or more nucleic acids of interest within a plant genome. Such a “gene stack” may be accomplished *via* conventional plant breeding using two or more

events, transformation of a plant with a construct that contains the sequences of interest, re-transformation of a transgenic plant, or addition of new traits through targeted integration *via* homologous recombination. Particular examples of such a stack include any combination of the following: a *dgt-28* nucleic acid; a Cry34Ab1
5 nucleic acid; a Cry35Ab1 nucleic acid; a Cry1F nucleic acid; a Cry1Ac nucleic acid; an *aad-12* nucleic acid; an *aad-1* nucleic acid; a *pat* nucleic acid; and a *DSM-2* nucleic acid.

In addition to the exemplary aspects and embodiments described above, further aspects and embodiments will become apparent by study of the following descriptions.

10

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 includes a partial sequence alignment of exemplary EPSP synthase enzymes (*i.e.*, DGT-28, DGT-32, DGT-33, and others). All three of these exemplary DGT enzymes share a conserved alanine at the *aroA* EPSP synthase enzyme amino
15 acid position 96. The location of this amino acid is indicated by an asterisk, and the amino acid residue is underlined.

FIG. 2 includes an alignment of exemplary DGT enzymes (*i.e.*, DGT-1, DGT-3, and DGT-7). The location of a mutated amino acid residue that was changed from a glycine to an alanine is indicated by the first asterisk. The location of a second
20 mutated amino acid residue that was changed from a threonine to an isoleucine is indicated by the second asterisk. The location of a third mutated amino acid residue that was changed from a proline to a serine is indicated by the third asterisk.

FIGs. 3-30 include maps of various exemplary plasmids: pDAB107527 (FIG. 3); pDAB105530 (FIG. 4); pDAB105531 (FIG. 5); pDAB105532 (FIG. 6);
25 pDAB105533 (FIG. 7); pDAB105534 (FIG. 8); pDAB4104 (FIG. 9); pDAB102715 (FIG. 10); pDAB107532 (FIG. 11); pDAB107534 (FIG. 12); pDAB102785 (FIG. 13); pDAB100445 (FIG. 14); pDAB102946 (FIG. 15); pDAB100469 (FIG. 16); pDAB102028 (FIG. 17); pDAB102029 (FIG. 18); pDAB102032 (FIG. 19); pDAB102034 (FIG. 20); pDAB100429 (FIG. 21); pDAB100442 (FIG. 22);
30 pDAB100430 (FIG. 23); pDAB102036 (FIG. 24); pDAB102038 (FIG. 25); pDAB102040 (FIG. 26); pDAB102042 (FIG. 27); pDAB107712 (FIG. 28); pDAB107713 (FIG. 29); and pDAB107714 (FIG. 30).

FIG. 31 includes IC₅₀ values obtained after introduction of various mutations within DGT-1 (A) and DGT-7 (B) using 1 mM PEP. For both FIG. 31(A) and FIG. 31(B) IC₅₀ curves, closed triangles represent wild-type, closed circles represent GA mutants, open squares represent GAPS mutants, and closed squares represent TIPS mutants.

FIGs. 32-46 include maps of various exemplary plasmids: pDAB102719 (FIG. 32); pDAB102718 (FIG. 33); pDAB107663 (FIG. 34); pDAB107664 (FIG. 35); pDAB107665 (FIG. 36); pDAB107666 (FIG. 37); pDAB109812 (FIG. 38); pDAB101556 (FIG. 39); pDAB107698 (FIG. 40); pDAB108384 (FIG. 41); pDAB108385 (FIG. 42); pDAB108386 (FIG. 43); pDAB108387 (FIG. 44); pDAB102716 (FIG. 45); pDAB102717 (FIG. 46); pDAB110828 (FIG. 47); pDAB110827 (FIG. 48); pDAB107545 (FIG. 49); pDAB107548 (FIG. 50); pDAB107553 (FIG. 51); pDAB102792 (FIG. 52); pDAB107602 (FIG. 53); and pDAB107533 (FIG. 54).

MODE(S) FOR CARRYING OUT THE INVENTION

I. Overview

Disclosed herein are novel polypeptides involved in metabolism of N-(phosphonomethyl) glycine, and nucleic acids encoding such polypeptides. In some examples, such a polypeptide confers (or increases) tolerance to glyphosate in a plant cell wherein the polypeptide is heterologously expressed, for example, without adversely affecting the binding of EPSP synthase with its natural substrate, phosphoenolpyruvate (PEP).

II. Terms

In order to further clarify the breadth of this disclosure, the following specific definitions, terms, and abbreviations are provided.

Unless specifically defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. Unless otherwise clear from the context in which it appears, a singular term shall include pluralities, and plural terms are understood to include the singular. Thus, the indefinite articles "a" and "an," as used preceding an element or component are non-restrictive regarding the number of instances (*i.e.*, occurrences) of the element or

component. Where ranges of numerical values are provided herein (*e.g.*, “less than about X,” “less than X,” and “for example, X₁... and X₂”), the ranges are understood to include all values and ranges of values included within the provided range, as if these included values and ranges had been expressly recited.

5 As used herein, the terms “comprising,” “including,” “having,” and “containing,” and variations thereof, are open-ended (*i.e.*, non-exclusive). For example, a composition or method that comprises a list of elements is not necessarily limited to only those elements. Such a composition or method may (or may not) include other elements not expressly listed or inherent to the composition or method.

10 Further, unless expressly stated to the contrary, “or” is used in the inclusive (and not the exclusive) sense. For example, a condition “A or B” is satisfied by any of the following: A is true (or present) and B is false (or not present); A is false (or not present) and B is true (or present); and both A and B are true (or present).

Plant: As used herein, the term “plant” includes a whole plant and any

15 descendant, cell, tissue, or part of a plant. The term “plant parts” include any part(s) of a plant, including, for example and without limitation: seed (including mature seed and immature seed); a plant cutting; a plant cell; a plant cell culture; a plant organ (*e.g.*, pollen, embryos, flowers, fruits, shoots, leaves, roots, stems, and explants). A plant tissue or plant organ may be a seed, protoplast, callus, or any other group of plant cells

20 that is organized into a structural or functional unit. A plant cell or tissue culture may be capable of regenerating a plant having the physiological and morphological characteristics of the plant from which the cell or tissue was obtained, and of regenerating a plant having substantially the same genotype as the plant. In contrast, some plant cells are not capable of being regenerated to produce plants. Regenerable

25 cells in a plant cell or tissue culture may be embryos, protoplasts, meristematic cells, callus, pollen, leaves, anthers, roots, root tips, silk, flowers, kernels, ears, cobs, husks, or stalks.

Plant parts include harvestable parts and parts useful for propagation of progeny plants. Plant parts useful for propagation include, for example and without

30 limitation: seed; fruit; a cutting; a seedling; a tuber; and a rootstock. A harvestable part of a plant may be any useful part of a plant, including, for example and without limitation: flower; pollen; seedling; tuber; leaf; stem; fruit; seed; and root.

A plant cell is the structural and physiological unit of the plant, comprising a protoplast and a cell wall. A plant cell may be in the form of an isolated single cell, or an aggregate of cells (*e.g.*, a friable callus and a cultured cell), and may be part of a higher organized unit (*e.g.*, a plant tissue, plant organ, and plant). Thus, a plant cell
5 may be a protoplast, a gamete producing cell, or a cell or collection of cells that can regenerate into a whole plant. As such, a seed, which comprises multiple plant cells and is capable of regenerating into a whole plant, is considered a “plant cell” in embodiments herein.

Herbicide resistance/tolerance: When referring to plants that are resistant or
10 tolerant to glyphosate, it is meant that an application of an amount of glyphosate on the plant does not significantly affect or kill the plant, wherein a wild-type plant of the same species would be significantly affected and/or killed by the application of the amount of glyphosate. A plant may be naturally tolerant to a particular herbicide, or a plant may be rendered herbicide tolerant as a result of genetic engineering, such as
15 for example, selective breeding; genetic transformation; and/or the introduction of a transgene within the genome of the plant. A “glyphosate resistant plant” refers to a plant containing a polypeptide or nucleic acid molecule that confers herbicide tolerance when provided to a heterologous plant or other organism expressing it (*i.e.*, that makes a plant or other organism herbicide-tolerant).

20 A plant that is resistant or tolerant to glyphosate may show some minimal impact from the application of glyphosate to the plant. For instance, there can be an alteration in the normal growth and development of the plant, wherein the plant may exhibit signs or symptoms that are associated with stress or disease. Such a minimal impact resulting from the application of glyphosate to plants that are resistant or
25 tolerant to glyphosate is in contrast to the adverse impact that results from application of glyphosate to plants that are susceptible to glyphosate. One of skill in the art can distinguish between plants that are resistant to glyphosate and plants that are susceptible to glyphosate. Application of glyphosate to plants comprising a nucleic acid that confers glyphosate tolerance results in significantly less impact than
30 application of the same amount of glyphosate to a plant of the same species that does not comprise a nucleic acid molecule that confers tolerance to glyphosate.

A plant that is tolerant to an herbicide or other chemical shows improved tolerance in comparison to an appropriate control plant. Damage resulting from

herbicide or other chemical treatment may be assessed by evaluating any parameter of plant growth or well-being. Such parameters are known to those of skill in the art, and their selection is within the discretion of the skilled person. Plant damage can be assessed by visual inspection and/or by statistical analysis of one or more
5 suitable parameter(s) of plant growth or well-being in individual plants or a group(s) of plants. Thus, damage may be assessed by evaluating parameters including, for example and without limitation: plant height; plant weight; leaf color; leaf length; flowering; fertility; silking; yield; and seed production. Damage may also be assessed by evaluating the time elapsed to a particular stage of development (*e.g.*,
10 silking, flowering, and pollen shed), or the time elapsed until a plant has recovered from treatment with a particular chemical and/or herbicide.

In making damage assessments, values may be assigned to particular degrees of damage so that statistical analysis or quantitative comparisons may be made. The use of ranges of values to describe particular degrees of damage is known in the art,
15 and any suitable range or scale may be used. For example, herbicide injury scores (also called tolerance scores) may be assigned. Accordingly, herbicide tolerance may also indicated by other ratings in this scale, where an appropriate control plant (or group of control plants) exhibits a statistically lower score on the scale in response to an herbicide treatment than a group of subject plants.

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

A herbicide does not “significantly affect” a plant when it either has no effect on the plant, when it has some effect on the plant from which the plant later recovers, or when it has an effect on the plant that is detrimental but which is offset,

for example, by the impact of the particular herbicide on weeds. Thus, for example, a crop plant may not be “significantly affected” by a herbicide or other treatment if the plant exhibits less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% decrease in at least one suitable parameter that is indicative of plant health and/or productivity, in comparison to an appropriate control plant (*e.g.*, an untreated plant of the same species). In particular embodiments, a plant is tolerant to a herbicide or other chemical if it shows damage in comparison to an appropriate control plant that is less than the damage exhibited by the control plant by at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or 1000% or more. A crop plant that is not significantly affected by an herbicide or other treatment may exhibit a decrease in at least one parameter, but the decrease is temporary in nature, and the plant recovers fully within, for example, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, or about 6 weeks. In particular embodiments, a plant that is tolerant to a herbicide or other chemical may be characterized by the fact that the plant is not significantly affected by application of the herbicide or other chemical.

Suitable parameters that are indicative of plant health and/or productivity include, for example and without limitation: plant height; plant weight; leaf length; time elapsed to a particular stage of development; flowering; yield; and seed production. The evaluation of a parameter may be performed by visual inspection and/or by statistical analysis of the parameter. Once evaluated in a subject plant and a control plant, a comparison may be made so as to determine if the subject plant is significantly affected by the herbicide or other treatment. if it.

Appropriate control plants that may be used to determine resistance to an herbicide (or other chemical) include plants of the same species that do not comprise a putative heterologous herbicide tolerance nucleic acid and/or polypeptide, and plants that do comprise the putative heterologous herbicide tolerance nucleic acid and/or polypeptide, but which have not been treated with the herbicide.

Herbicide: A “herbicide” is a chemical that causes temporary or permanent injury to a plant. Non-limiting examples of herbicides are listed and discussed in

further detail elsewhere herein. A herbicide may be incorporated into a plant or its cells, or it may act on the plant or cells without being incorporated. An “active ingredient” is a chemical in a herbicide formulation that is responsible for the phytotoxicity of the formulation. Active ingredients in commercial herbicide formulations are typically identified as an active ingredient on the product label. Product label information is available from the U.S. Environmental Protection Agency, and is updated online at oaspub.epa.gov/pestlabl/ppls.own. Product label information is also available online at www.cdms.net.

When used in regard to an herbicide, the term “acid equivalent” refers to the rate or quantity as the herbicidal active parent acid.

Isolated: An “isolated” biological component (such as a nucleic acid or polypeptide) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs (*i.e.*, other chromosomal and extra-chromosomal DNA and RNA, and proteins), while effecting a chemical or functional change in the component (*e.g.*, a nucleic acid may be isolated from a chromosome by breaking chemical bonds connecting the nucleic acid to the remaining DNA in the chromosome). Nucleic acid molecules and proteins that have been “isolated” include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell, as well as chemically-synthesized nucleic acid molecules, proteins, and peptides.

Nucleic acid: The terms “polynucleotide,” “nucleic acid,” and “nucleic acid molecule” are used interchangeably herein, and encompass a singular nucleic acid; plural nucleic acids; a nucleic acid fragment, variant, or derivative thereof; and nucleic acid construct (*e.g.*, messenger RNA (mRNA) and plasmid DNA (pDNA)). A polynucleotide or nucleic acid may contain the nucleotide sequence of a full-length cDNA sequence, or a fragment thereof, including untranslated 5' and/or 3' sequences and coding sequence(s). A polynucleotide or nucleic acid may be comprised of any polyribonucleotide or polydeoxyribonucleotide, which may include unmodified ribonucleotides or deoxyribonucleotides or modified ribonucleotides or deoxyribonucleotides. For example, a polynucleotide or nucleic acid may be comprised of single- and double-stranded DNA; DNA that is a mixture of single- and double-stranded regions; single- and double-stranded RNA; and RNA that is mixture

of single- and double-stranded regions. Hybrid molecules comprising DNA and RNA may be single-stranded, double-stranded, or a mixture of single- and double-stranded regions. The foregoing terms also include chemically, enzymatically, and metabolically modified forms of a polynucleotide or nucleic acid.

5 It is understood that a specific DNA refers also to the complement thereof, the sequence of which is determined according to the rules of deoxyribonucleotide base-pairing.

 As used herein, the term “gene” refers to a nucleic acid that encodes a functional product (RNA or polypeptide/protein). A gene may include regulatory
10 sequences preceding (5' non-coding sequences) and/or following (3' non-coding sequences) the sequence encoding the functional product.

 As used herein, the term “coding sequence” refers to a nucleic acid sequence that encodes a specific amino acid sequence. A “regulatory sequence” refers to a nucleotide sequence located upstream (*e.g.*, 5' non-coding sequences), within, or
15 downstream (*e.g.*, 3' non-coding sequences) of a coding sequence, which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences include, for example and without limitation: promoters; translation leader sequences; introns; polyadenylation recognition sequences; RNA processing sites; effector binding sites; and stem-loop structures.

20 As used herein, the term “codon degeneracy” refers to redundancy in the genetic code that permits variation of a particular nucleotide sequence without affecting the amino acid sequence of the encoded polypeptide. Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino
25 acids (the remaining three codons encode signals ending translation). As a result, many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six, whereas tryptophan and methionine are coded by just one triplet. The “genetic code” that shows which codons encode which amino acids is commonly known in the art.
30 The degeneracy therein allows for the bases of a DNA to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA.

 In some embodiments herein, when designing a coding sequence for improved expression in a host cell, the gene is designed such that the frequency of codon usage

therein approaches the frequency of the preferred codon usage of the host cell. Accordingly, the term “codon-optimized” refers to genes or coding sequences of nucleic acids for transformation of various hosts, wherein codons in the gene or coding sequence has been altered to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the nucleic acid. In examples, such optimization includes replacing at least one, more than one, a significant number, and/or all of the codons in the gene or coding sequence with one or more codons that are more frequently used in the genes of that organism.

Many organisms display a bias for use of particular codons to code for insertion of a particular amino acid in a growing peptide chain. Codon preference, or codon bias, differences in codon usage between organisms, is afforded by degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, *inter alia*, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored or designed for optimal gene expression in a given organism based on codon optimization.

Given the large number of gene sequences available for a wide variety of animal, plant and microbial species, it is possible to calculate the relative frequencies of codon usage. Codon usage tables are readily available, for example, at the “Codon Usage Database” available on the internet at kazusa.or.jp/codon/, and these tables can be adapted in a number of ways. See Nakamura *et al.* (2000) Nucl. Acids Res. 28:292. By utilizing a codon usage table, one of skill in the art can apply the frequencies corresponding to a given species to any given polypeptide sequence, to design and produce a synthetic nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide, but which uses codons optimal for the species.

Codon bias is reflected in the mean base composition of protein coding regions. For example, organisms having genomes with relatively low G+C contents utilize more codons having A or T in the third position of synonymous codons, whereas those having higher G+C contents utilize more codons having G or C in the third position. Further, 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 reasoning 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
5 could be reflected by correspondingly low levels of the encoded protein.

The codon bias can be calculated as the frequency at which a single codon is used relative to the codons for all amino acids. Alternatively, the codon bias may be calculated as the frequency at which a single codon is used to encode a particular amino acid, relative to all the other codons for that amino acid (synonymous codons).

10 The term "percent identity" (or "% identity") refers to a relationship between two or more polypeptide sequences (or polynucleotide sequences), as determined by comparing the sequences. The percent identity may express the degree of sequence relatedness between polypeptide (or polynucleotide) sequences, as may be determined by the match between strings of such sequences. In general, identity refers to an exact
15 nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. *See* Russell and Barton (1994) J. Mol. Biol. 244:332-50.

20 Techniques for aligning nucleic acid and amino acid sequences and determining identity are known in the art, and include, for example and without limitation, those provided in: Computational Molecular Biology (1988) (Lesk, A. M., Ed.) Oxford University, NY; Biocomputing: Informatics and Genome Projects (1993) (Smith, D. W., Ed.) Academic, NY; Computer Analysis of Sequence Data, Part I
25 (1994) (Griffin, A. M., and Griffin, H. G., Eds.) Humana, NJ; Sequence Analysis in Molecular Biology (1987) (von Heinje, G., Ed.) Academic, NY; and Sequence Analysis Primer (1991) (Gribskov, M. and Devereux, J., Eds.) Stockton, NY. A technique for determining the percent identity between two sequences may include providing the nucleotide sequence of an mRNA or gene and/or providing or inferring
30 the amino acid sequence encoded thereby, and comparing the sequence(s) to a second nucleotide and/or amino acid sequence. Genomic sequences can also be determined and compared in this fashion.

In addition, methods for aligning nucleic acid and amino acid sequences and determining identity are incorporated in various publicly available computer software programs. Sequence alignments and percent identity calculations can be performed, for example, using the AlignX™ program of the Vector NTI® suite (Invitrogen, Carlsbad, CA) or MegAlign™ program of the LASERGENE™ bioinformatics computing suite (DNASTAR™ Inc., Madison, WI). Multiple alignment of sequences may be performed using the Clustal™ method, which encompasses several varieties of an alignment algorithm, including Clustal™ V and Clustal™ W (Higgins and Sharp (1989) CABIOS 5:151-3; Higgins *et al.* (1992) Comput. Appl. Biosci. 8:189-91). For multiple alignments in Clustal™ V, default values that may be used include GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for multiple alignment in Clustal™ W include (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs(%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB). Default parameters for pairwise alignments and calculation of percent identity between protein sequences that may be used in a Clustal™ method are KTUPLE=1, GAP PENALTY=3, WINDOW=5, and DIAGONALS SAVED=5. For nucleic acids, these default parameters may be KTUPLE=2, GAP PENALTY=5, WINDOW=4, and DIAGONALS SAVED=4. After alignment of sequences using a Clustal™ program, it is possible to obtain a “percent identity” by viewing the “sequence distances” table in the same program.

In some embodiments, a nucleic acid encodes a polypeptide having a sequence identity (when compared to a reference polypeptide; *e.g.*, a DGT polypeptide) of, for example and without limitation: at least about 55%; at least about 60%; at least about 65%; at least about 70%; at least about 75%; at least about 80%; at least about 85%; at least about 90%; and at least about 95%, has the same or similar function as the reference polypeptide. Accordingly, any integer percentage of identity from, for example, 55% to 100% may be useful in describing particular nucleic acids herein, for example and without limitation: 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%, and 99%. Certain nucleic acid fragments not only have the foregoing sequence identity, but may encode a polypeptide having, for example and

without limitation: at least 50 amino acids; at least 100 amino acids; at least 150 amino acids; at least 200 amino acids; and at least 250 amino acids. Particular embodiments include a nucleic acid having at least about 90% identity to SEQ ID NO:1 (*e.g.*, at least 89% identity; at least about 90% identity; at least about 91% identity; at least about 92% identity; at least about 93% identity; at least about 94% identity; at least about 95% identity; at least about 96% identity; at least about 97% identity; at least about 98% identity; at least about 99% identity; and at least about 99.5% identity).

The term “sequence analysis software” refers to a computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. “Sequence analysis software” may be commercially available or independently developed. Non-limiting examples of sequence analysis software includes: the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI); BLASTP[™], BLASTN[™], and BLASTX[™] (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10); DNASTAR[™] (DNASTAR[™], Inc. Madison, WI); Sequencher[™] (Gene Codes Corporation, Ann Arbor, MI); and the FASTA[™] program incorporating the Smith-Waterman algorithm (Pearson (1994) Comput. Methods Genome Res. [Proc. Int. Symp.], Meeting Date 1992 (Suhai and Sandor, Eds.), Plenum: New York, NY, pp. 111-20). Where sequence analysis software has been used to analyze a nucleotide or amino acid sequence herein, the results of the analysis shown have been generated using default values of the program referenced, unless otherwise specified. As used herein, the term “default values” refers to a set of values or parameters that originally loads with the sequence analysis software when it is first initialized.

Hybridization: A nucleic acid comprising all or part of a nucleotide sequence may be used as a probe that selectively “hybridizes” to nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (*e.g.*, genomic or cDNA libraries from a chosen organism) that have a significant amount of sequence identity to the probe sequence. A hybridization probe may be a genomic DNA fragment; a plasmid DNA fragment; a cDNA fragment; an RNA fragment; a PCR amplified DNA fragment; an oligonucleotide; or other polynucleotide, and a probe may be labeled with a detectable group (*e.g.*, ³²P), or any other detectable marker. Thus, for example and without limitation, a probe for hybridization may be made by labeling a synthetic oligonucleotide that specifically hybridizes to a nucleic acid herein (*e.g.*, a

nucleic acid having at least about 90% identity to SEQ ID NO:1). Methods for preparation of probes for hybridization, and for construction of cDNA and genomic libraries, are known in the art. Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. An extensive guide to the hybridization of nucleic acids can be found in Sambrook *et al.* (1989), *supra*; and Ausubel *et al.* (1997) Short Protocols in Molecular Biology, Third Edition, Wiley, NY, New York, pp. 2-40.

In some embodiments, nucleic acid hybridization (*e.g.*, to amplified DNA) may be used to identify the presence of a transgenic event in a sample. Nucleic acid molecules or fragments thereof are capable of “specifically hybridizing” to another nucleic acid molecule under certain circumstances. In some examples, a nucleic acid specifically hybridizes under stringent conditions to a target nucleic acid. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure under stringent (*e.g.*, high-stringency) conditions.

A nucleic acid is said to be the “complement” of another nucleic acid molecule if the two nucleic acid molecules exhibit complete sequence complementarity. As used herein, nucleic acids are said to exhibit “complete complementarity” when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Molecules that exhibit complete complementarity will generally hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional “high-stringency” conditions. Conventional high-stringency conditions are described by Sambrook *et al.* (1989), *supra*.

Two molecules are said to exhibit “minimal complementarity” if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional “low-stringency” conditions. Conventional low-stringency conditions are also described by Sambrook *et al.* (1989), *supra*. In order for a nucleic acid molecule to serve as a primer or probe, it need only exhibit the minimal complementarity of sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

Factors that affect the stringency of hybridization are well-known to those of skill in the art and include, for example: temperature; pH; ionic strength; and

concentration of organic solvents (*e.g.*, formamide and dimethylsulfoxide). As is known to those of skill in the art, hybridization stringency is increased by higher temperatures, lower ionic strength, and lower solvent concentrations. Stringent conditions may also be achieved with the addition of destabilizing agents such as
5 formamide.

The term "stringent condition" or "stringency conditions" is defined with regard to the hybridization of one nucleic acid to another target nucleic acid (*i.e.*, to a nucleic acid molecule comprising a particular nucleotide sequence of interest) by the specific hybridization procedure discussed in Sambrook *et al.* (1989), *supra* (at
10 9.52-9.55). See also Sambrook *et al.* (1989) at 9.47-9.52 and 9.56-9.58.

Specificity in many applications is related to the conditions of post-hybridization washes, wherein factors include the ionic strength and temperature of the wash solution. For DNA-DNA hybrids, the thermal melting point (T_m) can be approximated from the equation:

$$15 \quad T_m = 81.5^{\circ}\text{C} + 16.6 (\log M) + 0.41(\%GC) - 0.61(\%\text{form}) - 500/L, \quad (1)$$

where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, %form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. Meinkoth
20 and Wahl (1984) Anal. Biochem. 138:267-84.

The T_m is the temperature (under a particular ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. The T_m is reduced by about 1°C for each 1% of mismatching. Thus, T_m , hybridization, and/or wash conditions can be adjusted for sequences of the desired identity to
25 hybridize. For example, if hybridization of sequences with 90% identity are sought, the T_m can be decreased 10°C (under a particular ionic strength and pH). Stringent conditions may, for example, be selected to be about 5°C lower than the thermal melting point (T_m) for a specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization
30 and/or wash at 1, 2, 3, or 4°C lower than the T_m ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the T_m ; low stringency conditions can utilize a hybridization and/or wash at 11 to 20°C lower than the T_m .

In some examples, stringent conditions are those in which the salt concentration is less than about 1.5 M Na⁺ (*e.g.*, about 0.01 to 1.0 M Na⁺) at pH 7.0 to 8.3, and the temperature is at least about 30°C for short nucleic acids (*e.g.*, 10 to 50 nucleotides in length) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides in length). Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1.0 M NaCl, 0.1% sodium dodecyl sulfate (SDS) at 37°C, and a wash in 1X to 2X SSC (20X SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 0.1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in about 50% formamide, about 1.0 M Na salt, about 0.1% SDS at about 37°C, and a wash in about 0.1X SSC at about 60 to 65°C.

As used herein, the term “polypeptide” includes a singular polypeptide, plural polypeptides, and fragments thereof. This term refers to a molecule comprised of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term “polypeptide” refers to any chain or chains of two or more amino acids, and does not refer to a specific length or size of the product. Accordingly, peptides, dipeptides, tripeptides, oligopeptides, protein, amino acid chain, and any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of “polypeptide,” and the foregoing terms are used interchangeably with “polypeptide” herein. A polypeptide may be isolated from a natural biological source or produced by recombinant technology, but a specific polypeptide is not necessarily translated from a specific nucleic acid. A polypeptide may be generated in any appropriate manner, including for example and without limitation, by chemical synthesis.

Endogenous and Heterologous: As used herein, the term “native” refers to the form of a polynucleotide, gene or polypeptide that is found in nature with its own regulatory sequences, if present. The term “endogenous” refers to the native form of the polynucleotide, gene or polypeptide in its natural location in the organism or in the genome of the organism.

In contrast, the term “heterologous” refers to a polynucleotide, gene or polypeptide that is not normally found at its location in the reference (host) organism. For example, a heterologous nucleic acid may be a nucleic acid that is normally found

in the reference organism at a different genomic location. By way of further example, a heterologous nucleic acid may be a nucleic acid that is not normally found in the reference organism. A host organism comprising a heterologous polynucleotide, gene or polypeptide may be produced by introducing the heterologous polynucleotide, gene or polypeptide into the host organism. In particular examples, a heterologous polynucleotide comprises a native coding sequence, or portion thereof, that is reintroduced into a source organism in a form that is different from the corresponding native polynucleotide. In particular examples, a heterologous gene comprises a native coding sequence, or portion thereof, that is reintroduced into a source organism in a form that is different from the corresponding native gene. For example, a heterologous gene may include a native coding sequence that is a portion of a chimeric gene including non-native regulatory regions that is reintroduced into the native host. In particular examples, a heterologous polypeptide is a native polypeptide that is reintroduced into a source organism in a form that is different from the corresponding native polypeptide.

A heterologous gene or polypeptide may be a gene or polypeptide that comprises a functional polypeptide or nucleic acid sequence encoding a functional polypeptide that is fused to another genes or polypeptide to produce a chimeric or fusion polypeptide, or a gene encoding the same. Genes and proteins of particular embodiments include specifically exemplified full-length sequences and portions, segments, fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules), variants, mutants, chimerics, and fusions of these sequences.

Modification: As used herein, the term "modification" may refer to a change in a particular reference polynucleotide that results in reduced, substantially eliminated, or eliminated activity of a polypeptide encoded by the reference polynucleotide. A modification may also refer to a change in a reference polypeptide that results in reduced, substantially eliminated, or eliminated activity of the reference polypeptide. Alternatively, the term "modification" may refer to a change in a reference polynucleotide that results in increased or enhanced activity of a polypeptide encoded by the reference polynucleotide, as well as a change in a reference polypeptide that results in increased or enhanced activity of the reference polypeptide. Changes such as the foregoing may be made by any of several methods well-known in the art including,

for example and without limitation: deleting a portion of the reference molecule; mutating the reference molecule (*e.g.*, *via* spontaneous mutagenesis, *via* random mutagenesis, *via* mutagenesis caused by mutator genes, and *via* transposon mutagenesis); substituting a portion of the reference molecule; inserting an element
5 into the reference molecule; down-regulating expression of the reference molecule; altering the cellular location of the reference molecule; altering the state of the reference molecule (*e.g.*, *via* methylation of a reference polynucleotide, and *via* phosphorylation or ubiquitination of a reference polypeptide); removing a cofactor of the reference molecule; introduction of an antisense RNA/DNA targeting the reference
10 molecule; introduction of an interfering RNA/DNA targeting the reference molecule; chemical modification of the reference molecule; covalent modification of the reference molecule; irradiation of the reference molecule with UV radiation or X-rays; homologous recombination that alters the reference molecule; mitotic recombination that alters the reference molecule; replacement of the promoter of the reference
15 molecule; and/or combinations of any of the foregoing.

Guidance in determining which nucleotides or amino acid residues may be modified in a specific example may be found by comparing the sequence of the reference polynucleotide or polypeptide with that of homologous (*e.g.*, homologous yeast or bacterial) polynucleotides or polypeptides, and maximizing the number of
20 modifications made in regions of high homology (conserved regions) or consensus sequences.

Derivative and Variant: The term “derivative,” as used herein, refers to a modification of an exemplary sequence herein. Such modifications include the substitution, insertion, and/or deletion of one or more bases of a coding sequence
25 herein that preserve, slightly alter, or increase the function of the coding sequence in a crop species. Such derivatives can be readily determined by one skilled in the art, for example and without limitation, by using computer modeling techniques for predicting and optimizing sequence structure. The term “derivative” thus also includes heterologous nucleic acids comprising a sequence having substantial sequence identity
30 with an exemplary sequence herein, such that they may have the same, slightly altered, or increased functionality for use in expressing DGT-28 in a crop plant.

As used herein, the term “variant” refers to a polypeptide differing from an exemplary polypeptide herein by amino acid insertions, deletions, mutations, and/or

substitutions, as may be introduced using, for example and without limitation, recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added, or deleted within a reference amino acid sequence may be found by comparing the sequence of the particular reference polypeptide with that of
5 homologous polypeptides, and minimizing the number of amino acid sequence changes made in regions of high homology (conserved regions), or by replacing amino acids with a consensus sequence. A variant polypeptide may have substituted amino acids, and yet retain the functional activity of the reference polypeptide. "Variant" genes comprise a nucleotide sequence that encodes the same polypeptide as a reference
10 gene or an equivalent polypeptide that has an activity equivalent or similar to the reference polypeptide.

In some embodiments, variant genes can be used to produce variant proteins, and recombinant hosts can be used to produce the variant proteins. For example, variant genes and proteins can be constructed that comprise contiguous residues (amino
15 acid or nucleotide) of any exemplified sequence herein. A variant gene or protein may have, for example and without limitation: 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,
20 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, 170, 171, 172, 173, 174,
25 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259,
30 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, and 293 contiguous residues (amino acid or nucleotide) that correspond to a segment (of

the same size) in the exemplified sequence. Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

It is understood by those of skill in the art that many levels of sequence identity are useful in identifying polypeptides (*e.g.*, from other species) that have the same or similar function or activity as a reference polypeptide. In some embodiments, a variant polypeptide having a sequence identity (when compared to a reference polypeptide; *e.g.*, a DGT-28 polypeptide) of, for example and without limitation: at least about 55%; at least about 60%; at least about 65%; at least about 70%; at least about 75%; at least about 80%; at least about 85%; at least about 90%; and at least about 95%, has the same or similar function as the reference polypeptide.

Strategies for designing and constructing variant genes and proteins that comprise contiguous residues of a particular molecule can be determined by obtaining and examining the structure of a protein of interest (*e.g.*, atomic 3-D (three dimensional) coordinates from a crystal structure and/or a molecular model). In some examples, a strategy may be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and not internal segments that are involved with protein folding and essential 3-D structural integrity. U.S. Patent No. 5,605,793, for example, relates to 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 process may improve the activity of a protein encoded by a subject gene. The result may be a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

An amino acid "substitution" can be the result of replacing one amino acid in a reference sequence with another amino acid having similar structural and/or chemical properties (*i.e.*, conservative amino acid substitution), or it can be the result of replacing one amino acid in a reference sequence with an amino acid having different structural and/or chemical properties (*i.e.*, non-conservative amino acid substitution). Amino acids can be placed in the following structural and/or chemical classes: non-polar; uncharged polar; basic; and acidic. Accordingly, "conservative" amino acid substitutions can be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of the residues involved. For

example, non-polar (hydrophobic) amino acids include glycine, alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; uncharged (neutral) polar amino acids include serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Alternatively, "non-conservative" amino acid substitutions can be made by selecting the differences in the polarity, charge, solubility, hydrophobicity, hydrophilicity, or amphipathic nature of any of these amino acids. "Insertions" or "deletions" can be within the range of variation as structurally or functionally tolerated by the recombinant proteins.

In some embodiments, a variant protein is "truncated" with respect to a reference, full-length protein. In some examples, a truncated protein retains the functional activity of the reference protein. By "truncated" protein, it is meant that a portion of a protein may be cleaved off, for example, while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage may be achieved by any of various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques, wherein the DNA bases encoding a portion of the protein are removed from the coding sequence, either through digestion with restriction endonucleases or other techniques available to the skilled artisan. A truncated protein may be expressed in a heterologous system, for example, *E. coli*, baculoviruses, plant-based viral systems, and yeast. Truncated proteins conferring herbicide tolerance may be confirmed by using the heterologous system expressing the protein in a herbicide tolerance bioassay, such as described herein. It is well-known in the art that truncated proteins can be successfully produced so that they retain the functional activity of the full-length reference protein. For example, Bt proteins can be used in a truncated (core protein) form. See, e.g., Hofte and Whiteley (1989) Microbiol. Rev. 53(2):242-55; and Adang *et al.* (1985) Gene 36:289-300.

In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Truncated genes may encode a polypeptide comprised of, for example, 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.

The variant genes and proteins that retain the function of the reference sequence from which they were designed may be determined by one of skill in the art, for example, by assaying recombinant variants for activity. If such an activity assay is known and characterized, then the determination of functional variants requires only
5 routine experimentation.

Specific changes to the “active site” of an enzyme may be made to affect the its inherent functionality with respect to activity or stereospecificity. *See Muller et al.* (2006) *Protein Sci.* 15(6):1356-68. For example, the known tauD structure has been used as a model dioxygenase to determine active site residues while bound to its
10 inherent substrate, taurine. *See Elkins et al.* (2002) *Biochemistry* 41(16):5185-92. Further information regarding sequence optimization and designability of enzyme active sites can be found in Chakrabarti *et al.* (2005) *Proc. Natl. Acad. Sci. USA* 102(34):12035-40.

Various structural properties and three-dimensional features of a protein may
15 be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be made that do not adversely affect the activity and/or three- dimensional configuration of the molecule (“tolerated” substitutions). Variant proteins can also be designed that differ at the sequence level from the reference protein, but which retain the same or similar overall essential
20 three-dimensional structure, surface charge distribution, and the like. *See, e.g., U.S. Patent 7,058,515; Larson et al.* (2002) *Protein Sci.* 11:2804-13; Crameri *et al.* (1997) *Nat. Biotechnol.* 15:436-8; Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-51; Stemmer (1994) *Nature* 370:389-91; Stemmer (1995) *Bio/Technology* 13:549-53; Crameri *et al.* (1996) *Nat. Med.* 2:100-3; and Crameri *et al.* (1996) *Nat. Biotechnol.* 14:
25 315-9.

Computational design of 5' or 3' UTRs (*e.g., synthetic hairpins*) that are suitable for use in an expression construct (*e.g., a DGT-28 expression construct*) may also be performed, and may be used to design elements within nucleic acids of some embodiments herein. Computer modeling and UTRs and computer modeling
30 techniques for use in predicting/evaluating 5' and 3' UTR derivatives include, for example and without limitation: MFold™ version 3.1 (available from Genetics Corporation Group, Madison, WI; *see Zucker et al.* “Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide,” in RNA Biochemistry

and *Biotechnology*, 11-43, J. Barciszewski & B.F.C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, Dordrecht, NL, 1999; Zucker *et al.* (1999) *J. Mol. Biol.* 288:911-40; Zucker *et al.* "RNA Secondary Structure Prediction," in Current Protocols in Nucleic Acid Chemistry, S. Beaucage, D.E. Bergstrom, G.D. Glick, and R.A. Jones eds., John Wiley & Sons, New York, 11.2.1-11.2.10, 2000); and COVE™ (RNA structure analysis using covariance models (stochastic context free grammar methods)) v.2.4.2 (Eddy and Durbin (1994) *Nucl. Acids Res.* 22:2079-88), which is freely distributed as source code and which can be downloaded by accessing the website, genetics.wustl.edu/eddy/software/; and FOLDALIGN™ (see Gorodkin *et al.* (1997) *Nucleic Acids Res.* 25(18):3724-32 and Gorodkin *et al.* (1997) *Proceedings International Conference on Intelligent Systems for Molecular Biology ISMB International Conference on Intelligent Systems for Molecular Biology* 5:120-123), also freely distributed and available for downloading at the website, foldalign.ku.dk/software/index.html.

15 Promoter: The term "promoter" refers to a DNA sequence capable of controlling the expression of a nucleic acid coding sequence or functional RNA. In examples, the controlled coding sequence is located 3' to a promoter sequence. A promoter may be derived in its entirety from a native gene, a promoter may be comprised of different elements derived from different promoters found in nature, or a
20 promoter may even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters can direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Examples of all of the foregoing promoters are known and used in the art to control the expression of heterologous nucleic acids.
25 Promoters that direct the expression of a gene in most cell types at most times are commonly referred to as "constitutive promoters." Furthermore, while those in the art have (in many cases unsuccessfully) attempted to delineate the exact boundaries of regulatory sequences, it has come to be understood that DNA fragments of different lengths may have identical promoter activity. The promoter activity of a particular
30 nucleic acid may be assayed using techniques familiar to those in the art.

Operably linked: The term "operably linked" refers to an association of nucleic acid sequences on a single nucleic acid, wherein the function of one of the nucleic acid sequences is affected by another. For example, a promoter is operably linked with a

coding sequence when the promoter is capable of effecting the expression of that coding sequence (*e.g.*, the coding sequence is under the transcriptional control of the promoter). A coding sequence may be operably linked to a regulatory sequence in a sense or antisense orientation.

5 Expression: The term “expression,” as used herein, may refer to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from a DNA. Expression may also refer to translation of mRNA into a polypeptide. As used herein, the term “overexpression” refers to expression that is higher than endogenous expression of the same gene or a related gene. Thus, a heterologous gene
10 is “overexpressed” if its expression is higher than that of a comparable endogenous gene.

Transformation: As used herein, the term “transformation” refers to the transfer and integration of a nucleic acid or fragment thereof into a host organism, resulting in genetically stable inheritance. Host organisms containing a transforming
15 nucleic acid are referred to as “transgenic,” “recombinant,” or “transformed” organisms. Known methods of transformation include, for example: *Agrobacterium tumefaciens*- or *A. rhizogenes*-mediated transformation; calcium phosphate transformation; polybrene transformation; protoplast fusion; electroporation; ultrasonic methods (*e.g.*, sonoporation); liposome transformation; microinjection; transformation
20 with naked DNA; transformation with plasmid vectors; transformation with viral vectors; biolistic transformation (microparticle bombardment); silicon carbide WHISKERS-mediated transformation; aerosol beaming; and PEG-mediated transformation.

Introduced: As used herein, the term “introduced” (in the context of
25 introducing a nucleic acid into a cell) includes transformation of a cell, as well as crossing a plant comprising the nucleic acid with a second plant, such that the second plant contains the nucleic acid, as may be performed utilizing conventional plant breeding techniques. Such breeding techniques are known in the art. For a discussion of plant breeding techniques, see Poehlman (1995) Breeding Field Crops, 4th Edition,
30 AVI Publication Co., Westport CT.

Backcrossing methods may be used to introduce a nucleic acid into a plant. This technique has been used for decades to introduce traits into plants. An example of a description of backcrossing (and other plant breeding methodologies) can be found

in, for example, Poelman (1995), *supra*; and Jensen (1988) Plant Breeding Methodology, Wiley, New York, NY. In an exemplary backcross protocol, an original plant of interest (the “recurrent parent”) is crossed to a second plant (the “non-recurrent parent”) that carries the a nucleic acid be introduced. The resulting progeny from this cross are then crossed again to the recurrent parent, and the process is repeated until a converted plant is obtained, wherein essentially all of the desired morphological and physiological characteristics of the recurrent parent are recovered in the converted plant, in addition to the nucleic acid from the non-recurrent parent.

Plasmid/vector: The terms “plasmid” and “vector,” as used herein, refer to an extra chromosomal element that may carry one or more gene(s) that are not part of the central metabolism of the cell. Plasmids and vectors typically are circular double-stranded DNA molecules. However, plasmids and vectors may be linear or circular nucleic acids, of a single- or double-stranded DNA or RNA, and may be derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction that is capable of introducing a promoter fragment and a coding DNA sequence along with any appropriate 3' untranslated sequence into a cell. In examples, plasmids and vectors may comprise autonomously replicating sequences, genome integrating sequences, and/or phage or nucleotide sequences.

III. DGT-28 and DGT-28-encoding sequences

Some embodiments herein provide an isolated polypeptide having at least about 90% identity (*e.g.*, 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, and at least 99% identity) to SEQ ID NO:1. Such a polypeptide is referred to herein as a DGT-28 polypeptide. Some embodiments herein provide a nucleic acid encoding a polypeptide having at least about 90% identity to SEQ ID NO:1. Particular examples of such a nucleic acid are referred to herein as a “*dgt-28*” nucleic acid. *Dgt-28* nucleic acids may be useful in any of a wide variety of applications (*e.g.*, introducing glyphosate resistance) in which modified glyphosate metabolism is desired in a plant cell.

Particular examples of *dgt-28* nucleic acids provided for illustrative purposes herein are SEQ ID NOs:2 and 3. Accordingly, some embodiments provide a nucleic acid comprising a nucleotide sequence having at least about 80% sequence identity

(e.g., 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, and at least 99% identity) to SEQ ID NO:2 or SEQ ID NO:3, wherein the nucleic acid encodes a polypeptide having at least about 90% identity to SEQ ID NO:1. Particular examples of *dgt-28* nucleic acids include nucleic acids that specifically hybridize to a nucleic acid having SEQ ID NO:2 or SEQ ID NO:3 under stringent (e.g., highly-stringent) conditions.

In some embodiments, codon-optimized *dgt-28* nucleic acids are provided. For example, to obtain high expression of a heterologous gene in a plant it may be desirable to design and reengineer the gene so that it is more efficiently expressed in a cell of the plant. This strategy may be particularly desirable in the circumstance where a bacterial gene is desired to be expressed in a plant cell.

Thus, some examples herein provide a plant-optimized gene encoding a DGT-28 protein, and methods for the desing thereof, to generate a DNA sequence that can be expressed optimally in dicotyledonous or monocotyledonous plants, and in which the sequence modifications do not hinder translation or transcription. Design of an optimized *dgt-28* gene for expression of the same DGT-28 protein in both monocotyledonous and dicotyledonous plants is exemplified herein with a reengineering of the protein coding region of this gene for optimal expression. Exemplary plant-optimized *dgt-28* nucleic acids herein include SEQ ID NO:2 and SEQ ID NO:3.

In engineering a gene encoding a DGT-28 protein for expression in dicotyledonous or monocotyledonous plants (e.g., cotton, canola, tobacco, corn, soybean, wheat and rice), the codon bias of the prospective host plant(s) may be determined, for example, through use of publicly available DNA sequence databases to find information about the codon distribution of plant genomes or the protein coding regions of various plant genes.

In designing coding regions in a nucleic acid for plant expression, the primary ("first choice") codons preferred by the plant should be determined, as well may be the second, third, fourth, etc. choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino acid sequence of the same peptide (e.g., a DGT-28 protein), but the new DNA sequence differs from the

original DNA sequence by the substitution of plant (first preferred, second preferred, third preferred, or fourth preferred, etc.) codons to specify the amino acid at each position within the amino acid sequence.

The new sequence may then be analyzed for restriction enzyme sites that might have been created by the modifications. The identified sites may be further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence that could affect transcription or translation of the gene of interest are stem-loop structures, exon:intron junctions (5' or 3'), poly A addition signals, and RNA polymerase termination signals; these sites may be removed by the substitution of plant codons. The sequence may be further analyzed and modified to reduce the frequency of TA or CG doublets. In addition to the doublets, G or C sequence blocks that have more than about six residues that are the same can affect transcription or translation of the sequence. Therefore, these blocks may be modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

SEQ ID NO: 2 (*dgt-28* (v5)) was optimized for expression in dicotyledonous plants. SEQ ID NO: 3 (*dgt-28* (v6)) was optimized for expression in monocotyledonous plants. The codon usage in these synthetic sequences was selected based upon preferred codon usage; *i.e.*, the expression products of each are encoded by codons having a bias toward either monocot or dicot plant usage, and deleterious sequences and superfluous restriction sites were removed to increase the efficiency of transcription/translation of the DGT-28 polypeptide and to facilitate DNA manipulation steps.

Likewise, the nucleic acid molecule of SEQ ID NO: 4 (*dgt-28* (v1)) was optimized to improve expression in *Escherichia coli*. Codon usage in SEQ ID NO:4 was selected based upon preferred *E.coli* codon usage; the expressed protein is encoded by codons having a bias toward *E.coli* usage. During the redesign, deleterious sequences and superfluous restriction sites were removed to increase the efficiency of transcription/translation of the DGT-28 coding sequence and to facilitate DNA manipulation steps. Thus, expression of DGT-28 from a nucleic acid comprising SEQ ID NO:4 in *E. coli* may result in robust protein expression, for example, for enzymatic characterization of DGT-28.

Once an optimized (*e.g.*, a plant-optimized) DNA sequence has been designed on paper, or *in silico*, actual DNA molecules may be synthesized in the laboratory to correspond in sequence precisely to the designed sequence. Such synthetic nucleic acid molecule molecules can be cloned and otherwise manipulated
5 exactly as if they were derived from natural or native sources.

A nucleic acid herein may be cloned into a vector for transformation into prokaryotic or eukaryotic cells for replication and/or expression. Vectors may be prokaryotic vectors; *e.g.*, plasmids, or shuttle vectors, insect vectors, or eukaryotic vectors. A nucleic acid herein may also be cloned into an expression vector, for
10 example, for administration to a plant cell. In certain applications, it may be preferable to have vectors that are functional in *E. coli* (*e.g.*, production of protein for raising antibodies, DNA sequence analysis, construction of inserts, obtaining quantities of nucleic acids).

To express a DGT-28 protein in a cell, a nucleic acid encoding the protein is
15 typically subcloned into an expression vector that contains a promoter to direct transcription. Suitable bacterial and eukaryotic promoters are well known in the art and described, *e.g.*, in Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989; 3rd ed., 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*,
20 *supra.*). Bacterial expression systems for expressing a nucleic acid herein are available in, for example, *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known by those of skill in the art and are also commercially available.

25 The particular expression vector used to transport the genetic information into the cell is selected with regard to the intended use of the DGT-28 protein (*e.g.*, expression in plants, animals, bacteria, fungus, and protozoa). Standard bacterial and animal expression vectors are known in the art and are described in detail, for example, U.S. Patent Publication 20050064474A1 and International Patent
30 Publications WO 05/084190, WO05/014791 and WO03/080809. Standard transfection methods can be used to produce bacterial cell lines that express large quantities of protein, which can then be purified using standard techniques.

The selection of a promoter used to direct expression of a nucleic acid herein depends on the particular application. A number of promoters that direct expression of a gene in a plant may be employed in embodiments herein. Such promoters can be selected from constitutive, chemically-regulated, inducible, tissue-specific, and seed-preferred promoters. For example, a strong constitutive promoter suited to the host cell may be used for expression and purification of DGT-28 proteins. Non-limiting examples of plant promoters include promoter sequences derived from *A. thaliana* ubiquitin-10 (ubi-10) (Callis, *et al.*, 1990, *J. Biol. Chem.*, 265:12486-12493); *A. tumefaciens* mannopine synthase (Δ mas) (Petolino *et al.*, U.S. Patent No. 6,730,824); and/or Cassava Vein Mosaic Virus (CsVMV) (Verdaguer *et al.*, 1996, *Plant Molecular Biology* 31:1129-1139).

Constitutive promoters include, for example, the core Cauliflower Mosaic Virus 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); Rice Actin promoter (McElroy *et al.* (1990) *Plant Cell* 2:163-171); Maize Ubiquitin promoter (U.S. Patent Number 5,510,474; Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU promoter (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); ALS promoter (U.S. Patent Number 5,659,026); Maize Histone promoter (Chabouté *et al.* *Plant Molecular Biology*, 8:179-191 (1987)); and the like.

The range of available plant compatible promoters includes tissue specific and inducible promoters. An inducible regulatory element is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor that binds specifically to an inducible regulatory element to activate transcription is present in an inactive form, which is then directly or indirectly converted to the active form by the inducer. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. Typically, the protein factor that binds specifically to an inducible regulatory element to activate transcription is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. A plant cell containing an inducible regulatory element may be exposed to an inducer

by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods.

Any inducible promoter can be used in embodiments herein. See Ward *et al.* Plant Mol. Biol. 22: 361-366 (1993). Inducible promoters include, for example and without limitation: ecdysone receptor promoters (U.S. Patent Number 6,504,082); promoters from the ACE1 system which respond to copper (Mett *et al.* PNAS 90: 4567-4571 (1993)); In2-1 and In2-2 gene from maize which respond to benzenesulfonamide herbicide safeners (US Patent Number 5,364,780; Hershey *et al.*, Mol. Gen. Genetics 227: 229-237 (1991) and Gatz *et al.*, Mol. Gen. Genetics 243: 32-38 (1994)); Tet repressor from Tn10 (Gatz *et al.*, Mol. Gen. Genet. 227: 229-237 (1991); promoters from a steroid hormone gene, the transcriptional activity of which is induced by a glucocorticosteroid hormone, Schena *et al.*, Proc. Natl. Acad. Sci. U.S.A. 88: 10421 (1991) and McNellis *et al.*, (1998) Plant J. 14(2):247-257; the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides (*see* U.S. Patent No. 5,965,387 and International Patent Application, Publication No. WO 93/001294); and the tobacco PR-1a promoter, which is activated by salicylic acid (*see* Ono S, Kusama M, Ogura R, Hiratsuka K., "Evaluation of the Use of the Tobacco PR-1a Promoter to Monitor Defense Gene Expression by the Luciferase Bioluminescence Reporter System," Biosci Biotechnol Biochem. 2011 Sep 23;75(9):1796-800). Other chemical-regulated promoters of interest include tetracycline-inducible and tetracycline-repressible promoters (*see*, for example, Gatz *et al.*, (1991) Mol. Gen. Genet. 227:229-237, and U.S. Patent Numbers 5,814,618 and 5,789,156).

Other regulatable promoters of interest include a cold responsive regulatory element or a heat shock regulatory element, the transcription of which can be effected in response to exposure to cold or heat, respectively (Takahashi *et al.*, Plant Physiol. 99:383-390, 1992); the promoter of the alcohol dehydrogenase gene (Gerlach *et al.*, PNAS USA 79:2981-2985 (1982); Walker *et al.*, PNAS 84(19):6624-6628 (1987)), inducible by anaerobic conditions; the light-inducible promoter derived from the pea rbcS gene or pea psaDb gene (Yamamoto *et al.* (1997) Plant J. 12(2):255-265); a light-inducible regulatory element (Feinbaum *et al.*, Mol. Gen. Genet. 226:449, 1991; Lam and Chua, Science 248:471, 1990;

Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138); a plant hormone inducible regulatory element (Yamaguchi-Shinozaki *et al.*, *Plant Mol. Biol.* 15:905, 1990; Kares *et al.*, *Plant Mol. Biol.* 15:225, 1990), and the like. An inducible regulatory element also
5 can be the promoter of the maize In2-1 or In2-2 gene, which responds to benzenesulfonamide herbicide safeners (Hershey *et al.*, *Mol. Gen. Genet.* 227:229-237, 1991; Gatz *et al.*, *Mol. Gen. Genet.* 243:32-38, 1994), and the Tet repressor of transposon Tn10 (Gatz *et al.*, *Mol. Gen. Genet.* 227:229-237, 1991).

Stress inducible promoters include salt/water stress-inducible promoters such
10 as P5CS (Zang *et al.* (1997) *Plant Sciences* 129:81-89); cold-inducible promoters, such as cor15a (Hajela *et al.* (1990) *Plant Physiol.* 93:1246-1252), cor15b (Wilhelm *et al.* (1993) *Plant Mol Biol* 23:1073-1077), wsc120 (Ouellet *et al.* (1998) *FEBS Lett.* 423-324-328), ci7 (Kirch *et al.* (1997) *Plant Mol Biol.* 33:897-909), and ci21A (Schneider *et al.* (1997) *Plant Physiol.* 113:335-45); drought-inducible promoters,
15 such as Trg-31 (Chaudhary *et al.* (1996) *Plant Mol. Biol.* 30:1247-57) and rd29 (Kasuga *et al.* (1999) *Nature Biotechnology* 18:287-291); osmotic inducible promoters, such as Rab17 (Vilardell *et al.* (1991) *Plant Mol. Biol.* 17:985-93) and osmotin (Raghothama *et al.* (1993) *Plant Mol Biol* 23:1117-28); heat inducible promoters, such as heat shock proteins (Barros *et al.* (1992) *Plant Mol.* 19:665-75;
20 Marrs *et al.* (1993) *Dev. Genet.* 14:27-41), smHSP (Waters *et al.* (1996) *J. Experimental Botany* 47:325-338); and the heat-shock inducible element from the parsley ubiquitin promoter (WO 03/102198). Other stress-inducible promoters include rip2 (U.S. Patent Number 5,332,808 and U.S. Publication No. 2003/0217393) and rd29a (Yamaguchi-Shinozaki *et al.* (1993) *Mol. Gen. Genetics*
25 236:331-340). Certain promoters are inducible by wounding, including the *Agrobacterium* pMAS promoter (Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505) and the *Agrobacterium* ORF13 promoter (Hansen *et al.*, (1997) *Mol. Gen. Genet.* 254(3):337-343).

Tissue-preferred promoters may be utilized to target enhanced transcription
30 and/or expression within a particular plant tissue. Examples of these types of promoters include seed-preferred expression, such as that provided by the phaseolin promoter (Bustos *et al.* 1989. *The Plant Cell* Vol. 1, 839-853), and the maize globulin-1 gene, Belanger, *et al.* 1991 *Genetics* 129:863-972. For dicots,

seed-preferred promoters include, but are not limited to, bean β -phaseolin, napin, β -conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-preferred promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, γ -zein, waxy, shrunken 1, shrunken 2, globulin 1, etc. Seed-preferred promoters also include those promoters that direct gene expression predominantly to specific tissues within the seed such as, for example, the endosperm-preferred promoter of γ -zein, the cryptic promoter from tobacco (Fobert *et al.* 1994. T-DNA tagging of a seed coat-specific cryptic promoter in tobacco. *Plant J.* 4: 567-577), the P-gene promoter from corn (Chopra *et al.* 1996. Alleles of the maize P gene with distinct tissue specificities encode Myb-homologous proteins with C-terminal replacements. *Plant Cell* 7:1149-1158, Erratum in *Plant Cell*.1997, 1:109), the globulin-1 promoter from corn (Belenger and Kriz.1991. Molecular basis for Allelic Polymorphism of the maize Globulin-1 gene. *Genetics* 129: 863-972), and promoters that direct expression to the seed coat or hull of corn kernels, for example the pericarp-specific glutamine synthetase promoter (Muhitch *et al.*, 2002. Isolation of a Promoter Sequence From the Glutamine Synthetase₁₋₂ Gene Capable of Conferring Tissue-Specific Gene Expression in Transgenic Maize. *Plant Science* 163:865-872).

In addition to the promoter, an expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in host cells, either prokaryotic or eukaryotic. A typical expression cassette thus contains a promoter operably-linked, *e.g.*, to a nucleic acid sequence encoding the protein, and signals required, *e.g.*, for efficient polyadenylation of the transcript, transcriptional termination, ribosome binding sites, or translation termination. Additional elements of the cassette may include, *e.g.*, enhancers and heterologous splicing signals.

Other components of the vector may be included, also depending upon intended use of the gene. Examples include selectable markers, targeting or regulatory sequences, transit peptide sequences such as the optimized transit peptide sequence (*see* U.S. Patent Number 5,510,471) stabilizing sequences such as RB7 MAR (*see* Thompson and Myatt, (1997) *Plant Mol. Biol.*, 34: 687-692 and WO9727207) or leader sequences, introns etc. General descriptions and examples of plant expression vectors and reporter genes can be found in Gruber, *et al.*,

“Vectors for Plant Transformation” in Methods in Plant Molecular Biology and Biotechnology, Glick *et al.* eds; CRC Press pp. 89-119 (1993).

The selection of an appropriate expression vector will depend upon the host and the method of introducing the expression vector into the host. The expression
5 cassette may include, at the 3' terminus of a heterologous nucleotide sequence of interest, a transcriptional and translational termination region functional in plants. The termination region can be native with the DNA sequence of interest or can be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase
10 (nos) termination regions (Depicker *et al.*, Mol. and Appl. Genet. 1:561-573 (1982) and Shaw *et al.* (1984) Nucleic Acids Research vol. 12, No. 20 pp7831-7846(nos)); see also Guerineau *et al.* Mol. Gen. Genet. 262:141-144 (1991); Proudfoot, Cell 64:671-674 (1991); Sanfacon *et al.* Genes Dev. 5:141-149 (1991); Mogen *et al.* Plant Cell 2:1261-1272 (1990); Munroe *et al.* Gene 91:151-158 (1990); Ballas *et al.*
15 Nucleic Acids Res. 17:7891-7903 (1989); Joshi *et al.* Nucleic Acid Res. 15:9627-9639 (1987).

An expression cassette may contain a 5' leader sequence. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include by way of example, picornavirus leaders, EMCV leader
20 (Encephalomyocarditis 5' noncoding region), Elroy-Stein *et al.* Proc. Nat. Acad. Sci. USA 86:6126-6130 (1989); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) Carrington and Freed Journal of Virology, 64:1590-1597 (1990), MDMV leader (Maize Dwarf Mosaic Virus), Allison *et al.*, Virology 154:9-20 (1986); human immunoglobulin heavy-chain binding protein (BiP), Macejak *et al.*
25 Nature 353:90-94 (1991); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), Jobling *et al.* Nature 325:622-625 (1987); Tobacco mosaic virus leader (TMV), Gallie *et al.* (1989) Molecular Biology of RNA, pages 237-256; and maize chlorotic mottle virus leader (MCMV) Lommel *et al.* Virology 81:382-385 (1991). See also Della-Cioppa *et al.* Plant Physiology 84:965-968
30 (1987).

The construct may also contain sequences that enhance translation and/or mRNA stability such as introns. An example of one such intron is the first intron of

gene II of the histone H3.III variant of *Arabidopsis thaliana*. Chaubet *et al.* Journal of Molecular Biology, 225:569-574 (1992).

In those instances where it is desirable to have the expressed product of the heterologous nucleotide sequence directed to a particular organelle, particularly the plastid, amyloplast, or to the endoplasmic reticulum, or secreted at the cell's surface or extracellularly, the expression cassette may further comprise a coding sequence for a transit peptide. Such transit peptides are well known in the art and include, but are not limited to, the transit peptide for the acyl carrier protein, the small subunit of RUBISCO, plant EPSP synthase and *Helianthus annuus* (see Lebrun *et al.* US Patent 5,510,417), *Zea mays* Brittle-1 chloroplast transit peptide (Nelson *et al.* Plant Physiol 117(4):1235-1252 (1998); Sullivan *et al.* Plant Cell 3(12):1337-48; Sullivan *et al.*, Planta (1995) 196(3):477-84; Sullivan *et al.*, J. Biol. Chem. (1992) 267(26):18999-9004) and the like. In addition, chimeric chloroplast transit peptides are known in the art, such as the Optimized Transit Peptide (see, U.S. Patent Number 5,510,471). Additional chloroplast transit peptides have been described previously in U.S. Patent Nos. 5,717,084; 5,728,925. One skilled in the art will readily appreciate the many options available in expressing a product to a particular organelle. For example, the barley alpha amylase sequence is often used to direct expression to the endoplasmic reticulum. Rogers, J. Biol. Chem. 260:3731-3738 (1985).

It will be appreciated by one skilled in the art that use of recombinant DNA technologies can improve control of expression of transfected nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within the host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Additionally, the promoter sequence might be genetically engineered to improve the level of expression as compared to the native promoter. Recombinant techniques useful for controlling the expression of nucleic acid molecules include, but are not limited to, stable integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (*e.g.*, promoters, operators, enhancers), substitutions or modifications of translational control signals (*e.g.*, ribosome binding

sites, Shine-Dalgarno or Kozak sequences), modification of nucleic acid molecules to correspond to the codon usage of the host cell, and deletion of sequences that destabilize transcripts.

Reporter or marker genes for selection of transformed cells or tissues or plant parts or plants may be included in the transformation vectors. Examples of selectable markers include those that confer resistance to anti-metabolites such as herbicides or antibiotics, for example, dihydrofolate reductase, which confers resistance to methotrexate (Reiss, *Plant Physiol. (Life Sci. Adv.)* 13:143-149, 1994; see also Herrera Estrella *et al.*, *Nature* 303:209-213, 1983; Meijer *et al.*, *Plant Mol. Biol.* 16:807-820, 1991); neomycin phosphotransferase, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, *EMBO J.* 2:987-995, 1983 and Fraley *et al.* *Proc. Natl. Acad. Sci USA* 80:4803 (1983)); hygromycin phosphotransferase, which confers resistance to hygromycin (Marsh, *Gene* 32:481-485, 1984; see also Waldron *et al.*, *Plant Mol. Biol.* 5:103-108, 1985; Zhijian *et al.*, *Plant Science* 108:219-227, 1995); *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman, *Proc. Natl. Acad. Sci., USA* 85:8047, 1988); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627); ornithine decarboxylase, which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine (DFMO; McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.); and deaminase from *Aspergillus terreus*, which confers resistance to Blasticidin S (Tamura, *Biosci. Biotechnol. Biochem.* 59:2336-2338, 1995).

Additional selectable markers include, for example, a mutant acetolactate synthase, which confers imidazolinone or sulfonylurea resistance (Lee *et al.*, *EMBO J.* 7:1241-1248, 1988), a mutant *psbA*, which confers resistance to atrazine (Smeda *et al.*, *Plant Physiol.* 103:911-917, 1993), or a mutant protoporphyrinogen oxidase (see U.S. Pat. No. 5, 767, 373), or other markers conferring resistance to an herbicide such as glufosinate. Examples of suitable selectable marker genes include, but are not limited to, genes encoding resistance to chloramphenicol (Herrera Estrella *et al.*, *EMBO J.* 2:987-992, 1983); streptomycin (Jones *et al.*, *Mol. Gen. Genet.* 210:86-91, 1987); spectinomycin (Bretagne-Sagnard *et al.*, *Transgenic Res.* 5:131-137, 1996); bleomycin (Hille *et al.*, *Plant Mol. Biol.* 7:171-176, 1990);

sulfonamide (Guerineau *et al.*, Plant Mol. Biol. 15:127-136, 1990); bromoxynil (Stalker *et al.*, Science 242:419-423, 1988); glyphosate (Shaw *et al.*, Science 233:478-481, 1986); phosphinothricin (DeBlock *et al.*, EMBO J. 6:2513-2518, 1987), and the like.

5 One option for use of a selective gene is a glufosinate-resistance encoding DNA and in one embodiment can be the phosphinothricin acetyl transferase (*pat*), maize optimized *pat* gene or *bar* gene under the control of the Cassava Vein Mosaic Virus promoter. These genes confer resistance to bialaphos. See, (*see*, Wohlleben *et al.*, (1988) *Gene* 70: 25-37); Gordon-Kamm *et al.*, Plant Cell 2:603; 1990; 10 Uchimiya *et al.*, BioTechnology 11:835, 1993; White *et al.*, Nucl. Acids Res. 18:1062, 1990; Spencer *et al.*, Theor. Appl. Genet. 79:625-631, 1990; and Anzai *et al.*, Mol. Gen. Gen. 219:492, 1989). A version of the *pat* gene is the maize optimized *pat* gene, described in U.S. Patent No. 6,096,947.

15 In addition, markers that facilitate identification of a plant cell containing the polynucleotide encoding the marker may be employed. Scorable or screenable markers are useful, where presence of the sequence produces a measurable product and can produce the product without destruction of the plant cell. Examples include a β -glucuronidase, or *uidA* gene (GUS), which encodes an enzyme for which various chromogenic substrates are known (for example, US Patents 5,268,463 and 20 5,599,670); chloramphenicol acetyl transferase (Jefferson *et al.* The EMBO Journal vol. 6 No. 13 pp. 3901-3907); and alkaline phosphatase. In a preferred embodiment, the marker used is beta-carotene or provitamin A (Ye *et al.*, Science 287:303-305- (2000)). The gene has been used to enhance the nutrition of rice, but in this instance it is employed instead as a screenable marker, and the presence of the gene linked to 25 a gene of interest is detected by the golden color provided. Unlike the situation where the gene is used for its nutritional contribution to the plant, a smaller amount of the protein suffices for marking purposes. Other screenable markers include the anthocyanin/flavonoid genes in general (See discussion at Taylor and Briggs, The Plant Cell (1990)2:115-127) including, for example, a R-locus gene, which encodes 30 a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, in Chromosome Structure and Function, Kluwer Academic Publishers, Appels and Gustafson eds., pp. 263-282 (1988)); the genes which control biosynthesis of flavonoid pigments, such as the maize C1 gene (Kao *et al.*, Plant

Cell (1996) 8: 1171-1179; Scheffler *et al.*, Mol. Gen. Genet. (1994) 242:40-48) and maize C2 (Wienand *et al.*, Mol. Gen. Genet. (1986) 203:202-207); the B gene (Chandler *et al.*, Plant Cell (1989) 1:1175-1183), the p1 gene (Grotewold *et al.*, Proc. Natl. Acad. Sci USA (1991) 88:4587-4591; Grotewold *et al.*, Cell (1994) 76:543-553; Sidorenko *et al.*, Plant Mol. Biol. (1999)39:11-19); the bronze locus genes (Ralston *et al.*, Genetics (1988) 119:185-197; Nash *et al.*, Plant Cell (1990) 2(11): 1039-1049), among others.

Further examples of suitable markers include the cyan fluorescent protein (CYP) gene (Bolte *et al.* (2004) J. Cell Science 117: 943-54 and Kato *et al.* (2002) Plant Physiol 129: 913-42), the yellow fluorescent protein gene (PHIYFP™ from Evrogen; see Bolte *et al.* (2004) J. Cell Science 117: 943-54); a lux gene, which encodes a luciferase, the presence of which may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry (Teeri *et al.* (1989) EMBO J. 8:343); a green fluorescent protein (GFP) gene (Sheen *et al.*, Plant J. (1995) 8(5):777-84); and DsRed2 where plant cells transformed with the marker gene are red in color, and thus visually selectable (Dietrich *et al.* (2002) Biotechniques 2(2):286-293). Additional examples include a β -lactamase gene (Sutcliffe, Proc. Nat'l. Acad. Sci. U.S.A. (1978) 75:3737), which encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a xyle gene (Zukowsky *et al.*, Proc. Nat'l. Acad. Sci. U.S.A. (1983) 80:1101), which encodes a catechol dioxygenase that can convert chromogenic catechols; an α -amylase gene (Ikuta *et al.*, Biotech. (1990) 8:241); and a tyrosinase gene (Katz *et al.*, J. Gen. Microbiol. (1983) 129:2703), which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone, which in turn condenses to form the easily detectable compound melanin. Clearly, many such markers are available and known to one skilled in the art.

IV. Cells and organisms comprising DGT-28

In some embodiments, a cell and/or organism (e.g., a plant cell or plant) is provided that comprises a heterologous polypeptide having at least 90% identity to SEQ ID NO:1. In particular embodiments, a cell and/or organism is provided that comprises a heterologous nucleic acid encoding a polypeptide having at least 90%

identity to SEQ ID NO:1; *e.g.*, a *dgt-28* nucleic acid. Some embodiments include a cell and/or organism comprising a heterologous nucleic acid that hybridizes to another nucleic acid having SEQ ID NO:2 or SEQ ID NO:3 under high-stringency conditions.

A plant cell, plant part, and/or plant may be genetically modified to comprise a heterologous polypeptide (*e.g.*, a DGT-28 protein) and/or heterologous nucleic acid (*e.g.*, a *dgt-28* nucleic acid) by any of several methods of introducing a heterologous molecule known in the art. In particular embodiments herein, a heterologous molecule is introduced into a plant cell, plant part, and/or plant by a method selected from, for example and without limitation: transformation and selective breeding (*e.g.*, backcross breeding).

Any plant species or plant cell may be genetically modified to comprise a heterologous polypeptide and/or nucleic acid herein. In some embodiments, the plant cell that is so genetically modified is not capable of regeneration to produce a plant. In some embodiments, plants which are genetically modified in accordance with the present disclosure (*e.g.*, plant host cells) includes, but is not limited to, a higher plant, a dicotyledonous plant, a monocotyledonous plants, a consumable plant, a crop plant, and a plant utilized for its oils (*e.g.*, an oilseed plant). Such plants include, for example and without limitation: alfalfa; soybean; cotton; rapeseed (canola); linseed; corn; rice; brachiaria; wheat; safflower; sorghum; sugarbeet; sunflower; tobacco; and grasses (*e.g.*, turf grass). In particular examples, a genetically modified plant cell or plant herein includes, for example and without limitation: *Brassica napus*; indian mustard (*Brassica juncea*); Ethiopian mustard (*Brassica carinata*); turnip (*Brassica rapa*); cabbage (*Brassica oleracea*); *Glycine max*; *Linum usitatissimum*; *Zea mays*; *Carthamus tinctorius*; *Helianthus annuus*; *Nicotiana tabacum*; *Arabidopsis thaliana*, Brazil nut (*Betholettia excelsa*); castor bean (*Ricinus communis*); coconut (*Cocos nucifera*); coriander (*Coriandrum sativum*); *Gossypium spp.*; groundnut (*Arachis hypogaea*); jojoba (*Simmondsia chinensis*); oil palm (*Elaeis guineensis*); olive (*Olea europaea*); *Oryza sativa*; squash (*Cucurbita maxima*); barley (*Hordeum vulgare*); sugarcane (*Saccharum officinarum*); *Triticum spp.* (including *Triticum durum* and *Triticum aestivum*); and duckweed (*Lemnaceae sp.*). In some embodiments, the plant may have a particular genetic background, as for elite cultivars, wild-type cultivars, and commercially distinguishable varieties.

Nucleic acids introduced into a plant cell may be used to confer desired traits on essentially any plant. A wide variety of plants and plant cell systems may be engineered for the desired physiological and agronomic characteristics described herein using a nucleic acid encoding a DGT polypeptide and various transformation methods.

5 Embodiments herein may use any of many methods for the transformation of plants (and production of genetically modified plants) that are known in the art. Numerous methods for plant transformation have been developed, including biological and physical transformation protocols for dicotyledenous plants, as well as monocotyledenous plants (*See, e.g., Goto-Fumiyuki et al. (1999) Nat. Biotechnol.*
10 *17:282-6; Miki et al. (1993) Methods in Plant Molecular Biology and Biotechnology (Glick, B. R. and Thompson, J. E., Eds.), CRC Press, Inc., Boca Raton, FL, pp. 67-88.* In addition, vectors and *in vitro* culture methods for plant cell and tissue transformation and regeneration of plants are described, for example, in Gruber *et al. (1993), supra*, at pp. 89-119.

15 Plant transformation techniques available for introducing a nucleic acid into a plant host cell include, for example and without limitation: transformation with disarmed T-DNA using *Agrobacterium tumefaciens* or *A. rhizogenes* as the transformation agent; calcium phosphate transfection; polybrene transformation; protoplast fusion; electroporation (D'Halluin *et al. (1992) Plant Cell 4:1495-505*);
20 ultrasonic methods (*e.g., sonoporation*); liposome transformation; microinjection; contact with naked DNA; contact with plasmid vectors; contact with viral vectors; biolistics (*e.g., DNA particle bombardment (see, e.g., Klein et al. (1987) Nature 327:70-3) and microparticle bombardment (Sanford et al. (1987) Part. Sci. Technol. 5:27; Sanford (1988) Trends Biotech. 6:299, Sanford (1990) Physiol. Plant 79:206; and*
25 *Klein et al. (1992) Biotechnology 10:268*); silicon carbide WHISKERS-mediated transformation (Kaeppler *et al. (1990) Plant Cell Rep. 9:415-8*); nanoparticle transformation (*see, e.g., U.S. Patent Publication No. US2009/0104700A1*); aerosol beaming; and polyethylene glycol (PEG)-mediated uptake. In specific examples, a heterologous nucleic acid may be introduced directly into the genomic DNA of a plant
30 cell.

A widely utilized method for introducing an expression vector into a plant is based on the natural transformation system of *Agrobacterium*. Horsch *et al. (1985) Science 227:1229*. *A. tumefaciens* and *A. rhizogenes* are plant pathogenic soil bacteria

known to be useful to genetically transform plant cells. The Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, carry genes responsible for genetic transformation of the plant. Kado (1991) Crit. Rev. Plant. Sci. 10:1. Details regarding *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer are also available in, for example, Gruber *et al.*, *supra*, Miki *et al.*, *supra*, Moloney *et al.* (1989) Plant Cell Reports 8:238, and U.S. Patent Nos. 4,940,838 and 5,464,763.

If *Agrobacterium* is used for the transformation, the DNA to be inserted typically is cloned into special plasmids; either into an intermediate vector or a binary vector. Intermediate vectors cannot replicate themselves in *Agrobacterium*. The intermediate vector may be transferred into *A. tumefaciens* by means of a helper plasmid (conjugation). The Japan Tobacco Superbinary system is an example of such a system (reviewed by Komari *et al.* (2006) Methods in Molecular Biology (K. Wang, ed.) No. 343; Agrobacterium Protocols, 2nd Edition, Vol. 1, Humana Press Inc., Totowa, NJ, pp.15-41; and Komori *et al.* (2007) Plant Physiol. 145:1155-60). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacterium*. Binary vectors 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 *Agrobacterium* (Holsters, 1978). The *Agrobacterium* comprises a plasmid carrying a *vir* region. The Ti or Ri plasmid also comprises the *vir* region necessary for the transfer of the T-DNA. The *vir* region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained.

The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of a T-strand containing the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria using a binary T DNA vector (Bevan (1984) Nuc. Acid Res. 12:8711-21) or the co-cultivation procedure (Horsch *et al.* (1985) Science 227:1229-31). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants. Bevan *et al.* (1982) Ann. Rev. Genet 16:357-84; Rogers *et al.* (1986) Methods Enzymol. 118:627-41. The *Agrobacterium* transformation system may also be used to transform, as well as transfer, nucleic acids to monocotyledonous plants and plant cells. See U.S. Patent No. 5,591,616; Hernalsteen *et al.* (1984) EMBO J 3:3039-41; Hooykass-Van Slogteren *et al.* (1984) Nature 311:763-4; Grimsley *et al.* (1987) Nature 325:1677-9; Boulton *et al.* (1989) Plant Mol. Biol. 12:31-40; and Gould *et al.* (1991) Plant Physiol. 95:426-34.

The genetic manipulations of a recombinant host herein may be performed using standard genetic techniques and screening, and may be carried out in any host cell that is suitable to genetic manipulation. In some embodiments, a recombinant host cell may be any organism or microorganism host suitable for genetic modification and/or recombinant gene expression. In some embodiments, a recombinant host may be a plant. Standard recombinant DNA and molecular cloning techniques used here are well-known in the art and are described in, for example and without limitation: Sambrook *et al.* (1989), *supra*; Silhavy *et al.* (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; and Ausubel *et al.* (1987) *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience, New York, NY.

Following the introduction of a nucleic acid into a plant cell, the plant cell may be grown, and upon emergence of differentiating tissue such as shoots and roots, mature plants can be generated. In some embodiments, a plurality of plants can be generated. Methodologies for regenerating plants are known to those of ordinary skill in the art and can be found, for example, in: Plant Cell and Tissue Culture, 1994, Vasil and Thorpe Eds. Kluwer Academic Publishers and in: Plant Cell Culture Protocols (Methods in Molecular Biology 111, 1999 Hall Eds Humana Press). Genetically modified plants described herein may be cultured in a fermentation medium or grown in a suitable medium such as soil. In some embodiments, a suitable growth medium for higher plants may be any growth medium for plants, including, but not limited to, soil, sand, any other particulate media that support root growth (*e.g.*, vermiculite, perlite, *etc.*) or hydroponic culture, as well as suitable light, water and nutritional supplements that facilitate the growth of the higher plant.

Transformed plant cells which are produced by any of the above transformation techniques can be cultured to regenerate a whole plant that possesses the transformed genotype, and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans, *et al.*, "Protoplasts Isolation and Culture" in *Handbook of Plant Cell Culture*, pp. 124-176, Macmillian Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca

Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, pollens, embryos or parts thereof. Such regeneration techniques are described generally in Klee *et al.* (1987) *Ann. Rev. of Plant Phys.* 38:467-486.

5 In other embodiments, the plant cells which are transformed are not capable of regeneration to produce a plant. Such cells may be employed, for example, in developing a plant cell line having the relevant phenotype, for example, herbicide resistance.

10 A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection can be performed by growing the engineered plant material on media containing an inhibitory amount of the antibiotic or herbicide to which the transforming gene construct confers resistance. Further, transformed plants and plant cells can also be identified by screening for the activities of any visible marker genes (*e.g.*, the β -glucuronidase, luciferase, or gfp
15 genes) that may be present on the recombinant nucleic acid constructs. Such selection and screening methodologies are well known to those skilled in the art.

A transgenic plant containing a heterologous molecule herein can be produced through selective breeding, for example, by sexually crossing a first parental plant comprising the molecule, and a second parental plant, thereby producing a plurality of
20 first progeny plants. A first progeny plant may then be selected that is resistant to a selectable marker (*e.g.*, glyphosate, resistance to which may be conferred upon the progeny plant by the heterologous molecule herein). The first progeny plant may then be selfed, thereby producing a plurality of second progeny plants. Then, a second progeny plant may be selected that is resistant to the selectable marker. These steps
25 can further include the back-crossing of the first progeny plant or the second progeny plant to the second parental plant or a third parental plant.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating, added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for
30 both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Other breeding methods commonly used for different traits and crops are known in the art. Backcross breeding has been used to transfer genes for a simply inherited, highly

heritable trait into a desirable homozygous cultivar or inbred line, which is the recurrent parent. The resulting plant is expected to have the attributes of the recurrent parent (*e.g.*, cultivar) and the desirable trait transferred from the donor parent. After the initial cross, individuals possessing the phenotype of the donor parent are selected
5 and repeatedly crossed (backcrossed) to the recurrent parent. The resulting parent is expected to have the attributes of the recurrent parent (*e.g.*, cultivar) and the desirable trait transferred from the donor parent.

A nucleic acid may also be introduced into a predetermined area of the plant genome through homologous recombination. Methods to stably integrate a
10 polynucleotide sequence within a specific chromosomal site of a plant cell *via* homologous recombination have been described within the art. For instance, site specific integration as described in US Patent Application Publication No. 2009/0111188 A1 involves the use of recombinases or integrases to mediate the introduction of a donor polynucleotide sequence into a chromosomal target. In
15 addition, International Patent Application No. WO 2008/021207, describes zinc finger mediated-homologous recombination to stably integrate one or more donor polynucleotide sequences within specific locations of the genome. The use of recombinases such as FLP/FRT as described in US Patent No. 6,720,475, or CRE/LOX as described in US Patent No. 5,658,772, can be utilized to stably
20 integrate a polynucleotide sequence into a specific chromosomal site. Finally, the use of meganucleases for targeting donor polynucleotides into a specific chromosomal location was described in Puchta *et al.*, PNAS USA 93 (1996) pp. 5055-5060).

Other various methods for site specific integration within plant cells are
25 generally known and applicable (Kumar *et al.*, *Trends in Plant Sci.* 6(4) (2001) pp. 155-159). Furthermore, site-specific recombination systems that have been identified in several prokaryotic and lower eukaryotic organisms may be applied for use in plants. Examples of such systems include, but are not limited too; the R/RS recombinase system from the pSR1 plasmid of the yeast *Zygosaccharomyces rouxii*
30 (Araki *et al.* (1985) *J. Mol. Biol.* 182: 191-203), and the Gin/gix system of phage Mu (Maeser and Kahlmann (1991) *Mol. Gen. Genet.* 230: 170-176).

In some embodiments, a heterologous nucleic acid encoding a polypeptide having at least 90% identity to SEQ ID NO:1 may be optionally combined with

another nucleic acid in the host cell and/or organism. For example, in certain embodiments, the heterologous nucleic acid encoding a polypeptide having at least 90% identity to SEQ ID NO:1 may be combined or “stacked” with another that provides additional resistance or tolerance to glyphosate or another herbicide, and/or
5 another that provides resistance to select insects or diseases and/or nutritional enhancements, and/or improved agronomic characteristics, and/or another that provides proteins or other products useful in feed, food, industrial, pharmaceutical or other uses. The “stacking” of two or more nucleic acid sequences of interest within a plant genome may be accomplished, for example, *via* conventional plant breeding
10 using two or more events, transformation of a plant with a construct(s) that contain the nucleic acids, re-transformation of a transgenic plant, or addition of new traits through targeted integration *via* homologous recombination.

Nucleic acids that may be “stacked” with a heterologous nucleic acid encoding a polypeptide having at least 90% identity to SEQ ID NO:1 include, for
15 example and without limitation:

Genes or Coding Sequence (e.g., iRNA) That Confer Resistance to Pests or Disease

(A) Plant Disease Resistance Genes. Plant defenses are often activated by specific interaction between the product of a disease resistance gene (R) in the plant and the product of a corresponding avirulence (Avr) gene in the pathogen. A plant
20 variety can be transformed with cloned resistance gene to engineer plants that are resistant to specific pathogen strains. Examples of such genes include, the tomato Cf-9 gene for resistance to *Cladosporium fulvum* (Jones *et al.*, 1994 Science 266:789), tomato *Pto* gene, which encodes a protein kinase, for resistance to
25 *Pseudomonas syringae* pv. tomato (Martin *et al.*, 1993 Science 262:1432), and *Arabidopsis* RSSP2 gene for resistance to *Pseudomonas syringae* (Mindrinos *et al.*, 1994 Cell 78:1089).

(B) A *Bacillus thuringiensis* protein, a derivative thereof or a synthetic polypeptide modeled thereon, such as, a nucleotide sequence of a *Bt* δ -endotoxin
30 gene (Geiser *et al.*, 1986 Gene 48:109), and a vegetative insecticidal (VIP) gene (*see, e.g.*, Estruch *et al.* (1996) Proc. Natl. Acad. Sci. 93:5389-94). Moreover, DNA molecules encoding δ -endotoxin genes can be purchased from American Type

Culture Collection (Rockville, Md.), under ATCC accession numbers 40098, 67136, 31995 and 31998.

(C) A lectin, such as, nucleotide sequences of several *Clivia miniata* mannose-binding lectin genes (Van Damme *et al.*, 1994 Plant Molec. Biol. 24:825).

5 (D) A vitamin binding protein, such as avidin and avidin homologs which are useful as larvicides against insect pests. See U.S. Pat. No. 5,659,026.

(E) An enzyme inhibitor, e.g., a protease inhibitor or an amylase inhibitor. Examples of such genes include a rice cysteine proteinase inhibitor (Abe *et al.*, 1987 J. Biol. Chem. 262:16793), a tobacco proteinase inhibitor I (Huub *et al.*, 1993 Plant
10 Molec. Biol. 21:985), and an α -amylase inhibitor (Sumitani *et al.*, 1993 Biosci. Biotech. Biochem. 57:1243).

(F) An insect-specific hormone or pheromone such as an ecdysteroid and juvenile hormone a variant thereof, a mimetic based thereon, or an antagonist or agonist thereof, such as baculovirus expression of cloned juvenile hormone esterase,
15 an inactivator of juvenile hormone (Hammock *et al.*, 1990 Nature 344:458).

(G) An insect-specific peptide or neuropeptide which, upon expression, disrupts the physiology of the affected pest (J. Biol. Chem. 269:9). Examples of such genes include an insect diuretic hormone receptor (Regan, 1994), an allostatin identified in *Diploptera punctata* (Pratt, 1989), and insect-specific, paralytic
20 neurotoxins (U.S. Pat. No. 5,266,361).

(H) An insect-specific venom produced in nature by a snake, a wasp, etc., such as a scorpion insectotoxic peptide (Pang, 1992 Gene 116:165).

(I) An enzyme responsible for a hyperaccumulation of monoterpene, a sesquiterpene, a steroid, hydroxamic acid, a phenylpropanoid derivative or another
25 non-protein molecule with insecticidal activity.

(J) An enzyme involved in the modification, including the post-translational modification, of a biologically active molecule; for example, glycolytic enzyme, a proteolytic enzyme, a lipolytic enzyme, a nuclease, a cyclase, a transaminase, an esterase, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an
30 elastase, a chitinase and a glucanase, whether natural or synthetic. Examples of such genes include, a callas gene (PCT published application WO93/02197), chitinase-encoding sequences (which can be obtained, for example, from the ATCC under accession numbers 3999637 and 67152), tobacco hookworm chitinase

(Kramer *et al.*, 1993 Insect Molec. Biol. 23:691), and parsley ubi4-2 polyubiquitin gene (Kawalleck *et al.*, 1993 Plant Molec. Biol. 21:673).

(K) A molecule that stimulates signal transduction. Examples of such molecules include nucleotide sequences for mung bean calmodulin cDNA clones
5 (Botella *et al.*, 1994 Plant Molec. Biol. 24:757) and a nucleotide sequence of a maize calmodulin cDNA clone (Griess *et al.*, 1994 Plant Physiol. 104:1467).

(L) A hydrophobic moment peptide. See U.S. Pat. Nos. 5,659,026 and 5,607,914; the latter teaches synthetic antimicrobial peptides that confer disease resistance.

10 (M) A membrane permease, a channel former or a channel blocker, such as a cecropin- β lytic peptide analog (Jaynes *et al.*, 1993 Plant Sci. 89:43) which renders transgenic tobacco plants resistant to *Pseudomonas solanacearum*.

(N) A viral-invasive protein or a complex toxin derived therefrom. For example, the accumulation of viral coat proteins in transformed plant cells imparts
15 resistance to viral infection and/or disease development effected by the virus from which the coat protein gene is derived, as well as by related viruses. Coat protein-mediated resistance has been conferred upon transformed plants against alfalfa mosaic virus, cucumber mosaic virus, tobacco streak virus, potato virus X, potato virus Y, tobacco etch virus, tobacco rattle virus and tobacco mosaic virus.
20 See, for example, Beachy *et al.* (1990) Ann. Rev. Phytopathol. 28:451.

(O) An insect-specific antibody or an immunotoxin derived therefrom. Thus, an antibody targeted to a critical metabolic function in the insect gut would inactivate an affected enzyme, killing the insect. For example, Taylor *et al.* (1994) Abstract #497, Seventh Int'l. Symposium on Molecular Plant-Microbe Interactions
25 shows enzymatic inactivation in transgenic tobacco via production of single-chain antibody fragments.

(P) A virus-specific antibody. See, for example, Tavladoraki *et al.* (1993) Nature 266:469, which shows that transgenic plants expressing recombinant antibody genes are protected from virus attack.

30 (Q) A developmental-arrestive protein produced in nature by a pathogen or a parasite. Thus, fungal endo α -1,4-D polygalacturonases facilitate fungal colonization and plant nutrient release by solubilizing plant cell wall homo- α -1,4-D-galacturonase (Lamb *et al.*, 1992) Bio/Technology 10:1436. The

cloning and characterization of a gene which encodes a bean endopolygalacturonase-inhibiting protein is described by Toubart *et al.* (1992 Plant J. 2:367).

(R) A developmental-arrestive protein produced in nature by a plant, such as the barley ribosome-inactivating gene that provides an increased resistance to fungal disease (Longemann *et al.*, 1992). Bio/Technology 10:3305.

(S) RNA interference, in which an RNA molecule is used to inhibit expression of a target gene. An RNA molecule in one example is partially or fully double stranded, which triggers a silencing response, resulting in cleavage of dsRNA into small interfering RNAs, which are then incorporated into a targeting complex that destroys homologous mRNAs. See, e.g., Fire *et al.*, US Patent 6,506,559; Graham *et al.* 6,573,099.

Genes That Confer Resistance to a Herbicide

(A) Genes encoding resistance or tolerance to a herbicide that inhibits the growing point or meristem, such as an imidazalinone, sulfonanilide or sulfonylurea herbicide. Exemplary genes in this category code for a mutant ALS enzyme (Lee *et al.*, 1988 EMBOJ. 7:1241), which is also known as AHAS enzyme (Miki *et al.*, 1990 Theor. Appl. Genet. 80:449).

(B) One or more additional genes encoding resistance or tolerance to glyphosate imparted by mutant EPSP synthase and *aroA* genes, or through metabolic inactivation by genes such as GAT (glyphosate acetyltransferase) or GOX (glyphosate oxidase) and other phosphono compounds such as glufosinate (*pat* and *bar* genes; DSM-2), and aryloxyphenoxypropionic acids and cyclohexanediones (ACCase inhibitor encoding genes). See, for example, U.S. Pat. No. 4,940,835, which discloses the nucleotide sequence of a form of EPSP which can confer glyphosate resistance. A DNA molecule encoding a mutant *aroA* gene can be obtained under ATCC Accession Number 39256, and the nucleotide sequence of the mutant gene is disclosed in U.S. Pat. No. 4,769,061. European patent application No. 0 333 033 and U.S. Pat. No. 4,975,374 disclose nucleotide sequences of glutamine synthetase genes which confer resistance to herbicides such as L-phosphinothricin. The nucleotide sequence of a phosphinothricinacetyl-transferase gene is provided in European application No. 0 242 246. De Greef *et al.* (1989) Bio/Technology 7:61 describes the production of transgenic plants that express

chimeric *bar* genes coding for phosphinothricin acetyl transferase activity. Exemplary of genes conferring resistance to aryloxyphenoxypropionic acids and cyclohexanediones, such as sethoxydim and haloxyfop, are the Accl-S1, Accl-S2 and Accl-S3 genes described by Marshall *et al.* (1992) Theor. Appl. Genet. 83:435.

5 (C) Genes encoding resistance or tolerance to a herbicide that inhibits photosynthesis, such as a triazine (*psbA* and *gs+* genes) and a benzonitrile (nitrilase gene). Przibilla *et al.* (1991) Plant Cell 3:169 describe the use of plasmids encoding mutant *psbA* genes to transform *Chlamydomonas*. Nucleotide sequences for nitrilase genes are disclosed in U.S. Pat. No. 4,810,648, and DNA molecules containing these
10 genes are available under ATCC accession numbers 53435, 67441 and 67442. Cloning and expression of DNA coding for a glutathione S-transferase is described by Hayes *et al.* (1992) Biochem. J. 285:173.

(D) Genes encoding resistance or tolerance to a herbicide that bind to hydroxyphenylpyruvate dioxygenases (HPPD), enzymes which catalyze the reaction
15 in which para-hydroxyphenylpyruvate (HPP) is transformed into homogentisate. This includes herbicides such as isoxazoles (EP418175, EP470856, EP487352, EP527036, EP560482, EP682659, U.S. Pat. No. 5,424,276), in particular isoxaflutole, which is a selective herbicide for maize, diketonitriles (EP496630, EP496631), in particular 2-cyano-3-cyclopropyl-1-(2-SO₂CH₃-4-CF₃
20 phenyl)propane-1,3-dione and 2-cyano-3-cyclopropyl-1-(2-SO₂CH₃-4-2,3Cl₂phenyl)propane-1,3-dione, triketones (EP625505, EP625508, U.S. Pat. No. 5,506,195), in particular sulcotrione, and pyrazolines. A gene that produces an overabundance of HPPD in plants can provide tolerance or resistance to such herbicides, including, for example, genes described in U.S. Patent Nos. 6,268,549
25 and 6,245,968 and U.S. Patent Application, Publication No. 20030066102.

(E) Genes encoding resistance or tolerance to phenoxy auxin herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and which may also confer resistance or tolerance to aryloxyphenoxypropionate (AOPP) herbicides. Examples of such genes include the α -ketoglutarate-dependent dioxygenase enzyme (*aad-1*)
30 gene, described in U.S. Patent No. 7,838,733.

(F) Genes encoding resistance or tolerance to phenoxy auxin herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and which may also confer resistance or tolerance to pyridyloxy auxin herbicides, such as fluroxypyr or triclopyr. Examples

of such genes include the α -ketoglutarate-dependent dioxygenase enzyme gene (*aad-12*), described in WO 2007/053482 A2.

(G) Genes encoding resistance or tolerance to dicamba (*see, e.g.,* U.S. Patent Publication No. 20030135879).

5 (H) Genes providing resistance or tolerance to herbicides that inhibit protoporphyrinogen oxidase (PPO) (*see* U.S. Pat. No. 5,767,373).

(I) Genes providing resistance or tolerance to triazine herbicides (such as atrazine) and urea derivatives (such as diuron) herbicides which bind to core proteins of photosystem II reaction centers (PS II) (*See* Brussian *et al.*, (1989) EMBO J. 1989, 8(4): 1237-1245.

Genes That Confer or Contribute to a Value-Added Trait

(A) Modified fatty acid metabolism, for example, by transforming maize or *Brassica* with an antisense gene or stearoyl-ACP desaturase to increase stearic acid content of the plant (Knultzon *et al.*, 1992) Proc. Nat. Acad. Sci. USA 89:2624.

15 (B) Decreased phytate content

(1) Introduction of a phytase-encoding gene, such as the *Aspergillus niger* phytase gene (Van Hartingsveldt *et al.*, 1993 Gene 127:87), enhances breakdown of phytate, adding more free phosphate to the transformed plant.

(2) A gene could be introduced that reduces phytate content. In
20 maize, this, for example, could be accomplished by cloning and then reintroducing DNA associated with the single allele which is responsible for maize mutants characterized by low levels of phytic acid (Raboy *et al.*, 1990 Maydica 35:383).

(C) Modified carbohydrate composition effected, for example, by transforming plants with a gene coding for an enzyme that alters the branching
25 pattern of starch. Examples of such enzymes include, *Streptococcus mucus* fructosyltransferase gene (Shiroza *et al.*, 1988) J. Bacteriol. 170:810, *Bacillus subtilis* levansucrase gene (Steinmetz *et al.*, 1985 Mol. Gen. Genet. 200:220), *Bacillus licheniformis* α -amylase (Pen *et al.*, 1992 Bio/Technology 10:292), tomato invertase genes (Elliot *et al.*, 1993), barley amylase gene (Sogaard *et al.*, 1993 J.
30 Biol. Chem. 268:22480), and maize endosperm starch branching enzyme II (Fisher *et al.*, 1993 Plant Physiol. 102:10450).

Various assays can be employed in connection with the nucleic acid molecule of certain embodiments of the disclosure. The following techniques are

useful in a variety of situations, and in one embodiment, are useful in detecting the presence of the nucleic acid molecule and/or the polypeptide encoded in a plant cell. For example, the presence of the molecule can be determined in a variety of ways, including using a primer or probe of the sequence, ELISA assay to detect the encoded protein, a Western blot to detect the protein, or a Northern or Southern blot to detect RNA or DNA. Enzymatic assays for detecting enzyme DGT-28 can be employed. Further, an antibody which can detect the presence of the DGT-28 protein can be generated using art recognized procedures. Additional techniques, such as *in situ* hybridization, enzyme staining, and immunostaining, also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. A transgene may be selectively expressed in some tissues of the plant or at some developmental stages, or the transgene may be expressed in substantially all plant tissues, substantially along its entire life cycle. However, any combinatorial expression mode is also applicable.

Southern analysis is a commonly used detection method, wherein DNA is cut with restriction endonucleases and fractionated on an agarose gel to separate the DNA by molecular weight and then transferring to nylon membranes. It is then hybridized with the probe fragment which was radioactively labeled with ^{32}P (or other probe labels) and washed in an SDS solution.

Likewise, Northern analysis deploys a similar protocol, wherein RNA is cut with restriction endonucleases and fractionated on an agarose gel to separate the RNA by molecular weight and then transferring to nylon membranes. It is then hybridized with the probe fragment which was radioactively labeled with ^{32}P (or other probe labels) and washed in an SDS solution. Analysis of the RNA (e.g., mRNA) isolated from the tissues of interest can indicate relative expression levels. Typically, if the mRNA is present or the amount of mRNA has increased, it can be assumed that the corresponding transgene is being expressed. Northern analysis, or other mRNA analytical protocols, can be used to determine expression levels of an introduced transgene or native gene.

In the Western analysis, instead of isolating DNA/RNA, the protein of interest is extracted and placed on an acrylamide gel. The protein is then blotted onto a membrane and contacted with a labeling substance. *See e.g., Hood et al., "Commercial Production of Avidin from Transgenic Maize; Characterization of*

Transformants, Production, Processing, Extraction and Purification” *Molecular Breeding* 3:291-306 (1997); Towbin *et al.* (1979) “Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications” *Proc Natl Acad Sci USA* 76(9): 4350-4354; Renart *et al.* “Transfer of
5 proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure” *Proc Natl Acad Sci USA* 76(7): 3116-3120.

The nucleic acids herein, or segments thereof, may be used to design primers for PCR amplification. In performing PCR amplification, a certain degree of
10 mismatch can be tolerated between primer and template. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

Another example of method detection is the pyrosequencing technique as described by Winge (Innov. Pharma. Tech. 00:18-24, 2000). In this method, an
15 oligonucleotide is designed that overlaps the adjacent genomic DNA and insert DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking genomic sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin.
20 dNTPs are added individually and the incorporation results in a light signal that is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Molecular Beacons have been described for use in sequence detection.
25 Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking genomic and insert DNA junction. The unique structure of the FRET probe results in it containing a secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the
30 presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe(s) to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal indicates the presence of

the flanking genomic/transgene insert sequence due to successful amplification and hybridization.

Hydrolysis probe assay, otherwise known as TAQMAN[®] (Life Technologies, Foster City, Calif.), is a method of detecting and quantifying the presence of a DNA sequence. Briefly, a FRET oligonucleotide probe is designed with one oligo within the transgene and one in the flanking genomic sequence for event-specific detection. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

The ELISA or enzyme linked immunoassay has been known since 1971. In general, antigens solubilised in a buffer are coated on a plastic surface. When serum is added, antibodies can attach to the antigen on the solid phase. The presence or absence of these antibodies can be demonstrated when conjugated to an enzyme. Adding the appropriate substrate will detect the amount of bound conjugate which can be quantified. A common ELISA assay is one which uses biotinylated anti-(protein) polyclonal antibodies and an alkaline phosphatase conjugate. For example, an ELISA used for quantitative determination of laccase levels can be an antibody sandwich assay, which utilizes polyclonal rabbit antibodies obtained commercially. The antibody is conjugated to alkaline phosphatases for detection. In another example, an ELISA assay to detect trypsin or trypsinogen uses biotinylated anti-trypsin or anti-trypsinogen polyclonal antibodies and a streptavidin-alkaline phosphatase conjugate.

Certain embodiments relate to processes of making crosses using a plant of an embodiment of this disclosure as at least one parent. For example, particular embodiments relate to an F₁ hybrid plant having as one or both parents any of the plants exemplified herein. Other embodiments relate to seed produced by such F₁ hybrids. Still other embodiments relate to a method for producing an F₁ hybrid seed by crossing an exemplified plant with a different (*e.g.* in-bred parent) plant and harvesting the resultant hybrid seed. Other embodiments relate to an exemplified plant that is

either a female parent or a male parent. Characteristics of the resulting plants may be improved by careful consideration of the parent plants.

V. Glyphosate tolerance mediated by DGT-28

5 Polypeptides having at least 90% sequence identity to SEQ ID NO:1 may have EPSPS enzymatic activity. Thus, polypeptides having at least 90% identity to SEQ ID NO:1, nucleic acids encoding a polypeptide having at least 90% identity to SEQ ID NO:1 (*e.g.*, SEQ ID NOs:2-4), and a nucleic acids that hybridize to a nucleic acid having SEQ ID NO:2 or SEQ ID NO:3 under high-stringency conditions may be used
10 in some embodiments to confer glyphosate tolerance to an cell or organism (*e.g.*, a plant cell or plant). Providing a plant or plant cell that is resistant to glyphosate herbicide formulations may be useful in a variety of applications, where those plant cells having such resistance can tolerate exposure to a sufficient amount of glyphosate that is used to control at least some weeds in an area under cultivation.

15 Glyphosate, a composition comprising N- (phosphonomethyl) glycine, is a widely used component in herbicides. Glyphosate is typically formulated as a salt in an aqueous liquid concentrate, a solid concentrate, an emulsion or a microemulsion. Glyphosate can be applied over-the-top of plants from emergence throughout the various stages of plant development.

20 Glyphosate tolerant plant varieties used in combination with glyphosate herbicidal formulations have become the standard program for weed management in crop production in the United States and throughout the world. The primary advantage to growers in using a glyphosate tolerance trait is that it allows simple and convenient application of glyphosate; a broad spectrum, post- emergence herbicide, to control
25 unwanted plants and grasses (*i.e.*, “weeds”) with excellent crop safety and less dependence on pre-plant herbicide applications. Other benefits include a better fit into no-till and reduced tillage systems. Glyphosate tolerant crops have expanded the options for weed management and made the practice of weed control much easier, less expensive and more flexible. Growers have reported making fewer trips across fields
30 to apply herbicides as well as making fewer cultivation trips, which conserves fuel and reduces soil erosion. Glyphosate-tolerant crops, therefore, decrease the environmental risks posed by herbicides, while at the same time increasing the efficacy of necessary chemical weed control.

Accordingly, some embodiments herein provide for selectively controlling weeds in an area under cultivation containing a plant comprising a polypeptide having at least 90% identity to SEQ ID NO:1, a nucleic acid encoding a polypeptide having at least 90% identity to SEQ ID NO:1, and/or a nucleic acid that hybridizes to a nucleic acid having SEQ ID NO:2 or SEQ ID NO:3 under high-stringency conditions, wherein the plant has increased glyphosate tolerance when compared to a plant of the same species that does not comprise the polypeptide and/or nucleic acid(s). In some examples, a method provided herein comprises applying a sufficient amount of a herbicidal glyphosate to the crop foliage and weeds to control growth of the weeds.

Particular embodiments herein provide a method for killing or controlling weeds or unwanted vegetation in an area under cultivation containing a crop (*e.g.*, a plant comprising a polypeptide having at least 90% identity to SEQ ID NO:1, a nucleic acid encoding a polypeptide having at least 90% identity to SEQ ID NO:1, and/or a nucleic acid that hybridizes to a nucleic acid having SEQ ID NO:2 or SEQ ID NO:3 under high-stringency conditions). In some examples, the method comprises applying glyphosate to the crop and/or the area under cultivation; for example, applying an amount of the glyphosate to foliage of the crop plant, and simultaneously to weeds growing in close proximity to such plants, wherein the amount is sufficient to result in control of the weeds or unwanted vegetation, while leaving the crop plant substantially unharmed.

A glyphosate composition may be applied to plants at an application rate sufficient to give desired biological results, for example, control of weed growth without significantly affecting glyphosate tolerant crop plants. These application rates are usually expressed as amount of glyphosate per unit area treated, *e.g.*, grams per hectare (g/ha). What constitutes a "significant effect" varies according to the standards and practice of those who investigate, develop, market and use compositions, and the selection of application rates that are significantly effective for a composition is within the skill of those skilled in the art.

In certain examples, the amount of the glyphosate composition applied per unit area to give 85% control of a weed species as measured by growth reduction or mortality is used to define an application rate. The selection of a number of glyphosate herbicide application rates sufficient to control weeds in an area under cultivation is within the skill of the ordinary agricultural scientist. Those of skill in the art will

likewise recognize that individual plant conditions, weather and growing conditions, as well as the specific active ingredients and their weight ratio in the composition, may influence the degree of herbicidal effectiveness in a particular application.

5 In some embodiments, an aqueous glyphosate composition can be applied to the foliar tissues of plants to kill or control the growth of a wide variety of unwanted plants, including annual and perennial grass and broadleaf weed species, by applying to the foliar tissues of the plants aqueous glyphosate compositions. The relative amount of glyphosate present in a contemplated herbicidal composition (*e.g.*, a particulate solid concentrate, liquid concentrate, ready-to-use composition, and tank-mix composition) 10 may vary depending upon many factors including, for example, the weed species to be controlled and the method of application. Generally speaking, however, the concentration of glyphosate, and optionally a surfactant and/or some other adjuvant or additive (as described elsewhere herein) used in the herbicidal composition is sufficient to control weeds within an area under cultivation.

15 An herbicidal spray composition may be applied as an aqueous solution or dispersion, whether the composition is manufactured ready for application, or results from the further dilution of a liquid glyphosate concentrate or the addition of water to a particulate solid glyphosate concentrate. However, the term “aqueous,” as used herein, includes compositions comprising some small amount of non-aqueous solvent, so long 20 as the predominant solvent present is water. An herbicidal spray compositions may be applied to the foliage of the plants to be treated through any of the appropriate methods that are well known to those having skill in the art, including aerial application and ground application techniques (*e.g.*, a ground boom, a hand sprayer, and a rope-wick).

In some examples, a liquid concentrate composition is formulated to include 25 glyphosate in a concentration of at least about 50 grams, at least about 75 grams, or at least about 100, 125, 150, 175, 200, 225, 250, 275, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690 or 700 grams (acid equivalent or a.e.) per liter, or more. The glyphosate concentration range 30 may be, for example, from about 50 to about 680 grams (a.e.) per liter (gpl), from about 100 to about 600 gpl, from about 250 to about 600 gpl, and from about 360 to about 540 gpl.

When expressed as a weight percentage based on the total weight of the glyphosate concentrate, a liquid concentrate may comprise, for example, at least about 10 wt.% glyphosate (a.e.), at least about 15 wt.%, and at least about 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, 62, 63, 64, 65, 66, 67, or 68 wt.%, or more. The glyphosate concentration range may be, for example, from about 10 wt.% to about 70 wt.% a.e., from about 20 wt.% to about 68 wt.%, or from about 25 wt.% to about 45 wt.%.

If the glyphosate is applied as a ready-to-use composition, the glyphosate concentration may be, for example, from about 1 wt.% to about 3 wt.% a.e., and from about 1 wt.% to about 2 wt.%.

Spray compositions may be formulated for application of, for example, at least about 1 gallon of spray composition per acre, at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 gallons per acre, and more. The spray volume of the spray composition may range, for example, from about 1 gallon to about 100 gallons per acre, from about 2 gallons to about 40 gallons per acre, and from about 2 gallons to about 5 gallons per acre for an aerial application, and from about 5 gallons to about 20 gallons per acre for a ground application.

In some examples, a liquid concentrate formulation having an aqueous phase wherein glyphosate is present predominantly in the form of a salt, and a non-aqueous phase optionally containing a second herbicidal active ingredient that is relatively water-insoluble, may be employed. Such formulations may include, for example, emulsions (including macroemulsions and microemulsions, water-in-oil, oil-in-water and water-in-oil -in-water types), suspensions, and suspoemulsions. The non-aqueous phase may comprise in certain instances a microencapsulated component (*e.g.*, a microencapsulated herbicide). In formulations having a non-aqueous phase, the concentration of glyphosate a.e. in the composition as a whole may nonetheless be within the particular exemplary ranges recited herein for aqueous concentrate formulations.

Suitable salt forms of glyphosate which may be used in accordance with any of the formulations include, for example, alkali metal salts, for example sodium and potassium salts, ammonium salts, di-ammonium salts such as dimethylammonium, alkylamine salts, for example dimethylamine and isopropylamine salts, alkanolamine

salts, for example ethanolamine salts, alkylsulfonium salts, for example trimethylsulfonium salts, sulfoxonium salts, and mixtures or combinations thereof. Examples of commercial formulations of glyphosate include, without restriction: GLYPHOMAX™, GLYPHOMAX™ XRT, GLYPHOMAX™ PLUS, DURANGO™, ROUNDUP™ ULTRA, ROUNDUP™ ULTRAMAK, ROUNDUP™ CT, ROUNDUP™ EXTRA, ROUNDUP™ BIOACTIVE, ROUNDUP™ BIOFORCE, RODEO™, POLARIST™, SPARK™, ACCORD™ SP, ACCORD™ XRT, and ACCORD™ CONCENTRATE, all of which contain glyphosate as its isopropylammonium salt (IPA); ROUNDUP™ DRY and RIVAL™, which contain glyphosate as its ammonium salt; ROUNDUP™ GEOFORCE, a sodium glyphosate formulation; TOUCHDOWN™, a glyphosate trimesium salt formulation, TOUCHDOWN™ IQ, a glyphosate diammonium salt formulation, TOUCHDOWN™ TOTAL IQ, a potassium glyphosate formulation, and ROUNDUP™ WEATHERMAX, a potassium glyphosate formulation. Glyphosate formulations may include safening agents, surfactants, and/or adjuvants.

If desired, the user may mix one or more adjuvants with a glyphosate composition and the water of dilution when preparing a formulation for application. Such adjuvants may include additional surfactant and/or an inorganic salt (*e.g.*, ammonium sulfate) with the aim of further enhancing herbicidal efficacy.

If desired, the user may also employ appropriate safeners in a glyphosate formulation to further protect plants and/or to add cross resistance to more herbicides. Safeners are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism, without compromising weed control efficacy. Safeners typically act to increase a plant's immune system by activating/expressing cP450. Exemplary safeners include, for example and without limitation: benoxacor, cloquintocet, cyometrinil, dichlormid, dicyclonon, dietholate, fenchlorazole, fenclorim, flurazole, fluxofenim, furilazole, isoxadifen, mefenpyr, mephenate, naphthalic anhydride, and oxabetrinil.

Safeners may be used for the protection of large-seeded grass crops, for example and without limitation, corn, grain sorghum, and wet-sown rice, against preplant-incorporated or preemergence-applied herbicides of the thiocarbamate and chloroacetanilide families. Safeners also have been developed to protect winter cereal crops such as wheat against postemergence applications of aryloxyphenoxypropionate

and sulfonylurea herbicides. The use of safeners for the protection of corn and rice against sulfonylurea, imidazolinone, cyclohexanedione, isoxazole, and triketone herbicides is also well-established.

Plant activators (a new class of compounds that protect plants by activating their defense mechanisms) may also be used in embodiments herein. Exemplary plant activators include acibenzolar and probenazole.

Embodiments of the present invention are further defined in the following Examples. It should be understood that these Examples are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the embodiments of the invention to adapt it to various usages and conditions. Thus, various modifications of the embodiments of the invention, in addition to those shown and described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. The following is provided by way of illustration and not intended to limit the scope of the invention.

EXAMPLES

Example 1: Materials and Methods

Embodiments of the present disclosure are further described in the following examples, which are offered by way of illustration, and are not intended to limit the invention in any manner.

A single amino acid mutation (G96A) in the *Escherichia coli* 5-enolpyruvylshikimate 3-phosphate synthase enzyme (EPSP synthase) can result in glyphosate insensitivity (Padgett et al., (1991); Eschenburg *et al.*, (2002); Priestman *et al.*, (2005); Haghani *et al.*, (2008)). While this mutation confers tolerance to glyphosate, it is also known to adversely affect binding of EPSP synthase with its natural substrate, phosphoenolpyruvate (PEP). The resulting change in substrate binding efficiency can render a mutated enzyme unsuitable for providing *in planta* tolerance to glyphosate.

The NCBI Genbank database was screened *in silico* for EPSP synthase protein and polynucleotide sequences that naturally contain an alanine at an analogous position

within the EPSP synthase enzyme as that of the G96A mutation which was introduced into the *E. coli* version of the enzyme (Padgett *et al.*, (1991); Eschenburg *et al.*, (2002); Priestman *et al.*, (2005); Haghani *et al.*, (2008)).

One enzyme that was identified to contain a natural alanine at this position was
5 DGT-28 (GENBANK ACC NO: ZP_06917240.1) from *Streptomyces sviveus* ATCC29083. Further *in silico* data mining revealed three other unique *Streptomyces* enzymes with greater homology to DGT-28; DGT-31 (GENBANK ACC NO: YP_004922608.1); DGT-32 (GENBANK ACC NO: ZP_04696613); and DGT-33 (GENBANK ACC NO: NC_010572). Each of these enzymes contains a natural
10 alanine at an analogous position within the EPSP synthase enzyme as that of the G96A mutation that was introduced into the *E. coli* version of the enzyme. **FIG. 1.**

Because EPSP synthase proteins from different organisms are of different lengths, the numbering of the mutation for the *E. coli* version of the EPSP synthase enzyme does not necessarily correspond with the numbering of the mutation for the
15 EPSP synthase enzymes from the other organisms. These identified EPSP synthase enzymes were not previously characterized in regard to glyphosate tolerance or PEP substrate affinity. Furthermore, these EPSP synthase enzymes represent a new class of EPSP synthase enzymes and do not contain any sequence motifs that have been used to characterize previously described Class I (plant derived sequences further described in
20 US Patent No. RE39247), II (bacterially derived sequences further described in US Patent No. RE39247), and III (bacterially derived sequences further described in International Patent Application WO 2006/110586) EPSP synthase enzymes.

The novel DGT-28, DGT-31, DGT-32, and DGT-33 enzymes were characterized for glyphosate tolerance and PEP substrate affinity by comparison to
25 Class I EPSP synthase enzymes. The following Class I enzymes; DGT-1 from *Glycine max*, DGT-3 from *Brassica napus* (GENBANK ACC NO: P17688), and DGT-7 from *Triticum aestivum* (GENBANK ACC NO: EU977181) were for comparison. The Class I EPSP synthase enzymes and mutant variants thereof were synthesized and evaluated. A mutation introduced into the plant EPSP synthase enzymes consisted of
30 the Glycine to Alanine mutation made within the EPSP synthase enzyme at a similar location as that of the G96A mutation from the *E. coli* version of the enzyme. In addition, Threonine to Isoleucine and Proline to Serine mutations were introduced within these Class I EPSP synthase enzymes at analogous positions as that of amino

acid 97 (T to I) and amino acid 101 (P to S) in the EPSP synthase of *E. coli* as described in Funke *et al.*, (2009).

FIG. 1 depicts a partial sequence alignment of DGT-28, DGT-31, DGT-32, and DGT-33 to other EPSP synthase enzymes. All four DGT enzymes share a conserved alanine at the *aroA* EPSP synthase enzyme amino acid position 96. The location of this amino acid is indicated by an asterisk, and the amino acid residue is underlined.

FIG. 2 shows an alignment of the DGT-1, DGT-3, and DGT-7 enzymes. The location of the amino acid residue that was mutated from glycine to alanine is indicated by the first asterisk. The location of the amino acid residue which was mutated from threonine to isoleucine is indicated by the second asterisk. The location of the third amino acid residue that was mutated from proline to serine is indicated by the third asterisk. These mutations were introduced into different versions of DGT-1, DGT-3, and DGT-7. The different versions (v1, v2, v3... vN) of the genes that contain the mutations are described in more detail below.

Example 2: Optimization of Sequence for Expression in Plants and Bacteria

Plant Optimization. Codon bias for dicots and monocots (maize) was calculated as the frequency at which a single codon is used relative to the codons for all amino acids. **Table 1.** Alternatively, the codon bias may be calculated as the frequency at which a single codon is used to encode a particular amino acid, relative to all the other codons for that amino acid (synonymous codons). In designing coding regions for plant expression, the primary ("first choice") codons preferred by the plant were determined, as well as the second, third, fourth, etc. choices of preferred codons when multiple choices existed.

Analysis of the DGT-28 coding sequence from *S. sviveus* revealed the presence of several sequence motifs that were believed to be detrimental to optimal plant expression, as well as a non-optimal codon composition for expression in dicotyledonous and monocotyledonous plants.

Table 1. Synonymous codon representation from coding regions of monocotyledonous (maize %) and dicotyledonous (dicot %) plant genes are shown in Columns D, E, I, and J. Values for a balanced-biased codon representation set for a plant-optimized synthetic gene design are in Columns C and H.

A	B	C	D	E	F	G	H	I	J
Amino Acid	Codon	Weighted Average	Maize %	Dicot %	Amino Acid	Codon	Weighted Average	Maize %	Dicot %
ALA (A) 100	GCA	25.5	18	25	LEU (L) 100	CTA	DNU	8	8
	GCC	35.6	34	27		CTC	34.3	26	19
	GCG	DNU	24	6		CTG	DNU	29	9
	GCT	39.0	24	42		CTT	34.3	17	28
ARG (R) 100	AGA	27.4	15	30		TTA	DNU	5	10
	AGG	31.5	26	25		TTG	31.4	15	26
	CGA	DNU	9	8	LYS (K) 100	AAA	30.6	22	39
	CGC	21.7	24	11		AAG	69.4	78	61
	CGG	DNU	15	4	MET (M)	ATG	100	100	100
ASN (N) 100	CGT	19.4	11	21		TTC	63.2	71	55
	AAC	61.4	68	55	PHE (F) 100	TTT	36.8	29	45
ASP (D) 100	AAT	38.6	32	45		CCA	41.4	26	42
	GAC	52.6	63	42		CCC	25.3	24	17
CYS (C) 100	GAT	47.4	37	58		CCG	DNU	28	9
	TGC	61.8	68	56	SER (S) 100	CCT	33.3	22	32
END 100	TGT	38.2	32	44		AGC	26.0	23	18
	TAA		20	48		AGT	DNU	9	14
	TAG		21	19		TCA	22.4	16	19
GLN (Q) 100	TGA		59	33		TCC	26.3	23	18
	CAA	48.4	38	59		TCG	DNU	14	6
GLU (E) 100	CAG	51.6	62	41		TCT	25.4	15	25
	GAA	38.8	29	49	THR (T) 100	ACA	28.0	21	27
GLY (G) 101	GAG	61.2	71	51		ACC	39.5	37	30
	GGA	28.5	19	38		ACG	DNU	22	8
	GGC	29.0	42	16		ACT	32.5	20	35
	GGG	16.0	20	12	TRP (W)	TGG	100	100	100
	GGT	26.6	20	33		TAC	65.0	73	57
HIS (H) 100	CAC	54.1	62	46	TYR (Y) 100	TAT	35.0	27	43
	CAT	45.9	38	54		GTA	DNU	8	12
ILE (I) 100	ATA	15.9	14	18		GTC	28.7	32	20
	ATC	47.9	58	37		GTG	38.0	39	29
	ATT	36.4	28	45		GTT	33.3	21	39

*DNU = Do Not Use

To engineer the plant-optimized genes encoding a DGT-28 protein, DNA sequences were designed to encode the amino acid sequences, utilizing a redundant genetic code established from the codon bias table compiled from the protein coding sequences for the particular host plants. In **Table 1**, Columns D and I present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the coding regions of monocotyledonous (maize)

plants. Columns E and J present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the coding regions of dicotyledonous plants. Some synonymous codons for some amino acids are found only rarely in plant genes (*e.g.* CGG). Usually, a codon was considered to be rarely
5 used if it is represented at about 10% or less of the time to encode the relevant amino acid in genes of either plant type (indicated by DNU in Columns C and H of Table 1). To balance the distribution of the remaining codon choices for an amino acid, a Weighted Average representation for each codon was calculated, using the formula:

10 Weighted Average % of C1 = $1/(\%C1 + \%C2 + \%C3 + \text{etc.}) \times \%C1 \times 100$, (1)
where C1 is the codon in question, and %C2, %C3, etc. represent the averages of the % values for dicots of remaining synonymous codons (average % values for the relevant codons are taken from Columns C and H) of **Table 1**.

15 The Weighted Average % value for each codon is given in Columns C and H of **Table 1**.

Using the foregoing procedure, a new DNA sequence that encodes essentially the amino acid sequence of the DGT-28 protein was designed for optimal expression in dicotyledonous plants, using a balanced codon distribution of frequently used codons
20 found in dicotyledonous plant genes. A second DNA sequence that encodes essentially the amino acid sequence of the DGT-28 protein was designed for optimal expression in monocotyledonous plants, using a balanced codon distribution of frequently used codons found in monocotyledonous plant genes. The two new DNA sequences differed from the original DNA sequences encoding *dgt-28* by the substitution of plant
25 (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the appropriate amino acid at each position within the protein amino acid sequence.

Design of the plant-optimized DNA sequences were initiated by reverse-translation of the protein sequences of the DGT-28 protein sequence (Genbank Accession No: ZP_06917240.1). SEQ ID NO:1 was reverse-translated using a dicot
30 codon bias table constructed from **Table 1**; Columns E and J. A second reverse-translation of SEQ ID NO:1 was completed using a monocot codon bias table constructed from **Table 1**; Columns D and I.

The initial reverse-translation sequences were then modified by compensating codon changes (while retaining overall weighted average codon representation) to remove or add restriction enzyme recognition sites, remove highly stable intrastrand secondary structures, and remove other sequences that might be detrimental to cloning manipulations or expression of the engineered gene in plants. The DNA sequence was then re-analyzed for restriction enzyme recognition sites that might have been created by the modifications. The identified sites were further modified by replacing the relevant codons with first, second, third, or fourth choice preferred codons. Other sites in the sequences that could affect transcription or translation of the gene of interest include the exon:intron junctions (5' or 3'), poly A addition signals, and RNA polymerase termination signals. The modified sequences were further analyzed and further modified to reduce the frequency of TA or CG doublets, and to increase the frequency of TG or CT doublets. In addition to these doublets, sequence blocks that have more than about six consecutive residues of [G+C] or [A+T] can affect transcription or translation of the sequence. Therefore, these sequence blocks were also modified by replacing the codons of first or second choice, etc. with other preferred codons of choice. Rarely used codons were not included to a substantial extent in the gene design, being used only when necessary to accommodate a different design criterion than codon composition, *per se* (e.g., addition or deletion of restriction enzyme recognition sites).

The newly-designed, dicotyledonous plant optimized *dgt-28 v5* polynucleotide sequence is listed in SEQ ID NO:2. The newly-designed, monocotyledonous plant optimized *dgt-28 v6* polynucleotide sequence is listed in SEQ ID NO:3; this sequence was slightly modified by including an alanine at the second amino acid position to introduce a restriction enzyme site. The resulting DNA sequences have a higher degree of codon diversity, a desirable base composition, contains strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA.

Synthesis of DNA fragments comprising SEQ ID NO:2 and SEQ ID NO:3 containing additional sequences, such as 6-frame stops (stop codons located in all six reading frames that are added to the 3' end of the coding sequence), and a 5' restriction site for cloning were performed by commercial suppliers (DNA2.0, Menlo Park, CA).

The synthetic nucleic acid molecule was then cloned into expression vectors and transformed into plants or bacteria as described in the Examples below.

Similar codon optimization strategies were used to design *dgt-1*, *dgt-3* v2 (G173A), *dgt-3* v3 (G173A; P178S), *dgt-3* v4 (T174I; P178S), *dgt-7* v4 (T168I; P172S), *dgt-32* v3, *dgt-33* v3, and *dgt-33* v3. The codon optimized version of these genes are listed as SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:144, respectively.

Bacterial Optimization. A new DNA sequence that encodes the DGT-28 protein of SEQ ID NO:1 that is optimized for expression in *Escherichia coli* cells was designed. Design of the *E. coli*-optimized DNA sequence was initiated by reverse translation of the protein sequence of SEQ ID NO:1, using a proprietary codon optimization protocol from GeneArt (Regensburg, Germany). The initial sequence was modified by compensating codon changes (while retaining overall weighted average representation) to remove or add restriction enzyme recognition sites, and remove highly stable intrastrand secondary structures and other sequences that might be detrimental to cloning manipulations or expression of the engineered gene. An example of such detrimental sequence to avoid within a coding region is a 16S ribosomal RNA binding sequence ("Shine-Dalgarno sequence") such as AGGAGG, which could encode, for example, two consecutive arginine amino acids, but which might also serve as an intragenic (and therefore undesirable) translation initiation signal. The *E. coli*-biased *dgt-28* DNA sequence (*dgt-28* v1) that encodes the protein of SEQ ID NO:1 is given as SEQ ID NO:4.

To facilitate cloning and to ensure efficient translation initiation, a 5' terminal *NdeI* restriction enzyme recognition sequence was placed upstream of the ATG translation start codon. Also to facilitate cloning, and to ensure proper translation termination, bases encoding two TAA translation stop codons and an *XhoI* restriction enzyme recognition site were included at the 3' end of the coding region. Synthesis of a DNA fragment comprising SEQ ID NO: 4 was performed by the commercial supplier, GeneArt™.

Similar *E. coli* codon optimization strategies were used to design *dgt-1* v5, *dgt-1* v6 (G112A), *dgt-1* v7 (G112A; P117S), *dgt-1* v8 (T113I; P117S), *dgt-3* v6 (G105A), *dgt-3* v7 (G105A; P112S), *dgt-3* v8 (T106I; P112S), *dgt-7* v5, *dgt-7* v6 (G113A), *dgt-7* v7 (G113A; P117S), *dgt-7* v8 (T114I; P117S), *dgt-32* v5, and *dgt-33*

v5. The *dgt-1*, *dgt-3*, and *dgt-7* sequence versions were modified by the removal of the chloroplast targeting sequence. The *E. coli*-codon optimized version of these genes are listed as SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:24, respectively.

Example 3: Vectors for Bacterial Expression of Glyphosate Tolerance Genes

Construction of pET Expression Vector, *dgt-28* for *E. coli* Expression. For *in vitro* testing, the *dgt-28 v1* *E. coli* optimized gene sequence (SEQ ID NO:4) was synthesized and cloned by GeneArt™ for synthesis and cloning. The synthesized *dgt-28 v1* gene sequence was cloned into the pET28 expression vector *via* added *Nde I* and *Xho I* restriction sites. The resulting construction introduced an N-terminal 6X His tag and was labeled as pDAB100445. FIG. 14.

Site Directed Mutagenesis of *dgt-28 v1*. Site directed mutagenesis was carried out on *dgt-28 v1* to assess the role of the alanine at position 84 in providing tolerance to glyphosate. This natural alanine was mutated to glycine to determine if the change would lower the enzyme's tolerance to glyphosate or affinity for PEP. This amino acid residue was selected, as it corresponds with the G96A mutation which was introduced into the *E. coli* EPSP synthase as previously described.

The Quick Change II™ kit from Stratagene™ (Santa Clara, CA) was used to perform the mutagenesis. PCR reactions were set up according to the QuickChange™ protocol using pDAB100445 (*dgt-28 v1*) as template DNA. The construct containing the mutated *dgt-28 v2* gene sequence was designated pDAB102946 (FIG. 15) and confirmed *via* DNA sequencing. The following primers were designed to make the amino acid switch:

DGT28	MutF	(SEQ	ID	NO:25;	5'
gATgTTTATTgCCgTgATggTggAACCAccgCACgTTTTTC)					
DGT28	MutR	(SEQ	ID	NO:26;	5'
gAAAACgTgCggTggTTCCACCATCACggCAATAAACATC)					

A second round of mutagenesis was carried out on *dgt-28 v2* in an attempt to further lower its tolerance to glyphosate. A second mutation, T172A, was introduced to the already mutagenized *dgt-28 v2*. The reciprocal alanine to threonine mutation of EPSP synthase at this position was previously described in Haghani *et al.*, (2008),

wherein it resulted in insensitivity to glyphosate. The end result was the production of a double A84G, T172A mutant which was designated as *dgt-28 v3*. PCR reactions were set up according to the QuickChange™ protocol using pDAB102946 (*dgt-28 v2*) as template DNA. The construct containing the mutated *dgt-28 v3* gene sequence was designated pDAB100469 (FIG. 16). The following primers were used to produce the T172A mutation:

DGT28 Mut2F (SEQ ID NO:27; 5' gggTCCgCTggCACgTCAgggTCTgCgTATTCg)
 DGT28 Mut2R (SEQ ID NO:28; 5'
 CgAATACgCAgACCCTgACgTgCCAgCggACCCAgCAgC)

10

Additional Constructions, pET Expression Vector for *E. coli* Expression. For *in vitro* testing, the *dgt-1 v5*, *dgt-1 v6*, *dgt-1 v7*, *dgt-1 v8*, *dgt-3 v6*, *dgt-3 v7*, *dgt-3 v8*, *dgt-7 v5*, *dgt-7 v6*, *dgt-7 v7*, *dgt-7 v8*, *dgt-32 v5*, and *dgt-33 v5* gene sequences were synthesized and cloned (GeneArt™). The synthesized genes were cloned into the pET28 expression vector. The resulting constructions were labeled as pDAB102028 (FIG. 17) containing *dgt-1 v5*, pDAB102029 (FIG. 18) containing *dgt-1 v6*, pDAB102032 (FIG. 19) containing *dgt-1 v7*, pDAB102034 (FIG. 20) containing *dgt-1 v8*, pDAB100429 (FIG. 21) containing *dgt-3 v6*, pDAB100442 (FIG. 22) containing *dgt-3 v7*, pDAB100430 (FIG. 23) containing *dgt-3 v8*, pDAB102036 (FIG. 24) containing *dgt-7 v5*, pDAB102038 (FIG. 25) containing *dgt-7 v6*, pDAB102040 (FIG. 26) containing *dgt-7 v7*, and pDAB102042 (FIG. 27) containing *dgt-7 v8*

Cloning of DGT-32, and DGT-33. For *in vitro* testing, the following plant optimized genes; *dgt-32 v3*, and *dgt-33 v3* were amplified out of the binary vectors pDAB107532 (FIG. 11) and pDAB107534 (FIG. 12), respectively. The following primer sets were used:

pMALDGT32F (SEQ ID NO:29; CATATGACCGTTATTGAAATTCCGGG) and
 pMALDGT32R (SEQ ID NO:30; GATATCCTATTATTAAACGACCTTCCAG) for *dgt-32*,
 and pMALDGT33F (SEQ ID NO:31; CATATGGGTGCAGTTACCGTTATTGA),
 pMALDGT33R (SEQ ID NO:32; GATATCCTATTATTATGCCTGCGGAC) for *dgt-33*.

30

Amplified sequences were then subcloned into pMAL-c5X so that each gene was an in-frame fusion with the *malE* coding sequence. The final expression constructs were pDAB107713 (FIG. 29) containing *dgt-32 v3*, and pDAB107714 (FIG. 30) containing *dgt-33 v3*.

Example 4: In-Vitro Biochemical Enzymatic Kinetic Assay

Overexpression and Purification of Recombinant DGT Enzymes. Recombinant DGT proteins were overexpressed in Rosetta2™ (DE3) cells (Novagen™, Madison, WI) from the constructs described above. A single colony was used to inoculate 50 mL starter cultures of LB containing chloramphenicol (25 µg/mL) and kanamycin (50 µg/mL) which were cultivated overnight at 37°C. The overnight cultures were used to inoculate 1L of LB containing chloramphenicol (25 µg/mL) and kanamycin (50 µg/mL). The cultures were grown at 37°C to an O.D.₆₀₀ = 0.6 then placed in an ice water bath for 10 minutes. Expression of the target proteins was achieved by addition of IPTG to a final concentration of 500 µM.

Induction was allowed to proceed overnight at 20°C followed by harvesting *via* centrifugation at 8,000 rpm for 20 minutes. The cell pellets were stored at -80°C until required for purification. All purification steps were carried out at 4°C. Cell pellets from 1 L cultures were resuspended in 20-30 mL Buffer A (50 mM HEPES pH 7.5, 150 mM KCl, 2 mM DTT, 1 mM EDTA, 20 mM imidazole, and 5% glycerol). COMPLETE™ protease inhibitor cocktail (1 tablet/50 mL, Roche, Indianapolis, IN) and lysozyme (1 mg/mL, Sigma-Aldrich, St. Louis, MO) were then added and the suspension was stirred for 20 minutes. Cell lysis was performed using a Branson™ Sonifier™ 250 (3 x 60 second bursts) followed by removal of the cell debris by centrifugation at 16,000 rpm for 45 minutes.

DGT enzymes were purified to homogeneity in one step *via* immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap FF crude column. The column was equilibrated in Buffer A and the sample was loaded in the same buffer. The column was then washed with 10 column volumes of Buffer A followed by elution in a 0-100 % Buffer B (50 mM HEPES pH 7.5, 200 mM KCl, 2 mM DTT, 1 mM EDTA, 500 mM imidazole, and 5% glycerol) linear gradient over 25 column volumes. Fractions containing target protein, as judged by SDS-PAGE analysis, were concentrated to 2.5 mL using a Millipore ultracentrifugation device equipped with a 10 kDa molecular weight cut-off (MWCO). The purified DGT enzymes were buffer exchanged using PD-10 columns (GE Healthcare) into 50 mM HEPES pH 7.5, 150 mM KCl, 2 mM DTT, and 5% glycerol and subsequently concentrated ~1 mL. Samples were typically diluted 1:50 and the UV-visible spectrum was recorded from

240 – 700 nm on a Cary50™ Bio UV-visible spectrophotometer. A theoretical extinction coefficient was then used to calculate the protein concentration based on the absorbance at 280 nm (ExPASy, Geneva, Switzerland).

Expression and Purification of Recombinant DGT-32 and DGT-33 Fusions.

5 The DGT-32 and DGT-33 enzymes were constructed to contain a maltose fusion tag located at the amino terminus of the enzyme. *Escherichia coli* cells transformed with pDAB107712 (Fig. 28), pDAB107713, and pDAB107714 were isolated and confirmed. A single colony of each bacterial strain was used to inoculate 50 mL of LB media containing 100 µg/µL carbenicillin and 25 µg/µL chloramphenicol. The starter
10 culture was grown overnight at 37°C and subsequently used to inoculate 600 mL of LB media supplemented with 0.2% glucose, 100 µg/µL carbenicillin, and 25 µg/µL chloramphenicol. The cultures were grown at 37°C to an OD₆₀₀ = 0.4 at which time IPTG was added to a final concentration of 50 µM IPTG. The cultures were induced for 15 hours at 18°C. The following day the cultures were harvested by centrifugation
15 at 8,000 rpm for 20 minutes to pellet the cells. The cell paste was stored at -80°C until required for purification.

Frozen pellets were resuspended in 20-30 mL buffer A (50 mM HEPES pH 7.5, 100 mM KCl, 1 mM EDTA, 5% glycerol, and 1 mM DTT) and 1 tablet of protease inhibitor (Roche Complete). Once the pellet was completely resolubilized 1 mg/mL of
20 lysozyme was added and the sample was mixed at 4°C for 15-20 minutes. Following the incubation with the lysozyme the sample was transferred to a 50 mL centrifuge tube and placed on ice. The sample was then sonicated for 1 minute intervals followed by 4 minutes of cooling. This step was repeated two more times for a total of three sonication cycles. Cell debris was removed by centrifugation at 16,500 rpm for 45
25 minutes and the supernatant was loaded into a 50 mL injection loop. The crude lysate was applied to an amylose column, washed for 7 column volumes with buffer A, and eluted in 100% buffer B (Buffer A and 10 mM maltose). Target protein was pooled and concentrated to 2.5 mL using a 30 kDa MWCO centricon. The purified protein was buffer exchanged into 50 mM HEPES pH 7.5, 100 mM KCl, and 5% glycerol
30 using a PD-10 gel filtration column. Protein concentrations were determined *via* Bradford assay using BSA as a standard. The pure protein was frozen in liquid nitrogen and stored at -80°C.

In Vitro Kinetic Characterization of Plant and Bacterial DGT Enzymes. The enzyme activities of wild-type (WT) and mutant DGTs were measured by inorganic phosphate (P_i) production in a modified procedure described by Lanzetta *et al.* (1979) Anal. Bioch. 100:95-7. Assays were performed in 96-well plate format in a total of 5 50 μ L on a Spectra-Max 190 plate reader (Molecular Devices, Sunnyvale, CA). Typical assays contained 50 mM HEPES pH 7.5, 150 mM KCl, 2 mM DTT, and 1 mM S3P. PEP and glyphosate concentrations were varied as indicated. Glyphosate was obtained from Sigma as the free acid and was resuspended in ddH₂O. Glyphosate was solubilized by addition of KOH until the mixture was at a neutral pH. Assays 10 were initiated by addition of the DGT enzyme at concentrations that varied between 0.01-1 μ M. Reactions were terminated by the addition of 235 μ L of a 3:1 mixture of malachite green: ammonium molybdate solution. After complete color development (~1 minute), the absorbance change at 660 nm was recorded and the amount of P_i formed was calculated from a standard curve. Control reactions lacking enzyme were 15 used to correct for background absorbance. High concentrations of PEP (> 2 mM) and glyphosate (> 30 mM) contribute a significant amount of background absorbance using this detection method. The data were fitted to the Michaelis-Menten equation which allowed for the determination of K_m and V_{max} (Equation 3) while IC_{50} was determined from Equation 4, where y is the relative activity and s is the Hill coefficient. Data were 20 analyzed using GraFit™ version 5 software (Erithacus Software Limited, Horley, U.K.).

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad (3)$$

$$y = \frac{100\%}{1 + \left(\frac{x}{IC_{50}} \right)^s} \quad (4)$$

25 The IC_{50} value for a competitive inhibitor will change dependent on the concentration of substrate, therefore the IC_{50} values in **Table 2** were obtained at 1 mM PEP and at 60 μ M PEP (an estimate of the intracellular PEP concentrations in plants). Only IC_{50} values measured at the same concentration of PEP should be compared (K_m determinations for DGT-32 and DGT-33 were determined at 100 μ M PEP). 30 Additionally, IC_{50} values of highly tolerant enzymes could not accurately be

determined by the method of Lanzetta and were therefore estimated based on relative activity.

Kinetics of Plant DGTs. Two enzymes with un-mutated native sequences, DGT-1 v5 and DGT-7 v5, were tested first to establish baseline parameters for glyphosate sensitivity. Both proteins displayed low K_m values for PEP ($\sim 70 \mu\text{M}$) and were sensitive to glyphosate with IC_{50} values of $\sim 20 \mu\text{M}$ (**Table 2**) at 1 mM PEP. As observed for DGT-1 v6, DGT-3 v6, and DGT-7 v6, a single point mutation from G to A significantly improved tolerance to glyphosate (IC_{50} values of 8-21 mM) but also increased the K_m for PEP by ~ 8 -fold. The double mutation (GAPS), for all plant derived DGTs (DGT-1 v7, DGT-3 v7, and DGT-7 v7), also enhanced glyphosate tolerance, but once again resulted in a considerable elevation in the PEP K_m . **Table 2.** The TIPS mutants (DGT-1 v8, DGT-3 v8, and DGT-7 v8) were tolerant to modest concentrations of glyphosate (3-6 mM) but in contrast to the GA and GAPS mutants, the K_m levels remained close to the wild-type proteins between 60-200 μM . FIG. 31 demonstrates the shifts in glyphosate tolerance for DGT-1 (A) and DGT-7 (B) upon introduction of the specified mutations. The PEP concentration was held at 1 mM for the experiments resulting in the data shown in FIG. 31, which likely led to the elevated IC_{50} ($>80 \text{ mM}$) for DGT-7 v8. Further procedures were carried out to determine if lower levels of PEP altered the relative tolerance to glyphosate. Physiologically relevant levels of PEP range from 5 – 60 μM . With 60 μM PEP, the IC_{50} value decreased significantly (3.6 mM), suggesting the initial determination was influenced by excess PEP, as expected from Michaelis-Menten kinetics and noted in **Table 2.**

FIG. 31 shows IC_{50} values obtained after introduction of various mutations within DGT-1 (A) and DGT-7 (B) using 1 mM PEP. For both A and B IC_{50} curves closed triangles represent wild-type, closed circles represent GA mutants, open squares represent GAPS mutants, and closed squares represent TIPS mutants.

Table 2. Steady-state kinetic parameters for DGT enzymes. IC_{50} values greater than 50 are estimates due to limitations of the method used. * IC_{50} for glyphosate was determined at 100 μM PEP.

IC_{50} (mM Glyphosate)						
	Protein	Sequence Version	IC_{50} at 1	IC_{50} at 60 μM	K_M^{PEP} (μM)	K_{cat} / K_m

				mM PEP	PEP		(M ⁻¹ s ⁻¹)
Plant Enzymes	Soybean	DGT-1 v5	Native	0.0	0.0	73.0	7.41E+04
		DGT-1 v6	GA	21.1	17.3	608.2	1.34E+04
		DGT-1 v7	GA PS	>80.0	>80.0	1291.2	6.67E+03
		DGT-1 v8	TI PS	13.3	5.9	151.4	1.23E+04
	Canola	DGT-3 v6	GA	15.8	8.7	1073.4	1.39E+04
		DGT-3 v7	GA PS	>50.0	42.0	2728.3	2.28E+03
		DGT-3 v8	TI PS	13.3	4.8	196.8	3.29E+04
	Wheat	DGT-7 v5	Native	0.0	0.0	75.6	2.15E+05
		DGT-7 v6	GA	8.1	15.1	538.2	1.61E+04
		DGT-7 v7	GA PS	19.7	15.4	1103.2	1.46E+04
		DGT-7 v8	TIPS	>80.0	3.6	60.5	1.36E+04
Bacterial Enzymes		DGT-28v1	Native	>80.0	>80.0	91.6	1.32E+05
		DGT-28 v2	AG	>50.0	2.2	161.5	6.86E+04
		DGT-28 v3	AGTA	>50.0	5.2	27.3	6.01E+02
		DGT-32	Native	--	>50.0*	139.8	1.30E+03
		DGT-33	Native	--	>50.0*	114.2	2.40E+03

Kinetics of Bacterial DGTs. Of the bacterial enzymes, DGT-28 v1 possesses the most favorable overall kinetic parameters (Elevated IC₅₀ and k_{cat}/K_m values). The enzyme was tolerant to glyphosate at concentrations >80 mM and displayed a catalytic efficiency of $1.32 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. The A→G mutation in DGT-28 v2 lowered the IC₅₀ to 2.17 mM (at 60 μM PEP) and caused a slight elevation in the K_m for PEP (161 μM). This mutant enzyme retains the high catalytic efficiency seen in DGT-28 v1. Even with a lowered IC₅₀, this mutated enzyme is suitable for providing tolerance to glyphosate *in planta* in certain applications. The data suggest that in this new class of EPSP synthase, the alanine is not the sole determinant for tolerance to glyphosate. To explore other possible determinants an additional variant, DGT-28 v3 (A84G T172A

double mutant), was constructed. This enzyme displayed lowered tolerance to glyphosate with an IC_{50} value of 5.15 mM (at 60 μ M PEP). The decrease in IC_{50} for DGT-28 v3 was accompanied by a 200-fold decrease in catalytic efficiency, suggesting the second mutation led to unintended effects (**Table 2**). The higher identity DGT-28 v1 homologues (~75 % amino acid identity), DGT-32 and DGT-33, had low K_m 's for PEP (~114 - 139 μ M), however catalytic efficiencies were 100-fold lower than DGT-28 v1. This drop in catalytic efficiency is likely derived from the maltose binding protein (MBP) fusion. The enzymes are also insensitive to glyphosate displaying IC_{50} values of greater than 50 mM. As a result of these *in vitro* assays, which indicated that the various DGT enzymes provided tolerance to glyphosate, the DGT enzymes were tested *in planta*.

Example 5: Cloning of Plant Transformation Vectors

Plant Binary Vector Construction. Standard cloning methods were used in the construction of entry vectors containing a chloroplast transit peptide polynucleotide sequence joined to *dgt-28* as an in-frame fusion. The entry vectors containing a transit peptide (TraP) fused to *dgt-28* were assembled using the IN-FUSION™ Advantage Technology (Clontech, Mountain View, CA). As a result of the fusion, the first amino acid, methionine, was removed from *dgt-28*. Transit peptides TraP4 v2 (SEQ ID NO:33), TraP5 v2 (SEQ ID NO:34), TraP8 v2 (SEQ ID NO:35), TraP9 v2 (SEQ ID NO:36), TraP12 v2 (SEQ ID NO:37), and TraP13 v2 (SEQ ID NO:38) were each synthesized by DNA2.0 (Menlo Park, CA) and fused to the 5' end fragment of *dgt-28*, up to and including a unique *AccI* restriction endonuclease recognition site.

Binary plasmids which contained the various TraP and *dgt-28* expression cassettes were driven by the *Arabidopsis thaliana* Ubiquitin 10 promoter (AtUbi10 v2; Callis, *et al.*, (1990) *J. Biol. Chem.*, 265: 12486-12493) and flanked by the *Agrobacterium tumefaciens* open reading frame twenty-three 3' untranslated region (AtuORF23 3' UTR v1; U.S. Pat. No. 5,428,147).

The assembled TraP and *dgt-28* expression cassettes were engineered using GATEWAY® Technology (Invitrogen, Carlsbad, CA) and transformed into plants *via* *Agrobacterium*-mediated plant transformation. Restriction endonucleases were obtained from New England BioLabs (NEB; Ipswich, MA) and T4 DNA Ligase (Invitrogen) was used for DNA ligation. Gateway reactions were performed using

GATEWAY[®] LR CLONASE[®] enzyme mix (Invitrogen) for assembling one entry vector into a single destination vector which contained the selectable marker cassette Cassava Vein Mosaic Virus promoter (CsVMV v2; Verdaguer *et al.*, (1996) *Plant Mol. Biol.*, 31: 1129-1139) – DSM-2 (U.S. Pat. App. No. 2007/086813) - *Agrobacterium tumefaciens* open reading frame one 3' untranslated region (AtuORF1 3' UTR v6; Huang *et al.*, (1990) *J. Bacteriol.* 172:1814-1822). Plasmid preparations were performed using NUCLEOSPIN[®] Plasmid Kit (Macherey-Nagel Inc., Bethlehem, PA) or the Plasmid Midi Kit (Qiagen) following the instructions of the suppliers. DNA fragments were isolated using QIAquick[™] Gel Extraction Kit (Qiagen) after agarose
 5 Tris-acetate gel electrophoresis.

Colonies of all assembled plasmids were initially screened by restriction digestion of miniprep DNA. Plasmid DNA of selected clones was sequenced by a commercial sequencing vendor (Eurofins[™] MWG Operon, Huntsville, AL). Sequence data were assembled and analyzed using the SEQUENCHER[™] software (Gene Codes Corp., Ann Arbor, MI).
 15

The following binary constructs express the various TraP:*dgt-28* fusion gene sequences: pDAB107527 (FIG. 3) contains TraP4 v2:*dgt-28* v5 (SEQ ID NO:79); pDAB105530 (FIG. 4) contains TraP5 v2: *dgt-28* v5 (SEQ ID NO:80); pDAB105531 (FIG. 5) contains TraP8 v2: *dgt-28* v5 (SEQ ID NO:81); PDAB105532 (FIG. 6)
 20 contains TraP9 v2: *dgt-28* v5 (SEQ ID NO:82); pDAB105533 (FIG. 7) contains TraP12 v2: *dgt-28* v5 (SEQ ID NO:83); and pDAB105534 (FIG. 8) contains TraP13 v2:*dgt-28* v5 (SEQ ID NO:84). The *dgt-28* v5 sequence of pDAB105534 was modified wherein the first codon (GCA) was changed to (GCT).

Additional Plant Binary Vector Construction. Cloning strategies similar to
 25 those described above were used to construct binary plasmids which contain *dgt-31*, *dgt-32*, *dgt-33*, *dgt-1*, *dgt-3*, and *dgt-7*.

The microbially derived genes; *dgt-31*, *dgt-32*, and *dgt-33*, were fused with different chloroplast transit peptides than previously described. The following chloroplast transit peptides were used; TraP14 v2 (SEQ ID NO:39), TraP23 v2 (SEQ
 30 ID NO:40), TraP24 v2 (SEQ ID NO:41). pDAB107532 (FIG. 11) contains *dgt-32* v3 fused to TraP14 v2 (SEQ ID NO:42), pDAB107534 (FIG. 12) contains *dgt-33* v3 fused to TraP24 v2 (SEQ ID NO:43), and pDAB1017533 (FIG. 54) contains *dgt-33* v1 fused to TraP23 v2 (SEQ ID NO:143). The *dgt* expression cassettes were driven by the

Arabidopsis thaliana Ubiquitin 10 promoter (AtUbi10 promoter v2) and flanked by the *Agrobacterium tumefaciens* open reading frame twenty-three 3' untranslated region (AtuORF23 3' UTR v1). A *DSM-2* selectable marker cassette containing Cassava Vein Mosaic Virus promoter (CsVMV v2) – *DSM-2* – *Agrobacterium tumefaciens* open reading frame one 3' untranslated region (AtuORF1 3' UTR v6) was also present in the binary vector.

Additional binaries are constructed wherein *dgt-31 v3*, *dgt-32 v3*, and *dgt-33 v3* are fused to the previously described chloroplast transit peptide sequences. For example, the TraP8 v2 sequence is fused to *dgt-31 v3*, *dgt-32 v3*, and *dgt-33 v3*, and cloned into binary vectors as described above.

Binary vectors containing the Class I genes (*dgt-1*, *dgt-3*, and *dgt-7*) were constructed. The following binary vectors were constructed and transformed into plants: pDAB4104 (FIG. 9), which contains the *dgt-1 v4* sequence as described in U.S. Patent Application Publication No. 2011/0124503, which is flanked by the *Nicotiana tabacum* Osmotin sequences as described in U.S. Patent Application Publication No. 2009/0064376; pDAB102715 (FIG. 10); pDAB102716 (FIG. 45); pDAB102717 (FIG. 46); and pDAB102785 (FIG. 13). The various TraP chloroplast transit peptides that were fused to *dgt-28*, *dgt-31*, *dgt-32*, and *dgt-33* were not added to the Class I genes, as these plant derived sequences possess native plant chloroplast transit peptides. These vectors are described in further detail in **Table 3**.

Table 3. Description of the binary vectors which contain a Class I EPSP synthase gene (*i.e.*, *dgt-1*, *dgt-3*, or *dgt-7*).

Name	Description	EPSPS mutation
pDAB4104	RB7 MAR v2 :: CsVMV promoter v2 / NtOsm 5' UTR v2 / <i>dgt-1 v4</i> / NtOsm 3' UTR v2 / AtuORF24 3' UTR v2 :: AtUbi10 promoter v4 / <i>pat v3</i> / AtuORF1 3'UTR v3 binary vector	TI PS
pDAB102715	AtUbi10 promoter v2 / <i>dgt-3 v2</i> / AtuORF23 3'UTR v1 :: CsVMV promoter v2 / <i>pat v9</i> / AtuORF1 3'UTR v6 binary vector	GA
pDAB102716	AtUbi10 promoter v2 / <i>dgt-3 v3</i> / AtuORF23 3'UTR v1 :: CsVMV promoter v2 / <i>pat v9</i> / AtuORF1 3'UTR v6 binary vector	GA PS
pDAB102717	AtUbi10 promoter v2 / <i>dgt-3 v4</i> / AtuORF23 3'UTR v1 :: CsVMV promoter v2 / <i>pat v9</i> / AtuORF1 3'UTR v6 binary vector	TI PS

Name	Description	EPSPS mutation
pDAB102785	AtUbi10 promoter v2 / <i>dgt-7</i> v4 / AtuORF23 3'UTR :: CsVMV promoter v2 / <i>DSM-2</i> v2 / AtuORF1 3'UTR v6 binary vector	TI PS

Example 6: Transformation into *Arabidopsis* and Selection

Arabidopsis thaliana Transformation. *Arabidopsis* was transformed using the floral dip method from Clough and Bent (1998). A selected *Agrobacterium* colony containing one of the binary plasmids described above was used to inoculate one or more 100 mL pre-cultures of YEP broth containing spectinomycin (100 mg/L) and kanamycin (50 mg/L). The culture was incubated overnight at 28°C with constant agitation at 225 rpm. The cells were pelleted at approximately 5000 xg for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 400 mL dunking media containing: 5% (w/v) sucrose, 10 µg/L 6-benzylaminopurine, and 0.04% Silwet™ L-77. Plants approximately 1 month old were dipped into the media for 5-10 minutes with gentle agitation. The plants were laid down on their sides and covered with transparent or opaque plastic bags for 2-3 hours, and then 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.

Selection of Transformed Plants. Freshly harvested T₁ seed [containing the *dgt* and *DSM-2* expression cassettes] was allowed to dry for 7 days at room temperature. T₁ seed was sown in 26.5 x 51-cm germination trays, each receiving a 200 mg aliquot of stratified T₁ seed (~10,000 seeds) 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 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 for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *DSM-2* gene).

Seven days after planting (DAP) and again 11 DAP, T₁ plants (cotyledon and 2-4-leaf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 mL/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate 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 reared in the greenhouse (22±5°C, 50±30% RH, 14 h light:10 dark, minimum 500 µE/m²s⁻¹ natural + supplemental light). Molecular confirmation analysis was completed on the surviving T₁ plants to confirm that the glyphosate tolerance gene had stably integrated into the genome of the plants.

Molecular Confirmation. The presence of the *dgt-28* and *DSM-2* transgenes within the genome of *Arabidopsis* plants that were transformed with pDAB107527, pDAB105530, pDAB105531, pDAB105532, pDAB105533, or pDAB105534 was confirmed. The presence of these polynucleotide sequences was confirmed *via* hydrolysis probe assays, gene expression cassette PCR (also described as plant transcription unit PCR -- PTU PCR), Southern blot analysis, and Quantitative Reverse Transcription PCR analyses.

The T₁ *Arabidopsis* plants were initially screened *via* a hydrolysis probe assay, analogous to TAQMAN[™], to confirm the presence of the *DSM-2* and *dgt-28* transgenes. Events were screened *via* gene expression cassette PCR to determine whether the *dgt* expression cassette completely integrated into the plant genomes without rearrangement. The data generated from these studies were used to determine the transgene copy number and identify select *Arabidopsis* events for self fertilization and advancement to the T₂ generation. The advanced T₂ *Arabidopsis* plants were also screened *via* hydrolysis probe assays to confirm the presence and to estimate the copy number of the *DSM-2* and *dgt* genes within the plant chromosome. Finally, a Southern blot assay was used to confirm the estimated copy number on a subset of the T₁ *Arabidopsis* plants.

Similar assays were used to confirm the presence of the *dgt-1* transgene from plants transformed with pDAB4101, the presence of the *dgt-32* transgene from plants transformed with pDAB107532, the presence of the *dgt-33* transgene from plants transformed with pDAB107534, the presence of the *dgt-3* transgene from plants

transformed with pDAB102715, the presence of the *dgt-3* transgene from plants transformed with pDAB102716, the presence of the *dgt-3* transgene from plants transformed with pDAB102717, and the presence of the *dgt-7* transgene from plants transformed with pDAB102785.

- 5 Hydrolysis Probe Assay. Copy number was determined in the T₁ and T₂ *Arabidopsis* plants using the hydrolysis probe assay described below. Plants with varying numbers of transgenes were identified and advanced for subsequent glyphosate tolerance studies.

- Tissue samples were collected in 96-well plates and lyophilized for 2 days.
- 10 Tissue maceration was performed with a KLECO™ tissue pulverizer and tungsten beads (Environ Metal INC., Sweet Home, Oregon). Following tissue maceration, the genomic DNA was isolated in high-throughput format using the Biosprint™ 96 Plant kit (Qiagen™, Germantown, MD) according to the manufacturer's suggested protocol. Genomic DNA was quantified by QUANT-IT™ PICO GREEN DNA ASSAY KIT
- 15 (Molecular Probes, Invitrogen, Carlsbad, CA). Quantified genomic DNA was adjusted to around 2 ng/μL for the hydrolysis probe assay using a BIOROBOT3000™ automated liquid handler (Qiagen, Germantown, MD). Transgene copy number determination by hydrolysis probe assay was performed by real-time PCR using the LIGHTCYCLER®480 system (Roche Applied Science, Indianapolis, IN). Assays
- 20 were designed for *DSM-2*, *dgt-28* and the internal reference gene, *TAFIII5* (Genbank ID: NC 003075; Duarte et al., (201) *BMC Evol. Biol.*, 10:61).\

- For amplification, LIGHTCYCLER®480 Probes Master mix (Roche Applied Science, Indianapolis, IN) was prepared at a 1X final concentration in a 10 μL volume multiplex reaction containing 0.1 μM of each primer for *DSM-2* and *dgt-28*, 0.4 μM of
- 25 each primer for *TAFIII5* and 0.2 μM of each probe. **Table 4.** A two-step amplification reaction was performed with an extension at 60°C for 40 seconds with fluorescence acquisition. All samples were run and the averaged Cycle threshold (Ct) values were used for analysis of each sample. Analysis of real time PCR data was performed using LightCycler™ software release 1.5 using the relative quant module
- 30 and is based on the ΔΔCt method. For this, a sample of genomic DNA from a single copy calibrator and known 2 copy check were included in each run. The copy number results of the hydrolysis probe screen were determined for the T₁ and T₂ transgenic *Arabidopsis* plants.

Table 4. Primer and probe Information for hydrolysis probe assay of *DSM-2*, *dgt-28* and internal reference gene (*TAFII15*).

Primer Name	Sequence
DSM2A (SEQ ID NO:44)	5' AGCCACATCCCAGTAACGA 3'
DSM2S (SEQ ID NO:45)	5' CCTCCCTCTTTGACGCC 3'
DSM2 Cy5 probe (SEQ ID NO:46)	5' CAGCCCAATGAGGCATCAGC 3'
DGT28F (SEQ ID NO:47)	5' CTTCAAGGAGATTTGGGATTTGT 3'
DGT28R (SEQ ID NO:48)	5' GAGGGTCGGCATCGTAT 3'
UPL154 probe	Cat# 04694406001 (Roche, Indianapolis, IN)
TAFFY-HEX probe (SEQ ID NO:49)	5' AGAGAAGTTTCGACGGATTTCTGGGC 3'
TAFII15-F (SEQ ID NO:50)	5' GAGGATTAGGGTTTCAACGGAG 3'
TAFII15-R (SEQ ID NO:51)	5' GAGAATTGAGCTGAGACGAGG 3'

dgt-28 Integration Confirmation via Southern Blot Analysis. Southern blot analysis was used to establish the integration pattern of the inserted T-strand DNA fragment and identify events which contained *dgt-28*. Data were generated to demonstrate the integration and integrity of the transgene inserts within the *Arabidopsis* genome. Southern blot data were used to identify simple integration of an intact copy of the T-strand DNA. Detailed Southern blot analysis was conducted using a PCR amplified probe specific to the *dgt-28* gene expression cassette. The hybridization of the probe with genomic DNA that had been digested with specific restriction enzymes identified genomic DNA fragments of specific molecular weights, the patterns of which were used to identify full length, simple insertion T₁ transgenic events for advancement to the next generation.

Tissue samples were collected in 2 mL conical tubes (Eppendorf™) and lyophilized for 2 days. Tissue maceration was performed with a KLECKO™ tissue pulverizer and tungsten beads. Following tissue maceration, the genomic DNA was isolated using a CTAB isolation procedure. The genomic DNA was further purified using the Qiagen™ Genomic Tips kit. Genomic DNA was quantified by Quant-IT™ Pico Green DNA assay kit (Molecular Probes, Invitrogen, Carlsbad, CA). Quantified genomic DNA was adjusted to 4 µg for a consistent concentration.

For each sample, 4 µg of genomic DNA was thoroughly digested with the restriction enzyme *SwaI* (New England Biolabs, Beverley, MA) and incubated at 25°C overnight, then *NsiI* was added to the reaction and incubated at 37°C for 6 hours. The

digested DNA was concentrated by precipitation with Quick Precipitation Solution™ (Edge Biosystems, Gaithersburg, MD) according to the manufacturer's suggested protocol. The genomic DNA was then resuspended in 25 µL of water at 65°C for 1 hour. Resuspended samples were loaded onto a 0.8% agarose gel prepared in 1X TAE and electrophoresed overnight at 1.1 V/cm in 1X TAE buffer. The gel was sequentially subjected to denaturation (0.2 M NaOH / 0.6 M NaCl) for 30 minutes, and neutralization (0.5 M Tris-HCl (pH 7.5) / 1.5 M NaCl) for 30 minutes.

Transfer of DNA fragments to nylon membranes was performed by passively wicking 20 X SSC solution overnight through the gel onto treated IMMOBILON™ NY+ transfer membrane (Millipore, Billerica, MA) by using a chromatography paper wick and paper towels. Following transfer, the membrane was briefly washed with 2X SSC, cross-linked with the STRATALINKER™ 1800 (Stratagene, LaJolla, CA), and vacuum baked at 80°C for 3 hours.

Blots were incubated with pre-hybridization solution (Perfect Hyb plus, Sigma, St. Louis, MO) for 1 hour at 65°C in glass roller bottles using a model 400 hybridization incubator (Robbins Scientific, Sunnyvale, CA). Probes were prepared from a PCR fragment containing the entire coding sequence. The PCR amplicon was purified using QIAEX™ II gel extraction kit and labeled with α³²P-dCTP *via* the Random RT Prime IT™ labeling kit (Stratagene, La Jolla, CA). Blots were hybridized overnight at 65°C with denatured probe added directly to hybridization buffer to approximately 2 million counts per blot per mL. Following hybridization, blots were sequentially washed at 65°C with 0.1X SSC / 0.1% SDS for 40 minutes. Finally, the blots were exposed to storage phosphor imaging screens and imaged using a Molecular Dynamics Storm 860™ imaging system.

The Southern blot analyses completed in this study were used to determine the copy number and confirm that selected events contained the *dgt-28* transgene within the genome of *Arabidopsis*.

dgt-28 Gene Expression Cassette Confirmation *via* PCR analysis. The presence of the *dgt-28* gene expression cassette contained in the T₁ plant events was detected by an end point PCR reaction. Primers (**Table 5**) specific to the AtUbi10 promoter v2 and AtuORF23 3'UTR v1 regions of the *dgt-28* gene expression cassette were used for detection.

Table 5. Oligonucleotide primers used for *dgt-28* gene expression cassette confirmation.

Primer Name	Sequence
Forward oligo (SEQ ID NO:52)	5' CTGCAGGTCAACGGATCAGGATAT 3'
Reverse oligo (SEQ ID NO:53)	5' TGGGCTGAATTGAAGACATGCTCC 3'

The PCR reactions required a standard three step PCR cycling protocol to amplify the gene expression cassette. All of the PCR reactions were completed using the following PCR conditions: 94°C for three minutes followed by 35 cycles of 94°C for thirty seconds, 60°C for thirty seconds, and 72°C for three minutes. The reactions were completed using the EX-TAQ™ PCR kit (TaKaRa Biotechnology Inc. Otsu, Shiga, Japan) per manufacturer's instructions. Following the final cycle, the reaction was incubated at 72°C for 10 minutes. TAE agarose gel electrophoresis was used to determine the PCR amplicon size. PCR amplicons of an expected size indicated the presence of a full length gene expression cassette was present in the genome of the transgenic *Arabidopsis* events.

dgt-28 Relative Transcription Confirmation via Quantitative Reverse Transcription PCR analysis. Tissue samples of *dgt-28* transgenic plants were collected in 96-well plates and frozen at 80°C. Tissue maceration was performed with a KLECO™ tissue pulverizer and tungsten beads (Environ Metal INC., Sweet Home, Oregon). Following tissue maceration, the Total RNA was isolated in high-throughput format using the Qiagen™ Rneasy 96 kit (Qiagen™, Germantown, MD) according to the manufacturer's suggested protocol which included the optional DnaseI treatment on the column. This step was subsequently followed by an additional DnaseI (Ambion™, Austin, TX) treatment of the eluted total RNA. cDNA synthesis was carried out using the total RNA as template with the High Capacity cDNA Reverse Transcription™ kit (Applied Biosystems, Austin, TX) following the manufacturer's suggested procedure with the addition of the oligonucleotide, TVN. Quantification of expression was completed by hydrolysis probe assay and was performed by real-time PCR using the LIGHTCYCLER®480 system (Roche Applied Science, Indianapolis, IN). Assays were designed for *dgt-28* and the internal reference gene "unknown protein" (Genbank Accession Number: AT4G24610) using the LIGHTCYCLER® Probe Design Software 2.0. For amplification, LIGHTCYCLER®480 Probes Master mix (Roche Applied

Science, Indianapolis, IN) was prepared at 1X final concentration in a 10 μ L volume singleplex reaction containing 0.4 μ M of each primer, and 0.2 μ M of each probe.

Table 6.

Table 6. PCR primers used for quantitative reverse transcription PCR analysis of *dgt-28*.

Primer Name	Sequence
AT26410LP (SEQ ID NO:54)	5' CGTCCACAAAGCTGAATGTG 3'
AT26410RP (SEQ ID NO:55)	5' CGAAGTCATGGAAGCCACTT 3'
UPL146	Cat# 04694325001 (Roche, Indianapolis, IN)
DGT28F (SEQ ID NO:56)	5' CTTCAAGGAGATTTGGGATTTGT 3'
DGT28R (SEQ ID NO:57)	5' GAGGGTCGGCATCGTAT 3'
UPL154 probe	Cat# 04694406001 (Roche, Indianapolis, IN)

A two-step amplification reaction was performed with an extension at 60°C for 40 seconds with fluorescence acquisition. All samples were run in triplicate and the averaged Cycle threshold (Ct) values were used for analysis of each sample. A minus reverse transcription reaction was run for each sample to ensure that no gDNA contamination was present. Analysis of real time PCR data was performed based on the $\Delta\Delta$ Ct method. This assay was used to determine the relative expression of *dgt-28* in transgenic *Arabidopsis* events which were determined to be hemizygous and homozygous. The relative transcription levels of the *dgt-28* mRNA ranged from 2.5 fold to 207.5 fold higher than the internal control. These data indicate that *dgt-28* transgenic plants contained a functional *dgt-28* gene expression cassette, and the plants were capable of transcribing the *dgt-28* transgene.

Western Blotting Analysis. DGT-28 was detected in leaf samples obtained from transgenic *Arabidopsis thaliana* plants. Plant extracts from *dgt-28* transgenic plants and DGT-28 protein standards were incubated with NUPAGE[®] LDS sample buffer (Invitrogen, Carlsbad, CA) containing DTT at 90°C for 10 minutes and electrophoretically separated in an acrylamide precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using the manufacturer's protocol. After blocking with the WESTERNBREEZE[®] Blocking Mix (Invitrogen) the DGT-28 protein was detected by anti-DGT-28 antiserum followed by goat anti-rabbit phosphatase. The detected protein was visualized by chemiluminescence substrate

BCIP/NBT Western Analysis Reagent (KPL, Gaithersburg, MD). Production of an intact DGT-28 protein *via* Western blot indicated that the *dgt-28* transgenic plants which were assayed expressed the DGT-28 protein.

5

Example 7: Glyphosate Tolerance

Transgenic T₁ *Arabidopsis* plants containing the *dgt-28* transgene were sprayed with differing rates of glyphosate. Elevated rates were applied in this study to determine the relative levels of resistance (105, 420, 1,680 or 3,360 g ae/ha). A typical 1X usage rate of glyphosate that will control non-transformed *Arabidopsis* is 420 g
10 ae/ha. Glyphosate formulations with the addition of ammonium sulfate were applied to the T₁ plants with a track sprayer calibrated at 187 L/ha. The T₁ *Arabidopsis* plants that were used in this study were variable copy number for the *dgt-28* transgene. The low copy *dgt-28* T₁ *Arabidopsis* plants were self-pollinated and used to produce T₂ plants. **Table 7** shows the comparison of *dgt-28* transgenic plants, drawn to a
15 glyphosate herbicide resistance gene, *dgt-1*, and wildtype controls. **Table 8** shows the comparison of *dgt-32*, and *dgt-33* drawn to a glyphosate herbicide resistance gene, *dgt-1*, and wildtype controls. **Table 9** shows the comparison of the novel bacterial EPSP synthase enzymes to the Class I EPSP synthase enzymes and the controls at a glyphosate rate of 1,680 g ae/ha.

20

Results of Glyphosate Selection of Transformed *dgt-28* *Arabidopsis* Plants.

The *Arabidopsis* T₁ transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Three flats or 30,000 seed were analyzed for each T₁ construct. The T₁ plants selected above were molecularly characterized and representative plants with variable copy number were subsequently
25 transplanted to individual pots and sprayed with various rates of commercial glyphosate as previously described. The response of these plants is presented in terms of % visual injury 2 weeks after treatment (WAT). Data are presented in a table which shows individual plants exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). An arithmetic mean and standard deviation is
30 presented for each construct used for *Arabidopsis* transformation. The range in individual response is also indicated in the last column for each rate and transformation. Wild-type, non-transformed *Arabidopsis* (c.v. Columbia) served as a glyphosate sensitive control.

The level of plant response varied. This variance can be attributed to the fact each plant represents an independent transformation event and thus the copy number of the gene of interest varies from plant to plant. It was noted that some plants which contained the transgene were not tolerant to glyphosate; a thorough analysis to determine whether these plants expressed the transgene was not completed. It is likely that the presence of high copy numbers of the transgene within the T₁ *Arabidopsis* plants resulted in transgene silencing or other epigenetic effects which resulted in sensitivity to glyphosate, despite the presence of the *dgt-28* transgene.

An overall population injury average by rate is presented in **Table 9** for rates of glyphosate at 1,680 g ae/ha to demonstrate the significant difference between the plants transformed with *dgt-3*, *dgt-7*, *dgt-28*, *dgt-32*, and *dgt-33* versus the *dgt-1* and wild-type controls.

The tolerance provided by the novel bacterial EPSP synthases varied depending upon the specific enzyme. DGT-28, DGT-32, and DGT-33 unexpectedly provided significant tolerance to glyphosate. The *dgt* genes imparted herbicide resistance to individual T₁ *Arabidopsis* plants across all transit peptides tested. As such, the use of additional chloroplast transit peptides (*i.e.*, TraP8 – *dgt-32* or TraP8 – *dgt-33*) would provide protection to glyphosate with similar injury levels as reported within a given treatment.

Table 7. *dgt-28* transformed T₁ *Arabidopsis* response to a range of glyphosate rates applied postemergence, compared to a *dgt-1* (T₄) homozygous resistant population, and a non-transformed control. Visual % injury 14 days after application.

pDAB107527: TraP4 v2 -- <i>dgt-28</i> v5	% Injury			% Injury		
Averages	<20%	20- 40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha glyphosate	4	0	0	3.8	7.5	0-15
420 g ae/ha glyphosate	2	1	1	28.8	28.1	0-65
1680 g ae/ha glyphosate	0	2	2	55.0	26.8	35-85
3360 g ae/ha glyphosate	0	2	2	43.8	18.0	30-70
pDAB105530: TraP5 v2 – <i>dgt-28</i> v5	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	6	0	0	0.0	0.0	0
105 g ae/ha glyphosate	2	2	2	39.3	37.4	8-100
420 g ae/ha glyphosate	1	4	1	33.0	26.6	8-85
1680 g ae/ha glyphosate	0	4	2	47.5	27.5	25-85

3360 g ae/ha glyphosate	0	0	6		76.7	13.7	50-85
pDAB105531: TraP8 v2 -- <i>dgt-28 v5</i>							
	% Injury				% Injury		
Averages	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0		0.0	0.0	0
105 g ae/ha glyphosate	3	1	0		10.8	10.4	0-25
420 g ae/ha glyphosate	3	0	1		22.8	18.6	8-50
1680 g ae/ha glyphosate	4	0	0		5.3	3.8	0-8
3360 g ae/ha glyphosate	0	4	0		29.3	6.8	22-35
pDAB105532: TraP9 v2 -- <i>dgt-28 v5</i>							
	% Injury				% Injury		
Averages	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0		0.0	0.0	0
105 g ae/ha glyphosate	3	0	1		17.5	28.7	0-60
420 g ae/ha glyphosate	1	1	2		39.5	25.1	18-70
1680 g ae/ha glyphosate	3	0	1		26.3	36.1	5-80
3360 g ae/ha glyphosate	3	0	1		25.8	32.9	8-75
pDAB105533: TraP12 v2 -- <i>dgt-28 v5</i>							
	% Injury				% Injury		
Averages	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	5	0	0		0.0	0.0	0
105 g ae/ha glyphosate	4	1	0		10.0	10.0	0-25
420 g ae/ha glyphosate	1	1	3		53.6	34.6	8-85
1680 g ae/ha glyphosate	4	1	0		11.0	8.2	0-20
3360 g ae/ha glyphosate	0	2	3		55.0	25.5	25-80
pDAB105534: TraP13 v2 -- <i>dgt-28 v5</i>							
	% Injury				% Injury		
Averages	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	5	0	0		0.0	0.0	0
105 g ae/ha glyphosate	4	0	1		14.0	20.6	0-50
420 g ae/ha glyphosate	3	1	1		17.6	19.5	0-50
1680 g ae/ha glyphosate	3	0	2		39.0	47.1	5-100
3360 g ae/ha glyphosate	2	2	1		31.2	22.3	18-70
pDAB4104: <i>dgt-1</i> (transformed control)							
	% Injury				% Injury		
Averages	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	5	0	0		0.0	0.0	0
105 g ae/ha glyphosate	0	0	4		80.0	0.0	80
420 g ae/ha glyphosate	0	0	4		80.0	0.0	80
1680 g ae/ha glyphosate	0	0	4		80.0	0.0	80
3360 g ae/ha glyphosate	0	0	4		81.3	2.5	80-85

WT (non-transformed control)	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	5	0	0	0.0	0.0	0
105 g ae/ha glyphosate	0	0	4	100.0	0.0	100
420 g ae/ha glyphosate	0	0	4	100.0	0.0	100
1680 g ae/ha glyphosate	0	0	4	100.0	0.0	100
3360 g ae/ha glyphosate	0	0	4	100.0	0.0	100

Table 8. *dgt-32*, and *dgt-33* transformed T₁ *Arabidopsis* response to a range of glyphosate rates applied postemergence, compared to a *dgt-1* (T₄) homozygous resistant population, and a non-transformed control. Visual % injury 14 days after application.

pDAB107532: TraP14 v2 – <i>dgt-32</i> v3						
Averages	% Injury			% Injury		
	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	2	0	2	30.0	29.4	0-60
1680 g ae/ha glyphosate	3	0	1	17.5	21.8	5-50
3360 g ae/ha glyphosate	0	3	1	35.0	30.0	20-80
pDAB107534: TraP24 v2 -- <i>dgt-33</i> v3						
Averages	% Injury			% Injury		
	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha glyphosate	2	2	0	21.3	14.9	5-40
420 g ae/ha glyphosate	1	1	2	46.3	30.9	5-70
1680 g ae/ha glyphosate	1	0	3	62.5	38.8	5-90
3360 g ae/ha glyphosate	1	0	3	62.0	36.0	8-80
pDAB4104: <i>dgt-1</i> (transformed control)						
Averages	% Injury			% Injury		
	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha glyphosate	0	2	3	42.5	15.0	20-50
420 g ae/ha glyphosate	0	1	2	38.8	11.1	25-50
1680 g ae/ha glyphosate	0	0	4	79.0	19.4	50-90
3360 g ae/ha glyphosate	0	0	4	50.0	0.0	50

WT (non-transformed control)	% Injury			% Injury		
	<20 %	20-40%	>40%	Ave	Std dev	Range (%)
Averages						
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha glyphosate	0	0	4	85.0	0.0	85
420 g ae/ha glyphosate	0	0	4	100.0	0.0	100
1680 g ae/ha glyphosate	0	0	4	100.0	0.0	100
3360 g ae/ha glyphosate	0	0	4	100.0	0.0	100

Table 9. *dgt-28*, *dgt-32*, *dgt-33*, *dgt-3*, and *dgt-7* transformed T₁ *Arabidopsis* response to glyphosate applied postemergence at 1,680 g ae/ha, compared to a *dgt-1* (T₄) homozygous resistant population, and a non-transformed control. Visual % injury 5 14 days after application.

			% Injury			% Injury		
			<20%	20-40%	>40%	Ave	Std dev	Range (%)
Bacterial Enzymes	pDAB107527	TraP4 v2 -- <i>dgt-28</i> v5	0	2	2	55.0	26.8	35-85
	pDAB105530	TraP5 v2 -- <i>dgt-28</i> v5	0	4	2	47.5	27.5	25-85
	pDAB105531	TraP8 v2 -- <i>dgt-28</i> v5	4	0	0	5.3	3.8	0-8
	pDAB105532	TraP9 v2 -- <i>dgt-28</i> v5	3	0	1	26.3	36.1	5-80
	pDAB105533	TraP12 v2 -- <i>dgt-28</i> v5	4	1	0	11.0	8.2	0-20
	pDAB105534	TraP13 v2 -- <i>dgt-28</i> v5	3	0	2	39.0	47.1	5-100
	pDAB107532	TraP14 v2 -- <i>dgt-32</i> v3	3	0	1	17.5	21.8	5-50
	pDAB107534	TraP24 v2 -- <i>dgt-33</i> v3	1	0	3	62.5	38.8	5-90
Class I Enzymes	pDAB102715	<i>dgt-3</i> v2	4	0	3	42	48	0-100
	pDAB102716	<i>dgt-3</i> v3	2	1	0	14	23	0-40
	pDAB102717	<i>dgt-3</i> v4	3	2	1	28	35	10-100
	pDAB102785	<i>dgt-7</i> v4	0	1	1	45	21	30-60
	pDAB4104	<i>dgt-1</i> (transformed control)	0	0	4	80.0	0.0	80
	--	WT (non-transformed)	0	0	4	100.0	0.0	100

		control)							
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dgt-28 as a Selectable Marker. The use of *dgt-28* as a selectable marker for glyphosate selection agent is tested with the *Arabidopsis* transformed plants described above. Approximately 50 T₄ generation *Arabidopsis* seed (homozygous for *dgt-28*) are
5 spiked into approximately 5,000 wildtype (sensitive to glyphosate) seed. The seeds are germinated and plantlets are sprayed with a selecting dose of glyphosate. Several treatments of glyphosate are compared; each tray of plants receives either one or two application timings of glyphosate in one of the following treatment schemes: 7 DAP (days after planting), 11 DAP, or 7 followed by 11 DAP. Since all plants also contain a
10 glufosinate resistance gene in the same transformation vector, *dgt-28* containing plants selected with glyphosate can be directly compared to *DSM-2* or *pat* containing plants selected with glufosinate.

Glyphosate treatments are applied with a DeVilbiss™ spray tip as previously described. Transgenic plants containing *dgt-28* are identified as “resistant” or
15 “sensitive” 17 DAP. Treatments of 26.25-1680 g ae/ha glyphosate applied 7 and 11 days after planting (DAP), show effective selection for transgenic *Arabidopsis* plants that contain *dgt-28*. Sensitive and resistant plants are counted and the number of glyphosate tolerant plants is found to correlate with the original number of transgenic seed containing the *dgt-28* transgene which are planted. These results indicate that
20 *dgt-28* can be effectively used as an alternative selectable marker for a population of transformed *Arabidopsis*.

Heritability. Confirmed transgenic T₁ *Arabidopsis* events were self-pollinated to produce T₂ seed. These seed were progeny tested by applying Ignite™ herbicide containing glufosinate (200 g ae/ha) to 100 random T₂ siblings. Each individual T₂
25 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). The T₁ families (T₂ 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). The percentage of T₁ families that segregated with the expected Mendelian inheritance are illustrated in
30 **Table 10**, and demonstrate that the *dgt-28* trait is passed *via* Mendelian inheritance to the T₂ generation. Seed were collected from 5 to 15 T₂ individuals (T₃ seed). Twenty-five T₃ siblings from each of 3-4 randomly-selected T₂ families were progeny

tested as previously described. Data showed no segregation and thus demonstrated that *dgt-28* and *dgt-3* are stably integrated within the chromosome and inherited in a Mendelian fashion to at least three generations.

Table 10. Percentage of T₁ families (T₂ plants) segregating as single Mendelian inheritance for a progeny test of 100 plants.

Gene of Interest	T1 Families Tested Segregating at 1 Locus (%)
<i>dgt-3</i> v2	64%
<i>dgt-3</i> v3	60%
<i>dgt-3</i> v4	80%
<i>dgt-7</i> v4	63%
TraP5 v2 – <i>dgt-28</i> v5	100%
TraP8 v2 – <i>dgt-28</i> v5	100%
TraP9 v2 – <i>dgt-28</i> v5	100%
TraP12 v2 – <i>dgt-28</i> v5	50%
TraP13 v2 – <i>dgt-28</i> v5	75%
<i>yfp</i> Transgenic Control Plants	100%

T₂ Arabidopsis Data. The second generation plants (T₂) of selected T₁ *Arabidopsis* events which contained low copy numbers of the *dgt-28* transgene were further characterized for glyphosate tolerance. Glyphosate was applied as described previously. The response of the plants is presented in terms of % visual injury 2 weeks after treatment (WAT). Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). An arithmetic mean and standard deviation are presented for each construct used for *Arabidopsis* transformation. The range in individual response is also indicated in the last column for each rate and transformation. Wild-type, non-transformed *Arabidopsis* (cv. Columbia) served as a glyphosate sensitive control. In the T₂ generation hemizygous and homozygous plants were available for testing for each event and therefore were included for each rate of glyphosate tested. Hemizygous plants contain two different alleles at a locus as compared to homozygous plants which contain the same two alleles at a locus. Variability of response to glyphosate is expected in the T₂ generation as a result of the difference in gene dosage for hemizygous as compared to

homozygous plants. The variability in response to glyphosate is reflected in the standard deviation and range of response.

In the T₂ generation both single copy and multi-copy *dgt-28* events were characterized for glyphosate tolerance. Within an event, single copy plants showed similar levels of tolerance to glyphosate. Characteristic data for a single copy T₂ event are presented in **Table 11**. Events containing *dgt-28* linked with TraP5 v2 did not provide robust tolerance to glyphosate as compared with the *dgt-28* constructs which contained other TraP transit peptides. However, the *dgt-28* TraP5 constructs did provide a low level of glyphosate tolerance as compared to the non-transformed Columbia control. There were instances when events that were shown to contain two or more copies of *dgt-28* were more susceptible to elevated rates of glyphosate (data not shown). This increase in sensitivity to glyphosate is similar to the data previously described for the T₁ plants which also contained high copy numbers of the *dgt-28* transgene. It is likely that the presence of high copy numbers of the transgene within the *Arabidopsis* plants result in transgene silencing or other epigenetic effects which resulted in sensitivity to glyphosate, despite the presence of the *dgt-28* transgene.

These events contained *dgt-28* linked with TraP5 v2 (pDAB105530), TraP12 v2 (pDAB105533) and TraP13 v2 (pDAB105534).

In addition to *dgt-28*, T₂ *Arabidopsis* events transformed with *dgt-3* are presented in **Table 12**. As described for the *dgt-28* events in **Table 11**, the data table contains a representative event that is characteristic of the response to glyphosate for each construct. For the *dgt-3* characterization, constructs containing a single PTU (plant transformation unit) with the *dgt-3* gene being driven by the AtUbi10 promoter (pDAB102716, FIG. 45 and pDAB102715, FIG. 10) were compared to constructs with the same gene containing 2 PTUs of the gene (pDAB102719, FIG. 32; pDAB102718, FIG. 33). The constructs which contained 2 PTU used the AtUbi10 promoter to drive one copy of the gene and the CsVMV promoter to drive the other copy. The use of the double PTU was incorporated to compare the *dgt-3* transgenic plants with *dgt-28* transgenic plants which contained two copies of the transgene. Data demonstrated that single copy T₂ *dgt-3* events with only a single PTU were more susceptible to glyphosate than single copy *dgt-28* events tested, but were more tolerant than the non-transformed control. T₁ families containing 2 PTUs of the *dgt-3* gene provided a higher level of visual tolerance to glyphosate compared to the 1 PTU constructs. In

both instances the T_1 families were compared to the *dgt-1* and wildtype controls. T_2 data demonstrate that *dgt-28* provides robust tolerance as single copy events.

Table 11. Response of selected individual T_2 *Arabidopsis* events containing *dgt-28* to glyphosate applied postemergence at varying rates, compared to a *dgt-1* (T_4) homozygous resistant population, and a non-transformed control. Visual % injury 14 days after application.

pDAB105530: TraP5 v2 - <i>dgt-28</i> v5				% Injury		
1 copy	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	0	0	4	75.0	17.8	50-90
840 g ae/ha glyphosate	0	0	4	80.0	20.0	50-90
1680 g ae/ha glyphosate	0	0	4	75.0	10.8	60-85
3360 g ae/ha glyphosate	0	0	4	76.3	4.8	70-80
pDAB105531: TraP8 v2 - <i>dgt-28</i> v5				% Injury		
1 copy	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	0.5	1.0	0-2
840 g ae/ha glyphosate	4	0	0	1.3	2.5	0-5
1680 g ae/ha glyphosate	4	0	0	7.5	5.0	5-15
3360 g ae/ha glyphosate	4	0	0	7.5	6.5	0-15
pDAB105532: TraP9 v2 - <i>dgt-28</i> v5				% Injury		
1 copy	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	2.0	4.0	0-8
840 g ae/ha glyphosate	4	0	0	9.0	2.0	8-12
1680 g ae/ha glyphosate	4	0	0	7.3	4.6	2-12
3360 g ae/ha glyphosate	4	0	0	11.0	1.2	10-12
pDAB105533: TraP12 v2 - <i>dgt-28</i> v5				% Injury		
1 copy	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	0.0	0.0	0
840 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1680 g ae/ha glyphosate	4	0	0	0.0	0.0	0
3360 g ae/ha glyphosate	3	1	0	13.3	7.9	8-25
pDAB105534: TraP13 v2 - <i>dgt-28</i> v5				% Injury		
1 copy	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	0.0	0.0	0
840 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1680 g ae/ha glyphosate	4	0	0	0.0	0.0	0
3360 g ae/ha glyphosate	3	1	0	13.3	7.9	8-25

1 copy	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0		0.0	0.0	0
420 g ae/ha glyphosate	3	1	0		5.0	10.0	0-20
840 g ae/ha glyphosate	3	1	0		5.0	10.0	0-20
1680 g ae/ha glyphosate	2	2	0		10.0	11.5	0-20
3360 g ae/ha glyphosate	2	2	0		15.0	12.2	5-30
WT (non-transformed control)							
	% Injury				% Injury		
	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0		0.0	0.0	0
420 g ae/ha glyphosate	0	0	4		100.0	0.0	100
840 g ae/ha glyphosate	0	0	4		100.0	0.0	100
1680 g ae/ha glyphosate	0	0	4		100.0	0.0	100
3360 g ae/ha glyphosate	0	0	4		100.0	0.0	100
pDAB4104: <i>dgt-1</i> (transformed control)							
	% Injury				% Injury		
1 copy	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0		0.0	0.0	0
420 g ae/ha glyphosate	0	4	0		37.5	2.9	35-40
840 g ae/ha glyphosate	0	0	4		45.0	0.0	45
1680 g ae/ha glyphosate	0	0	4		47.5	2.9	45-50
3360 g ae/ha glyphosate	0	0	4		50.0	0.0	50

Table 12. Response of selected T₂ *Arabidopsis* events transformed with *dgt-3* to glyphosate applied postemergence at varying rates. Visual % injury 14 days after application.

pDAB102716: <i>dgt-3 v3</i> (1 PTU)	% Injury				% Injury		
1 copy seg	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0		0	0	0
420 g ae/ha glyphosate	1	1	2		39	25	15-65
840 g ae/ha glyphosate	0	2	2		50	23	30-70
1680 g ae/ha glyphosate	0	1	3		69	19	40-80
3360 g ae/ha glyphosate	0	0	4		79	6	70-85

pDAB102719: <i>dgt-3 v3</i> (2 PTU)	% Injury				% Injury		
1 copy seg	<20%	20-40%	>40%		Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0		0	0	0
420 g ae/ha glyphosate	0	4	0		20	0	20
840 g ae/ha glyphosate	0	3	1		38	5	35-45
1680 g ae/ha glyphosate	3	1	0		15	7	10-25
3360 g ae/ha glyphosate	2	2	0		21	8	15-30

pDAB102715: <i>dgt-3 v2</i> (1 PTU)	% Injury			% Injury		
1 copy seg	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0	0	0
420 g ae/ha glyphosate	2	2	0	26	16	10-40
840 g ae/ha glyphosate	0	2	2	55	17	40-70
1680 g ae/ha glyphosate	0	2	2	56	22	35-75
3360 g ae/ha glyphosate	0	0	4	65	17	50-80

pDAB102718: <i>dgt-3 v2</i> (2 PTU)	% Injury			% Injury		
1 copy seg	<20%	20-40%	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0	0	0
420 g ae/ha glyphosate	4	0	0	5	7	0-15
840 g ae/ha glyphosate	2	2	0	23	10	15-35
1680 g ae/ha glyphosate	3	0	1	20	20	5-50
3360 g ae/ha glyphosate	1	1	2	36	22	15-60

T₃ *Arabidopsis* Data. The third generation plants (T₃) of selected T₂ *Arabidopsis* events which contained low copy numbers of the *dgt-28* transgene were further characterized for glyphosate tolerance. Twenty-five plants per line were selected with glufosinate as previously described and lines from every construct tested did not segregate for the selectable marker gene. Glyphosate was applied as described previously. The response of the plants is presented in terms of % visual injury 2 weeks after treatment (WAT). Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). An arithmetic mean and standard deviation are presented for each construct used for *Arabidopsis* transformation. The range in individual response is also indicated in the last column for each rate and transformation. Wild-type, non-transformed *Arabidopsis* (cv. Columbia) served as a glyphosate sensitive control.

Table 13. Response of selected individual T₃ *Arabidopsis* events containing *dgt-28* to glyphosate applied postemergence at varying rates, compared to a *dgt-1* (T₄) homozygous resistant population, and a non-transformed control. Visual % injury 14 days after application.

<i>dgt-28</i> (pDAB107602)	% Injury Range (No. Replicates)			% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0

420 g ae/ha glyphosate	0	0	4	73.8	2.5	70-75
840 g ae/ha glyphosate	0	0	4	71.3	7.5	60-75
1680 g ae/ha glyphosate	0	0	4	77.5	2.9	75-80
3360 g ae/ha glyphosate	0	0	4	77.5	2.9	75-80

TraP4::dgt-28 (pDAB107527)		% Injury Range (No. Replicates)		% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	0.0	0.0	0
840 g ae/ha glyphosate	4	0	0	5.0	0.0	5
1680 g ae/ha glyphosate	4	0	0	10.0	0.0	10
3360 g ae/ha glyphosate	1	3	0	18.8	2.5	15-20

TraP5 v1::dgt-28 (pDAB102792)		% Injury Range (No. Replicates)		% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	3	0	0	0.0	0.0	0
840 g ae/ha glyphosate	3	0	0	0.0	0.0	0
1680 g ae/ha glyphosate	3	0	0	6.0	1.7	5-8
3360 g ae/ha glyphosate	2	0	0	6.5	2.1	5-8

TraP5 v2::dgt-28 (pDAB105530)		% Injury Range (No. Replicates)		% Injury Analysis		
Averages	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	6.0	1.7	5-8
840 g ae/ha glyphosate	4	0	0	8.0	0.0	8
1680 g ae/ha glyphosate	4	0	0	14.3	1.5	12-15
3360 g ae/ha glyphosate	1	3	0	18.7	2.5	15-20

TraP8 v2::dgt-28 (pDAB105531)		% Injury Range (No. Replicates)		% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	2.5	5.0	0-10
840 g ae/ha glyphosate	4	0	0	3.3	3.9	0-8
1680 g ae/ha glyphosate	4	0	0	2.5	2.9	0-5
3360 g ae/ha glyphosate	4	0	0	7.3	6.4	2-15

TraP9 v2::dgt-28 (pDAB105532)		% Injury Range (No. Replicates)		% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	1.3	2.5	0-5
840 g ae/ha glyphosate	4	0	0	1.8	2.4	0-5
1680 g ae/ha glyphosate	4	0	0	0.0	0.0	0
3360 g ae/ha glyphosate	4	0	0	10.0	4.4	5-15

TraP12 v2::dgt-28 (pDAB105533)		% Injury Range (No. Replicates)		% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	0.0	0.0	0
840 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1680 g ae/ha glyphosate	4	0	0	3.8	7.5	0-15
3360 g ae/ha glyphosate	4	0	0	6.3	4.8	0-10

TraP13 v2::dgt-28 (pDAB105534)		% Injury Range (No. Replicates)		% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	2	2	0	10.0	11.5	0-20
840 g ae/ha glyphosate	4	0	0	1.3	2.5	0-5
1680 g ae/ha glyphosate	4	0	0	2.8	1.5	2-5
3360 g ae/ha glyphosate	4	0	0	8.0	0.0	8

TraP23::dgt-28 (pDAB107553)		% Injury Range (No. Replicates)		% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	4	0	0	0.0	0.0	0
840 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1680 g ae/ha glyphosate	4	0	0	7.8	2.1	5-10
3360 g ae/ha glyphosate	4	0	0	10.8	3.0	8-15

WT (non-transformed control)		% Injury Range (No. Replicates)		% Injury Analysis		
Application Rate	<20%	20-40 %	>40%	Ave	Std dev	Range (%)

0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha glyphosate	0	0	4	100. 0	0.0	100
840 g ae/ha glyphosate	0	0	4	100. 0	0.0	100
1680 g ae/ha glyphosate	0	0	4	100. 0	0.0	100
3360 g ae/ha glyphosate	0	0	4	100. 0	0.0	100

Selection of transformed plants. Freshly harvested T₁ seed [*dgt-31*, *dgt-32*, and *dgt-33 v1* gene] were allowed to dry at room temperature and shipped to Indianapolis for testing. T₁ 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 T₁ 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 once plants had germinated prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *dsm-2* gene).

Six days after planting (DAP) and again 10 DAP, T₁ plants (cotyledon and 2-4-leaf stage, respectively) were sprayed with a 0.1% solution of IGNITE™ herbicide (280 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 mL/tray (703 L/ha) using a DeVilbiss™ compressed air spray tip to deliver an effective rate of 200 g ae/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying. Surviving plants were transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360™). Plants reared in the greenhouse at least 1 day prior to tissue sampling for copy number analyses.

T₁ plants were sampled and copy number analysis for the *dgt-31*, *dgt-32*, and *dgt-33 v1* gene were completed. T₁ plants were then assigned to various rates of glyphosate so that a range of copies were among each rate. For *Arabidopsis*, 26.25 g

ae/ha glyphosate is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were applied to determine relative levels of resistance (105, 420, 1680, or 3360 g ae/ha). **Table 15** shows the comparisons drawn to *dgt-1*.

5 All glyphosate herbicide applications were made by track sprayer in a 187 L/ha spray volume. Glyphosate used was of the commercial Durango dimethylamine salt formulation (480 g ae/L, Dow AgroSciences, LLC). Low copy T₁ plants that exhibited tolerance to either glufosinate or glyphosate were further accessed in the T₂ generation.

The first *Arabidopsis* transformations were conducted using *dgt-31*, *dgt-32*, and
10 *dgt-33 v1*. T₁ transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Three flats or 30,000 seed were analyzed for each T₁ construct. Transformation frequency was calculated and results of T₁ *dgt-31*, *dgt-32*, and *dgt-33* constructs are listed in **Table 14**.

Table 14. Transformation frequency of T₁ *dgt-31*, *dgt-32*, and *dgt-33*
15 *Arabidopsis* constructs selected with glufosinate for selection of the selectable marker gene DSM-2.

Construct	Cassette	Transformation Frequency (%)
pDAB107532	AtUbi10/TraP14 <i>dgt-32 v1</i>	0.47
pDAB107533	AtUbi10/TraP23 <i>dgt-31 v1</i>	0.36
pDAB107534	AtUbi10/TraP24 <i>dgt-33 v1</i>	0.68

T₁ plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial glyphosate. **Table 15** compares the response
20 of *dgt-31*, *dgt-32*, and *dgt-33 v1* and control genes to impart glyphosate resistance to *Arabidopsis* T₁ 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%). An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is
25 also indicated in the last column for each rate and transformation. Wild-type non-transformed *Arabidopsis* (cv. Columbia) served as a glyphosate sensitive control. The *DGT-31 (v1)* gene with transit peptide TraP23 imparted slight herbicide tolerance to individual T₁ *Arabidopsis* plants compared to the negative control, but the gene exhibited improved tolerance with transit peptide TraP8. Both DGT-32 and DGT-33

demonstrated robust tolerance to glyphosate at the rates tested with TraP8 and with their respective differing chloroplast transit peptide (TraP14 and TraP24 respectively). Within a given treatment, the level of plant response varied greatly, which can be attributed to the fact each plant represents an independent transformation event and thus the copy number of the gene of interest varies from plant to plant. Of important note, at each glyphosate rate tested, there were individuals that were more tolerant than others. An overall population injury average by rate is presented in **Table 15** to demonstrate the significant difference between the plants transformed with *dgt-31*, *dgt-32*, and *dgt-33 v1* versus the *dgt-1 v1* or Wild-type controls.

Table 15. *dgt-31*, *dgt-32*, and *dgt-33 v1* transformed T₁ *Arabidopsis* response to a range of glyphosate rates applied postemergence, compared to a *dgt-1* (T4) homozygous resistant population, or a non-transformed control. Visual % injury 2 weeks after treatment.

TraP23 dgt-31	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	0	0	4	81.3	2.5	80-85
420 g ae/ha	0	0	4	97.3	4.9	90-100
1680 g ae/ha	0	0	4	90.0	7.1	85-100
3360 g ae/ha	0	0	4	91.3	6.3	85-100
TraP14 dgt-32	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	4	0	0	0.0	0.0	0
420 g ae/ha	2	0	2	30.0	29.4	0-60
1680 g ae/ha	3	0	1	17.5	21.8	5-50
3360 g ae/ha	0	3	1	35.0	30.0	20-80
TraP24 dgt-33	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	2	2	0	21.3	14.9	5-40

420 g ae/ha	1	1	2	46.3	30.9	5-70
1680 g ae/ha	1	0	3	62.5	38.8	5-90
3360 g ae/ha	1	0	3	62.0	36.0	8-80
TraP8 dgt-31	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0		0.0	0.0
105 g ae/ha glyphosate	0	1	3		43.8	17.0
420 g ae/ha glyphosate	1	2	1		43.8	32.5
1680 g ae/ha glyphosate	0	1	3		71.3	27.8
3360 g ae/ha glyphosate	0	0	4		81.3	8.5
TraP8 dgt-32	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0		0.0	0.0
105 g ae/ha glyphosate	4	0	0		0.0	0.0
420 g ae/ha glyphosate	4	0	0		7.5	5.0
1680 g ae/ha glyphosate	3	1	0		10.8	9.6
3360 g ae/ha glyphosate	4	0	0		12.8	3.2
TraP8 dgt-33	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0		0.0	0.0
105 g ae/ha glyphosate	4	0	0		0.0	0.0
420 g ae/ha glyphosate	4	0	0		2.5	3.8
1680 g ae/ha glyphosate	4	0	0		6.3	2.5
3360 g ae/ha glyphosate	3	1	0		20.0	13.5
dgt-1 (transformed control)	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	0	1	3	42.5	15.0	20-50
420 g ae/ha	0	2	2	38.8	11.1	25-50
1680 g ae/ha	0	0	4	79.0	19.4	50-90

3360 g ae/ha	0	0	4	50.0	0.0	50
WT (non-transformed control)	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	0	0	4	100.0	0.0	100
420 g ae/ha	0	0	4	100.0	0.0	100
1680 g ae/ha	0	0	4	100.0	0.0	100
3360 g ae/ha	0	0	4	100.0	0.0	100
TraP23 <i>dgt-31</i>	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	0	0	4	81.3	2.5	80-85
420 g ae/ha	0	0	4	97.3	4.9	90-100
1680 g ae/ha	0	0	4	90.0	7.1	85-100
3360 g ae/ha	0	0	4	91.3	6.3	85-100
TraP14 <i>dgt-32</i>	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	4	0	0	0.0	0.0	0
420 g ae/ha	2	0	2	30.0	29.4	0-60
1680 g ae/ha	3	0	1	17.5	21.8	5-50
3360 g ae/ha	0	3	1	35.0	30.0	20-80
TraP24 <i>dgt-33</i>	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	2	2	0	21.3	14.9	5-40
420 g ae/ha	1	1	2	46.3	30.9	5-70
1680 g ae/ha	1	0	3	62.5	38.8	5-90
3360 g ae/ha	1	0	3	62.0	36.0	8-80
<i>dgt-1</i> (transformed control)	% Injury			% Injury		

Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	0	1	3	42.5	15.0	20-50
420 g ae/ha	0	2	2	38.8	11.1	25-50
1680 g ae/ha	0	0	4	79.0	19.4	50-90
3360 g ae/ha	0	0	4	50.0	0.0	50
WT (non-transformed control)	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
105 g ae/ha	0	0	4	100.0	0.0	100
420 g ae/ha	0	0	4	100.0	0.0	100
1680 g ae/ha	0	0	4	100.0	0.0	100
3360 g ae/ha	0	0	4	100.0	0.0	100

Example 8: *dgt-32* and *dgt-33* as Selectable Markers

dgt-32 and *dgt-33 v1* are used as selectable markers with glyphosate as the selection agent. The performance of these markers is analyzed with transformed *Arabidopsis*. Approximately 50 T₄ generation *Arabidopsis* seed (homozygous for *dgt-32* and *dgt-33 v1*) are spiked into approximately 5,000 wild-type (sensitive) seed. Several treatments are compared, each tray of plants receiving either one or two application timings of glyphosate in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 followed by 11 DAP. Since all individuals also contain the *dsm-2* gene in the same transformation vector, *dgt-32* and *dgt-33* selected with glyphosate are able to be directly compared to *dsm-2* selected with glufosinate.

Treatments are applied with a DeVilbiss™ spray tip. Plants are identified as Resistant or Sensitive 17 DAP. Treatments of 26.25 - 280 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), are equally effective in selection frequency. These results indicate that *dgt-32* and *dgt-33 v1* can be effectively used as a selectable marker.

Heritability. A variety of T₁ events were self-pollinated to produce T₂ seed. These seed were progeny tested by applying IGNITE™ (200 g ae/ha) to 100 random T₂ siblings. Each individual T₂ plant was transplanted to 7.5-cm square pots prior to

spray application (track sprayer at 187 L/ha applications rate). T_1 families (T_2 plants) that segregate in the anticipated 3 Resistant:1 Sensitive model for a dominantly-inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$) were determined.

- 5 Seed was collected from 5 to 15 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of 3 randomly-selected T_2 families were progeny tested. Data showing no segregation demonstrate that *dgt-32* and *dgt-33 v1* were each stably integrated and inherited in a Mendelian fashion to at least three generations.

- 10 Additional Herbicide Tolerance Characterization of T_3 DGT lines. T_3 generation *Arabidopsis* seed is stratified, and sown into selection trays. A transformed control line containing *dgt-1* and the non-transformed control are planted in a similar manner. Seedlings are transferred to individual 3-inch pots in the greenhouse. All plants are sprayed with the use of a track sprayer set at 187 L/ha. The plants are sprayed with a range of glyphosate from 420-3360 g ae/ha (DURANGO™ DMA, Dow
15 AgroSciences). All applications are formulated in water. Each treatment is replicated 4 times, and plants are evaluated at 7 and 14 days after treatment.

Example 9: Transformation of Additional Crop Species

- 20 Soybean is transformed with *dgt-28*, *dgt-31*, *dgt-32*, and/or *dgt-33* (with or without a chloroplast transit peptide) to provide high levels of resistance to the herbicide glyphosate, utilizing a method known to those of skill in the art, for example, substantially the same techniques previously described in Example 11 or Example 13 of PCT International Patent Publication No. WO 2007/053482.

- 25 Cotton is transformed with *dgt-28*, *dgt-31*, *dgt-32*, and/or *dgt-33* (with or without a chloroplast transit peptide) to provide high levels of resistance to the herbicide glyphosate by utilizing a method known to those of skill in the art, for example, substantially the same techniques previously described in Examples 14 of U.S. Patent 7,838,733, or Example 12 of PCT International Patent Publication No. WO 2007/053482.

- 30 Canola is transformed with *dgt-28*, *dgt-31*, *dgt-32*, and/or *dgt-33* (with or without a chloroplast transit peptide) to provide high levels of resistance to the herbicide glyphosate by utilizing a method known to those of skill in the art, for example, substantially the same techniques previously described in Example 26 of U.S.

Patent 7,838,733, or Example 22 of PCT International Patent Publication No. WO 2007/053482.

Example 10: Maize Transformation

5 DNA Constructs for Maize Transformation. Standard cloning methods, as described above, were used in the construction of binary vectors for use in *Agrobacterium tumefaciens*-mediated transformation of maize. **Table 16** lists the vectors which were constructed for maize transformation. The following gene elements were used in the vectors which contained *dgt-28*; the *Zea mays* Ubiquitin 1 promoter (ZmUbi1; U.S. Patent No. 5,510,474) was used to drive the *dgt-28* coding sequence which is flanked by a *Zea mays* Lipase 3' untranslated region (ZmLip 3'UTR; US Patent No. 7179902), the selectable marker cassette consists of the *Zea mays* Ubiquitin 1 promoter which was used to drive the *aad-1* coding sequence (US Patent No. 7,838,733) which is flanked by a *Zea mays* Lipase 3' untranslated region. 10 The *aad-1* coding sequence confers tolerance to the phenoxy auxin herbicides, such as, 2,4-dichlorophenoxyacetic acid (2,4-D) and to aryloxyphenoxypropionate (AOPP) herbicides. 15

The *dgt-28* constructs were built as standard binary vectors and *Agrobacterium* superbinary system vectors (Japan Tobacco, Tokyo, JP). The standard binary vectors include; pDAB107663, pDAB107664, pDAB107665, and pDAB107665. The 20 *Agrobacterium* superbinary system vectors include pDAB108384, pDAB108385, pDAB108386, and pDAB108387.

Additional constructs were completed which contain a *yellow fluorescent protein* (*yfp*; US Patent Application 2007/0298412) reporter gene. pDAB109812 25 contains a *yfp* reporter gene cassette which is driven by the *Zea mays* Ubiquitin 1 promoter and flanked by the *Zea mays* per 5 3' untranslated region (Zm per5 3'UTR; US Patent No. 7179902), the selectable marker cassette consists of the sugar cane bacilliform virus promoter (SCBV; US Patent No. 5,994,123) which is used to drive the expression of *aad-1* and is flanked by the *Zea mays* Lipase 3' untranslated region. 30 pDAB101556 contains a *yfp* cassette which is driven by the *Zea mays* Ubiquitin 1 promoter and flanked by the *Zea mays* per 5 3' untranslated region, the selectable marker cassette consists of the *Zea mays* Ubiquitin 1 promoter which is used to drive the expression of *aad-1* and is flanked by the *Zea mays* Lipase 3' untranslated region.

pDAB107698 contains a *dgt-28* cassette which is driven by the *Zea mays* Ubiquitin 1 promoter and is flanked by a *Zea mays* Lipase 3' untranslated region, an *yfp* cassette which is driven by the *Zea mays* Ubiquitin 1 promoter and flanked by the *Zea mays* per 5 3' untranslated region, the selectable marker cassette consists of the sugar cane bacilliform virus promoter which is used to drive the expression of *aad-1* and is flanked by the *Zea mays* Lipase 3' untranslated region. All three of these constructs are standard binary vectors.

Table 16. Maize Transformation Vectors

Plasmid No.	Fig. No:	Description of Gene Elements
pDAB1076 63	34	ZmUbi1/TraP4 <i>dgt-28</i> /ZmLip 3'UTR :: ZmUbi1/ <i>aad-1</i> /ZmLip 3'UTR binary vector
pDAB1076 64	35	ZmUbi1/TraP8 <i>dgt-28</i> /ZmLip 3'UTR :: ZmUbi1/ <i>aad-1</i> /ZmLip 3'UTR binary vector
pDAB1076 65	36	ZmUbi1/TraP23 <i>dgt-28</i> /ZmLip 3'UTR :: ZmUbi1/ <i>aad-1</i> /ZmLip 3'UTR binary vector
pDAB1076 66	37	ZmUbi1/TraP5 <i>dgt-28</i> /ZmLip 3'UTR :: ZmUbi1/ <i>aad-1</i> /ZmLip 3'UTR binary vector
pDAB1098 12	38	ZmUbi1/ <i>yfp</i> /ZmPer5 3'UTR :: SCBV / <i>aad-1</i> /ZmLip 3'UTR binary vector
pDAB1015 56	39	ZmUbi1/ <i>yfp</i> /ZmPer5 3'UTR :: ZmUbi1/ <i>aad-1</i> / ZmLip 3'UTR binary vector
pDAB1076 98	40	ZmUbi1/TraP8 <i>dgt-28</i> /ZmLip 3'UTR :: ZmUbi1/ <i>yfp</i> /ZmLip 3'UTR::SCBV/ <i>aad-1</i> /ZmLip 3'UTR
pDAB1083 84	41	ZmUbi1/TraP4 <i>dgt-28</i> /ZmLip 3'UTR:: ZmUbi1/ <i>aad-1</i> /ZmLip 3'UTR superbinary vector
pDAB1083 85	42	ZmUbi1/TraP8 <i>dgt-28</i> /ZmLip 3'UTR :: ZmUbi1/ <i>aad-1</i> /ZmLip 3'UTR superbinary precursor
pDAB1083 86	43	ZmUbi1/TraP23 <i>dgt-28</i> /ZmLip 3'UTR :: ZmUbi1/ <i>aad-1</i> /ZmLip 3'UTR superbinary precursor
pDAB1083 87	44	ZmUbi1/TraP5 <i>dgt-28</i> /ZmLip 3'UTR::ZmUbi1/ <i>aad-1</i> /ZmLip 3'UTR superbinary precursor

Ear sterilization and embryo isolation. To obtain maize immature embryos, plants of the *Zea mays* inbred line B104 were grown in the greenhouse and were self or sib-pollinated to produce ears. The ears were harvested approximately 9-12 days post-pollination. On the experimental day, ears were surface-sterilized by immersion in a 20% solution of sodium hypochlorite (5%) and shaken for 20-30 minutes, followed by three rinses in sterile water. After sterilization, immature zygotic embryos (1.5–2.4 mm) were aseptically dissected from each ear and randomly distributed into micro-centrifuge tubes containing liquid infection media (LS Basal Medium, 4.43 gm/L; N6 Vitamin Solution [1000X], 1.00 mL/L; L-proline, 700.0 mg/L; Sucrose,

68.5 gm/L; D(+) Glucose, 36.0 gm/L; 10mg/ml of 2,4-D, 150 μ L/L). For a given set of experiments, pooled embryos from three ears were used for each transformation.

***Agrobacterium* Culture Initiation:**

Glycerol stocks of *Agrobacterium* containing the binary transformation vectors described above were streaked on AB minimal medium plates containing appropriate antibiotics and were grown at 20 °C for 3-4 days. A single colony was picked and streaked onto YEP plates containing the same antibiotics and was incubated at 28 °C for 1-2 days.

Agrobacterium culture and Co-cultivation. *Agrobacterium* colonies were taken from the YEP plate, suspended in 10 mL of infection medium in a 50 mL disposable tube, and the cell density was adjusted to OD₆₀₀ nm of 0.2-0.4 using a spectrophotometer. The *Agrobacterium* cultures were placed on a rotary shaker at 125 rpm, room temperature, while embryo dissection was performed. Immature zygotic embryos between 1.5-2.4 mm in size were isolated from the sterilized maize kernels and placed in 1 mL of the infection medium) and washed once in the same medium. The *Agrobacterium* suspension (2 mL) was added to each tube and the tubes were placed on a shaker platform for 10-15 minutes. The embryos were transferred onto co-cultivation media (MS Salts, 4.33 gm/L; L-proline, 700.0 mg/L; Myo-inositol, 100.0 mg/L; Casein enzymatic hydrolysate 100.0 mg/L; 30 mM Dicamba-KOH, 3.3 mg/L; Sucrose, 30.0 gm/L; Gelzan™, 3.00 gm/L; Modified MS-Vitamin [1000X], 1.00 ml/L; 8.5 mg/ml AgNO₃, 15.0 mg/L; DMSO, 100 μ M), oriented with the scutellum facing up and incubated at 25°C, under 24-hour light at 50 μ mole m⁻² sec⁻¹ light intensity for 3 days.

Callus Selection and Regeneration of Putative Events. Following the co-cultivation period, embryos were transferred to resting media (MS Salts, 4.33 gm/L; L-proline, 700.0 mg/L; 1,2,3,5/4,6- Hexahydroxycyclohexane, 100 mg/L; MES [(2-(n-morpholino)-ethanesulfonic acid), free acid] 0.500 gm/L ; Casein enzymatic hydrolysate 100.0 mg/L; 30 mM Dicamba-KOH, 3.3 mg/L; Sucrose, 30.0 gm/L; Gelzan 2.30 gm/L; Modified MS-Vitamin [1000X], 1.00 ml/L; 8.5mg/ml AgNO₃, 15.0 mg/L; Carbenicillin, 250.0 mg/L) without selective agent and incubated under 24 hour light at 50 μ mole m⁻² sec⁻¹ light intensity and at 25°C for 3 days.

Growth inhibition dosage response experiments suggested that glyphosate concentrations of 0.25 mM and higher were sufficient to inhibit cell growth in the

untransformed B104 maize line. Embryos were transferred onto Selection 1 media containing 0.5mM glyphosate (MS Salts, 4.33 gm/L; L-proline, 700.0 mg/L; Myo-inositol, 100.0 mg/L; MES [(2-(n-morpholino)-ethanesulfonic acid), free acid] 0.500 gm/L; Casein enzymatic hydrolysate 100.0 mg/L; 30mM Dicamba-KOH, 3.3 mg/L; Sucrose, 30.0 gm/L; Gelzan™ 2.30 gm/L; Modified MS-Vitamin [1000X], 1.00 ml/L; 8.5mg/ml AgNo₃, 15.0 mg/L; Carbenicillin, 250.0 mg/L) and incubated in either dark and/or under 24-hour light at 50 $\mu\text{mole m}^{-2} \text{sec}^{-1}$ light intensity for 7-14 days at 28°C.

Proliferating embryogenic calli were transferred onto Selection 2 media containing 1.0 mM glyphosate (MS Salts, 4.33 gm/L; 1,2,3,5/4,6-Hexahydroxycyclohexane, 100mg/L; L-proline, 700.0 mg/L; MES [(2-(n-morpholino)-ethanesulfonic acid), free acid] 0.500 gm/L; Casein enzymatic hydrolysate 100.0 mg/L; 30 mM Dicamba-KOH, 3.3 mg/L; Sucrose, 30.0 gm/L; Gelzan™ 2.30 gm/L; Modified MS-Vitamin [1000X], 1.00 ml/L; 8.5mg/mL AgNo₃, 15.0 mg/L; Carbenicillin, 250.0 mg/L; R-Haloxyp acid 0.1810 mg/L), and were incubated in either dark and/or under 24-hour light at 50 $\mu\text{mole m}^{-2} \text{sec}^{-1}$ light intensity for 14 days at 28°C. This selection step allowed transgenic callus to further proliferate and differentiate. The callus selection period lasted for three to four weeks.

Proliferating, embryogenic calli were transferred onto PreReg media containing 0.5 mM glyphosate (MS Salts, 4.33 gm/L; 1,2,3,5/4,6- Hexahydroxycyclohexane, 100 mg/L; L-proline, 350.0 mg/L; MES [(2-(n-morpholino)-ethanesulfonic acid), free acid] 0.250 gm/L ; Casein enzymatic hydrolysate 50.0 mg/L; NAA-NaOH 0.500 mg/L; ABA-EtOH 2.50 mg/L; BA 1.00 mg/L; Sucrose, 45.0 gm/L; Gelzan™ 2.50 gm/L; Modified MS-Vitamin [1000X], 1.00 ml/L; 8.5mg/ml AgNo₃, 1.00 mg/L; Carbenicillin, 250.0 mg/L) and cultured under 24-hour light at 50 $\mu\text{mole m}^{-2} \text{sec}^{-1}$ light intensity for 7 days at 28°C.

Embryogenic calli with shoot-like buds were transferred onto Regeneration media containing 0.5 mM glyphosate (MS Salts, 4.33 gm/L; 1,2,3,5/4,6-Hexahydroxycyclohexane, 100.0 mg/L; Sucrose, 60.0 gm/L; Gellan Gum G434™ 3.00 gm/L; Modified MS-Vitamin [1000X], 1.00 ml/L; Carbenicillin, 125.0 mg/L) and cultured under 24-hour light at 50 $\mu\text{mole m}^{-2} \text{sec}^{-1}$ light intensity for 7 days.

Small shoots with primary roots were transferred to rooting media (MS Salts, 4.33 gm/L; Modified MS-Vitamin [1000X], 1.00 ml/L; 1,2,3,5/4,6-

Hexahydroxycyclohexane, 100 mg/L; Sucrose, 60.0 gm/L; Gellan Gum G434™ 3.00 gm/L; Carbenicillin, 250.0 mg/L) in phytotrays and were incubated under 16/8 hr. light/dark at 140-190 $\mu\text{mole m}^{-2} \text{sec}^{-1}$ light intensity for 7 days at 27°C. Putative transgenic plantlets were analyzed for transgene copy number using the protocols described above and transferred to soil.

Molecular Confirmation of the Presence of the *dgt-28* and *aad-1* transgenes within Maize Plants. The presence of the *dgt-28* and *aad-1* polynucleotide sequences were confirmed *via* hydrolysis probe assays. Isolated T₀ Maize plants were initially screened *via* a hydrolysis probe assay, analogous to TAQMAN™, to confirm the presence of a *aad-1* and *dgt-28* transgenes. The data generated from these studies were used to determine the transgene copy number and used to select transgenic maize events for back crossing and advancement to the T₁ generation.

Tissue samples were collected in 96-well plates, tissue maceration was performed with a KLECO™ tissue pulverizer and stainless steel beads (Hoover Precision Products, Cumming, GA), in Qiagen™ RLT buffer. Following tissue maceration, the genomic DNA was isolated in high-throughput format using the Biosprint 96™ Plant kit (Qiagen, Germantown, MD) according to the manufacturer's suggested protocol. Genomic DNA was quantified by Quant-IT™ Pico Green DNA assay kit (Molecular Probes, Invitrogen, Carlsbad, CA). Quantified genomic DNA was adjusted to around 2ng/ μL for the hydrolysis probe assay using a BIOROBOT3000™ automated liquid handler (Qiagen, Germantown, MD). Transgene copy number determination by hydrolysis probe assay, analogous to TAQMAN® assay, was performed by real-time PCR using the LIGHTCYCLER®480 system (Roche Applied Science, Indianapolis, IN). Assays were designed for *aad-1*, *dgt-28* and an internal reference gene Invertase (Genbank Accession No: U16123.1) using the LIGHTCYCLER® Probe Design Software 2.0. For amplification, LIGHTCYCLER®480 Probes Master mix (Roche Applied Science, Indianapolis, IN) was prepared at 1X final concentration in a 10 μL volume multiplex reaction containing 0.4 μM of each primer for *aad-1* and *dgt-28* and 0.2 μM of each probe (Table 17).

A two-step amplification reaction was performed with an extension at 60°C for 40 seconds with fluorescence acquisition. All samples were run and the averaged Cycle threshold (Ct) values were used for analysis of each sample. Analysis of real

time PCR data was performed using LightCycler[®] software release 1.5 using the relative quant module and is based on the $\Delta\Delta C_t$ method. Controls included a sample of genomic DNA from a single copy calibrator and known two copy check that were included in each run. **Table 18** lists the results of the hydrolysis probe assays.

- 5 **Table 17.** Primer and probe sequences used for hydrolysis probe assay of *aad-1*, *dgt-28* and internal reference (Invertase).

Oligonucleotide Name	Gene Detected	SEQ ID NO:	Oligo Sequence
GAAD1F	<i>aad-1</i> forward primer	58	TGTTTCGGTTCCCTCTACCAA
GAAD1P	<i>aad-1</i> probe	59	CACAGAACCGTCGCTTCAGCAACA
GAAD1R	<i>aad-1</i> reverse primer	60	CAACATCCATCACCTTGACTGA
IV-Probe	Invertase probe	61	CGAGCAGACCGCCGTGTACTTCTACC
IVF-Taq	Invertase forward primer	62	TGGCGGACGACGACTTGT
IVR-Taq	Invertase reverse primer	63	AAAGTTTGGAGGCTGCCGT
zmDGT28 F	<i>dgt-28</i> forward primer	64	TTCAGCACCCGTCAGAAT
zmDGT28 FAM	<i>dgt-28</i> probe	65	TGCCGAGAACTTGAGGAGGT
zmDGT28 R	<i>dgt-28</i> reverse primer	66	TGGTCGCCATAGCTTGT

- 10 **Table 18.** T_0 copy amount results for *dgt-28* events. Low copy events consisted of 1-2 transgene copies, single copy numbers are listed in parenthesis. High copy events contained 3 or more transgene copies.

Plasmid used for Transformation	# of Low Copy Events (single copy)	# of High Copy Events
pDAB107663	43 (31)	10
pDAB107664	30 (24)	5
pDAB107665	40 (27)	10
pDAB107666	24 (12)	12

pDAB109812	2 (1)	0
pDAB101556	25 (15)	10
pDAB107698	3 (1)	2

Example 11: Herbicide Tolerance in *dgt-28* Transformed Corn

Zea mays dgt-28 transformation events (T_0) were allowed to acclimate in the greenhouse and were grown until plants had transitioned from tissue culture to greenhouse growing conditions (*i.e.*, 2-4 new, normal looking leaves had emerged from the whorl). Plants were grown at 27°C under 16 hour light:8 hour dark conditions in the greenhouse. The plants were then treated with commercial formulations of DURANGO DMA™ (containing the herbicide glyphosate) with the addition of 2% w/v ammonium-sulfate. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. T_0 plants were sprayed with a range of glyphosate from 280 – 4480 g ae/ha glyphosate, which is capable of significant injury to untransformed corn lines. A lethal dose is defined as the rate that causes >95% injury to the B104 inbred.

The results of the T_0 *dgt-28* corn plants demonstrated that tolerance to glyphosate was achieved at rates up to 4480 g ae/ha. A specific media type was used in the T_0 generation. Minimal stunting and overall plant growth of transformed plants compared to the non-transformed controls demonstrated that *dgt-28* provides robust tolerance to glyphosate when linked to the TraP5, TraP8, and TraP23 chloroplast transit peptides.

Selected T_0 plants are selfed or backcrossed for further characterization in the next generation. 100 chosen *dgt-28* lines containing the T_1 plants are sprayed with 140-1120 g ae/ha glufosinate or 105-1680 g ae/ha glyphosate. Both the selectable marker and glyphosate resistant gene are constructed on the same plasmid. Therefore, if one herbicide tolerant gene is selected for by spraying with an herbicide, both genes are believed to be present. At 14 DAT, resistant and sensitive plants are counted to determine the percentage of lines that segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. These data demonstrate that *dgt-28* is inheritable as a robust glyphosate resistance gene in a monocot species. Increased rates of glyphosate are applied to the T_1 or F_1 survivors to further characterize the tolerance and protection that is provided by the *dgt-28* gene.

Post-emergence herbicide tolerance in *dgt-28* transformed T₀ Corn. T₀ events of *dgt-28* linked with TraP4, TraP5, TraP8 and TraP23 were generated by *Agrobacterium* transformation and were allowed to acclimate under controlled growth chamber conditions until 2-4 new, normal looking leaves had emerged from the whorl.

5 Plants were assigned individual identification numbers and sampled for copy number analyses of both *dgt-28* and *aad-1*. Based on copy number analyses, plants were selected for protein expression analyses. Plants were transplanted into larger pots with new growing media and grown at 27°C under 16 hour light:8 hour dark conditions in the greenhouse. Remaining plants that were not sampled for protein expression were

10 then treated with commercial formulations of DURANGO DMA™ (glyphosate) with the addition of 2% w/v ammonium-sulfate. Treatments were distributed so that each grouping of plants contained T₀ events of varying copy number. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. T₀ plants were sprayed with a range of glyphosate from 280-4480 g ae/ha

15 glyphosate capable of significant injury to untransformed corn lines. A lethal dose is defined as the rate that causes >95% injury to the B104 inbred. B104 was the genetic background of the transformants.

Results of T₀ *dgt-28* corn plants demonstrate that tolerance to glyphosate was achieved up to 4480 g ae/ha. **Table 19.** Minimal stunting and overall plant growth of

20 transformed plants compared to the non-transformed controls demonstrated that *dgt-28* provides robust protection to glyphosate when linked to TraP5, TraP8, and TraP23.

Table 19. Response of T₀ *dgt-28* events of varying copy numbers to rates of glyphosate ranging from 280-4480 g ae/ha + 2.0% w/v ammonium sulfate 14 days after treatment.

TraP4 <i>dgt-28</i>	% Injury			% Injury		
	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
Application Rate						
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
280 g ae/ha	5	0	0	1.0	2.2	0-5
560 g ae/ha	6	0	0	2.0	4.0	0-10
1120 g ae/ha	12	0	0	1.3	3.1	0-10
2240 g ae/ha	7	0	0	1.7	4.5	0-12
4480 g ae/ha	7	0	0	1.1	3.0	0-8

TraP8 <i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	6	0	0	0.0	0.0	0
280 g ae/ha	5	1	0	6.7	8.8	0-20
560 g ae/ha	0	2	0	20.0	0.0	20
1120 g ae/ha	7	0	0	1.4	2.4	0-5
2240 g ae/ha	3	1	0	7.5	15.0	0-30
4480 g ae/ha	6	0	0	1.7	4.1	0-10
TraP23 <i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	6	0	0	0.8	2.0	0-5
280 g ae/ha	7	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	1.3	2.5	0-5
1120 g ae/ha	10	2	0	3.3	7.8	0-20
2240 g ae/ha	6	0	0	1.3	3.3	0-8
4480 g ae/ha	6	1	0	4.3	7.9	0-20
TraP5 <i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
280 g ae/ha	7	1	0	5.0	14.1	0-40
560 g ae/ha	8	0	0	0.6	1.8	0-5
1120 g ae/ha	7	1	0	5.0	14.1	0-40
2240 g ae/ha	8	0	0	0.0	0.0	0
4480 g ae/ha	8	0	0	0.0	0.0	0

Protein expression analyses by standard ELISA demonstrated a mean range of DGT-28 protein from 12.6 - 22.5 ng/cm² across the constructs tested.

- 5 Confirmation of glyphosate tolerance in the F₁ generation under greenhouse conditions. Single copy T₀ plants that were not sprayed were backcrossed to the non-transformed background B104 for further characterization in the next generation. In the T₁ generation, glyphosate tolerance was assessed to confirm the inheritance of

the *dgt-28* gene. For T₁ plants, the herbicide ASSURE II™ (35 g ae/ha quizalofop-methyl) was applied at the V1 growth stage to select for the AAD-1 protein. Both the selectable marker and glyphosate resistant gene are constructed on the same plasmid. Therefore if one gene is selected, both genes are believed to be present. After 7 DAT, resistant and sensitive plants were counted and null plants were removed from the population. These data demonstrate that *dgt-28* (v1) is heritable as a robust glyphosate resistance gene in a monocot species. Plants were sampled for characterization of DGT-28 protein by standard ELISA and RNA transcript level. Resistant plants were sprayed with 560-4480 g ae/ha glyphosate as previously described. The data demonstrate robust tolerance of *dgt-28* linked with the chloroplast transit peptides TraP4, TraP5, TraP8 and TraP23 up to 4480 g ae/ha glyphosate.

Table 20.

Table 20. Response of F₁ single copy *dgt-28* events to rates of glyphosate ranging from 560-4480 g ae/ha + 2.0% w/v ammonium sulfate 14 days after treatment.

B104 / TraP4::<i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	9.0	1.2	8-10
2240 g ae/ha	4	0	0	2.5	2.9	0-5
4480 g ae/ha	4	0	0	0.0	0.0	0
B104 / TraP8::<i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	1.3	2.5	0-5
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	5.0	4.1	0-10
4480 g ae/ha	4	0	0	6.3	2.5	5-10
B104 / TraP23::<i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0

560 g ae/ha	3	1	0	10.0	10.0	5-25
1120 g ae/ha	2	2	0	18.8	11.8	10-35
2240 g ae/ha	4	0	0	12.5	2.9	10-15
4480 g ae/ha	3	1	0	10.0	7.1	5-20
B104 / TraP5::dgt-28	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	8.0	0.0	8
1120 g ae/ha	4	0	0	11.3	3.0	8-15
2240 g ae/ha	4	0	0	12.5	2.9	10-15
4480 g ae/ha	4	0	0	10.0	2.5	10-15
Non-transformed B104	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	0	0	4	100.0	0.0	100
1120 g ae/ha	0	0	4	100.0	0.0	100
2240 g ae/ha	0	0	4	100.0	0.0	100
4480 g ae/ha	0	0	4	100.0	0.0	100

Protein expression data demonstrate a range of mean DGT-28 protein from 42.2 – 88.2 ng/cm² across T₁ events and constructs tested, establishing protein expression in the T₁ generation.

- 5 Characterization of dgt-28 corn under field conditions. Single copy T₁ events were sent to a field location to create both hybrid hemizygous and inbred homozygous seed for additional characterization. Hybrid seeds were created by crossing T₁ events in the maize transformation line B104 to the inbred line 4XP811 generating hybrid populations segregating 1:1 (hemizygous:null) for the event. The resulting seeds were
- 10 shipped to 2 separate locations. A total of five single copy events per construct were planted at each location in a randomized complete block design in triplicate. The fields were designed for glyphosate applications to occur at the V4 growth stage and a separate grouping of plants to be applied at the V8 growth stage. The 4XP811/B104 conventional hybrid was used as a negative control.

Experimental rows were treated with 184 g ae/ha ASSURE II™ (106 g ai/L quizalofop-methyl) to eliminate null segregants. All experimental entries segregated 1:1 (sensitive:resistant) ($p=0.05$) with respect to the ASSURE II™ application. Selected resistant plants were sampled from each event for quantification of the DGT-28 protein by standard ELISA.

Quizalofop-methyl resistant plants were treated with the commercial herbicide DURANGO DMA™ (480 g ae/L glyphosate) with the addition of 2.5% w/v ammonium-sulfate at either the V4 or V8 growth stages. Herbicide applications were made with a boom sprayer calibrated to deliver a volume of 187 L/ha, 50-cm spray height. Plants were sprayed with a range of glyphosate from 1120 – 4480 g ae/ha glyphosate, capable of significant injury to untransformed corn lines. A lethal dose is defined as the rate that causes > 95% injury to the 4XP811 inbred. Visual injury assessments were taken for the percentage of visual chlorosis, percentage of necrosis, percentage of growth inhibition and total visual injury at 7, 14 and 21 DAT (days after treatment). Assessments were compared to the untreated checks for each line and the negative controls.

Visual injury data for all assessment timings demonstrated robust tolerance up to 4480 g ae/ha DURANGO DMA™ at both locations and application timings. Representative events for the V4 application are presented from one location and are consistent with other events, application timings and locations. **Table 21.** One event from the construct containing *dgt-28* linked with TraP23 (pDAB107665) was tolerant to the ASSURE II™ selection for the AAD-1 protein, but was sensitive to all rates of glyphosate applied.

Table 21. Response of *dgt-28* events applied with a range of glyphosate from 1120-4480 g ae/ha + 2.5% w/v ammonium sulfate at the V4 growth stage.

4XPB11//B104/TraP4::<i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	0.0	0.0	0
4480 g ae/ha	4	0	0	0.0	0.0	0
4XPB11//B104/TraP8::<i>dgt-28</i>	% Injury			% Injury		

Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	0.0	0.0	0
4480 g ae/ha	4	0	0	0.0	0.0	0
4XPB11//B104/TraP23::dgt-28	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	0.0	0.0	0
4480 g ae/ha	4	0	0	0.0	0.0	0
4XPB11//B104/TraP5::dgt-28	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	0.0	0.0	0
4480 g ae/ha	4	0	0	0.0	0.0	0
Non-transformed 4XPB11//B104	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	0	0	4	100.0	0.0	100
2240 g ae/ha	0	0	4	100.0	0.0	100
4480 g ae/ha	0	0	4	100.0	0.0	100

Additional assessments were made during the reproductive growth stage for the 4480 g ae/ha glyphosate rate. Visual assessments of tassels, pollination timing and ear fill were similar to the untreated checks of each line for all constructs, application

5 timings and locations. Quantification results for the DGT-28 protein demonstrated a range of mean protein expression from 186.4 – 303.0 ng/cm². Data demonstrates robust tolerance of *dgt-28* transformed corn under field conditions through the

reproductive growth stages up to 4480 g ae/ha glyphosate. Data also demonstrated DGT-28 protein detection and function based on spray tolerance results.

Confirmation of heritability and tolerance of *dgt-28* corn in the homozygous state. Seed from the T₁S₂ were planted under greenhouse conditions as previously described. The same five single copy lines that were characterized under field conditions were characterized in the homogeneous state. Plants were grown until the V3 growth stage and separated into three rates of glyphosate ranging from 1120-4480 g ae/ha glyphosate (DURANGO DMA™) and four replicates per treatment. Applications were made in a track sprayer as previously described and were formulated in 2.0% w/v ammonium sulfate. An application of ammonium sulfate served as an untreated check for each line. Visual assessments were taken 7 and 14 days after treatment as previously described. Data demonstrated robust tolerance up to 4480 g ae/ha glyphosate for all events tested. **Table 22.**

Table 22. Response of homozygous *dgt-28* events applied with a range of glyphosate from 1120-4480 g ae/ha + 2.0% w/v ammonium sulfate.

TraP4::<i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	3.8	2.5	0-5
4480 g ae/ha	4	0	0	14.3	1.5	12-15
TraP8::<i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	9.0	1.2	8-10
4480 g ae/ha	4	0	0	11.3	2.5	10-15
TraP23::<i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	4.5	3.3	0-8

2240 g ae/ha	4	0	0	7.5	2.9	5-10
4480 g ae/ha	4	0	0	15.0	0.0	15
TraP5::<i>dgt-28</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	1.3	2.5	0-5
2240 g ae/ha	4	0	0	9.0	2.0	8-12
4480 g ae/ha	4	0	0	15.0	2.4	12-18
Non-transformed B104	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
1120 g ae/ha	0	0	4	100.0	0.0	100
2240 g ae/ha	0	0	4	100.0	0.0	100
4480 g ae/ha	0	0	4	100.0	0.0	100

The line from pDAB107665 that was not tolerant under field conditions demonstrated no tolerance to glyphosate and therefore consistent with field observations (data not shown). With the exception of the one line previously mentioned, all replicates that were treated with glyphosate from the lines were not sensitive to glyphosate. Therefore data demonstrates heritability to a homogeneous population of *dgt-28* corn in a Mendelian fashion. Expression of the DGT-28 protein by standard ELISA demonstrated a range of mean protein expression from 27.5 – 65.8 ng/cm² across single copy events that were tolerant to glyphosate. Data demonstrates functional protein and stability of the DGT-28 protein across generations.

Example 12: Postemergence herbicide tolerance use of glyphosate as a selectable marker

As previously described, T₀ transformed plants were moved from tissue culture and acclimated in the greenhouse. The events tested contained *dgt-28* linked to TraP5, TraP8, and TraP23 chloroplast transit peptides. It was demonstrated that these T₀ plants provided robust tolerance up to 4480 g ae/ha glyphosate, and non-transformed plants were controlled with glyphosate at concentrations as low as 280 g ae/ha. These

data demonstrate that *dgt-28* can be utilized as a selectable marker using a concentration of glyphosate ranging from 280 – 4480 g ae/ha.

A number of seed from fixed lines of corn which contain the *dgt-28* transgene are spiked into a number of non-transformed corn seed. The seed are planted and allowed to grow to the V1-V3 developmental stage, at which time the plantlets are sprayed with a selecting dose of glyphosate in the range of 280 – 4480 g ae/ha. Following 7-10 days, sensitive and resistant plants are counted, and the amount of glyphosate tolerant plants correlates with the original number of transgenic seed containing the *dgt-28* transgene which are planted.

10

Example 13: Stacking of *dgt-28* Corn

The AAD-1 protein is used as the selectable marker in *dgt-28* transformed corn for research purposes. The *aad-1* gene can also be utilized as a herbicide tolerant trait in corn to provide robust 2,4-D tolerance up to a V8 application in a crop. Four events from the constructs pDAB107663 (TraP4::*dgt-28*), pDAB107664 (TraP8::*dgt-28*) and pDAB107666 (TraP5::*dgt-28*) were characterized for the tolerance of a tank mix application of glyphosate and 2,4-D. The characterization study was completed with F₁ seed under greenhouse conditions. Applications were made in a track sprayer as previously described at the following rates: 1120-2240 g ae/ha glyphosate (selective for the *dgt-28* gene), 1120-2240 g ae/ha 2,4-D (selective for the *aad-1* gene), or a tank mixture of the two herbicides at the rates described. Plants were graded at 7 and 14 DAT. Spray results for applications of the herbicides at 2240 g ae/ha are shown in Table 23.

Table 23. Response of F₁ *aad-1* and *dgt-28* corn sprayed with 2240 g ae/ha of 2,4-D, glyphosate and a tank mix combination of the two herbicides 14 days after treatment.

F ₁ Event	2240 g ae/ha 2,4-D		2240 g ae/ha glyphosate		2240 g ae/ha 2,4-D + 2240 g ae/ha glyphosate	
	Mean % injury	Std. Dev.	Mean % injury	Std. Dev.	Mean % injury	Std. Dev.
107663[3]-012.AJ001	5.0	4.1	3.8	4.8	8.8	3.0
107663[3]-029.AJ001	2.5	5.0	1.3	2.5	5.0	5.8
107663[3]-027.AJ001	2.5	2.9	11.8	2.9	13.8	2.5

107663[3]-011.AJ001	3.8	2.5	11.5	1.0	12.8	1.5
B104	27.5	17.7	100.0	0.0	100.0	0.0

The results confirm that *dgt-28* can be successfully stacked with *aad-1*, thus increasing the spectrum herbicides that may be applied to the crop of interest (glyphosate + phenoxyacetic acids for *dgt-28* and *aad-1*, respectively). In crop production where hard to control broadleaf weeds or resistant weed biotypes exist the stack can be used as a means of weed control and protection of the crop of interest. Additional input or output traits can also be stacked with the *dgt-28* gene in corn and other plants.

Example 14: Transformation of Other Crops

Additional crops are transformed using known techniques. For *Agrobacterium*-mediated transformation of rye, see, e.g., Popelka JC, Xu J, Altpeter F., "Generation of rye with low transgene copy number after biolistic gene transfer and production of (*Secale cereale* L.) plants instantly marker-free transgenic rye," Transgenic Res. 2003 Oct;12(5):587-96.). For *Agrobacterium*-mediated transformation of sorghum, see, e.g., Zhao *et al.*, "Agrobacterium-mediated sorghum transformation," Plant Mol Biol. 2000 Dec;44(6):789-98. For *Agrobacterium*-mediated transformation of barley, see, e.g., Tingay *et al.*, "Agrobacterium tumefaciens-mediated barley transformation," The Plant Journal, (1997) 11: 1369–1376. For *Agrobacterium*-mediated transformation of wheat, see, e.g., Cheng *et al.*, "Genetic Transformation of Wheat Mediated by *Agrobacterium tumefaciens*," Plant Physiol. 1997 Nov;115(3):971-980. For *Agrobacterium*-mediated transformation of rice, see, e.g., Hiei *et al.*, "Transformation of rice mediated by *Agrobacterium tumefaciens*," Plant Mol. Biol. 1997 Sep;35(1-2):205-18.

Other (non-*Agrobacterium*) transformation techniques are used to transform *dgt-28*, *dgt-32*, or *dgt-33*, for example, into Maize (*Zea mays*), Wheat (*Triticum* spp.), Rice (*Oryza* spp. and *Zizania* spp.), Barley (*Hordeum* spp.), Cotton (*Abroma augusta* and *Gossypium* spp.), Soybean (*Glycine max*), Sugar and table beets (*Beta* spp.), Sugar cane (*Arenga pinnata*), Tomato (*Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato (*Solanum tuberosum*), Sweet potato (*Ipomoea batatas*), Rye (*Secale* spp.), Peppers

(*Capsicum annuum*, *chinense*, and *frutescens*), Lettuce (*Lactuca sativa*, *perennis*, and *pulchella*), Cabbage (*Brassica* spp.), Celery (*Apium graveolens*), Eggplant (*Solanum melongena*), Peanut (*Arachis hypogea*), Sorghum (*Sorghum* spp.), Alfalfa (*Medicago sativa*), Carrot (*Daucus carota*), Beans (*Phaseolus* spp. and other genera), Oats (*Avena* 5 *sativa* and *strigosa*), Peas (*Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Helianthus annuus*), Squash (*Cucurbita* spp.), Cucumber (*Cucumis sativa*). Tobacco (*Nicotiana* spp.), Arabidopsis (*Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, *Poa*, *Cynodon*, and other genera), Clover (*Trifolium*), Vetch (*Vicia*).

Glyphosate resistance conferred by *dgt-28*, *dgt-32*, and *dgt-33* increases the 10 applicability of glyphosate herbicides for in-season use in many deciduous and evergreen timber cropping systems. Glyphosate herbicide resistant timber species increase the flexibility of over-the-top use of these herbicides without injury concerns. Thus, *dgt-28*, *dgt-32*, and/or *dgt-33* are transformed into the timber species: alder (*Alnus* spp.), ash (*Fraxinus* spp.), aspen and poplar species (*Populus* spp.), beech 15 (*Fagus* spp.), birch (*Betula* spp.), cherry (*Prunus* spp.), eucalyptus (*Eucalyptus* spp.), hickory (*Carya* spp.), maple (*Acer* spp.), oak (*Quercus* spp.), and pine (*Pinus* spp.).

Glyphosate herbicide resistance increases the applicability of glyphosate herbicides for the selective weed control in ornamental and fruit-bearing species. Thus, *dgt-28*, *dgt-32*, and/or *dgt-33* are transformed into the ornamental and fruit-bearing 20 species: rose (*Rosa* spp.), burning bush (*Euonymus* spp.), petunia (*Petunia* spp.), begonia (*Begonia* spp.), rhododendron (*Rhododendron* spp.), crabapple or apple (*Malus* spp.), pear (*Pyrus* spp.), peach (*Prunus* spp.), and marigolds (*Tagetes* spp.).

Example 15: Stacking With Other Traits

25 Transgenic crops containing insect resistance (IR) traits are prevalent in corn, soybean, and cotton plants throughout North America, and usage of these traits is expanding worldwide. Commercial transgenic crops combining insect resistant and herbicide tolerant (HT) traits have been developed by multiple seed companies. These include *Bacillus thuringiensis* traits (e.g., Bt toxins listed at the website 30 lifesci.sussex.ac.uk, 2006), non-Bt insect resistance traits, and any or all of the HT traits mentioned above. The ability to control multiple pest problems through IR traits is a valuable commercial product concept. However, the convenience of this product

concept will be restricted if weed control and insect control are independent of one another.

Dgt-28, dgt-31, dgt-32, or dgt-33, alone or stacked with one or more additional HT traits, are stacked with one or more additional input traits (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) (*see* www.isb.vt.edu), either through
5 conventional breeding or jointly as a novel transformation event. IR trait(s) is/are stacked with *dgt-28, dgt-31, dgt-32, or dgt-33*. Upon obtaining a coding sequence of an IR trait, expression elements (*e.g.*, promoter, intron, 3'UTR, etc.) are added and the IR trait is molecularly stacked with *dgt-28, dgt-31, dgt-32, or dgt-33* via recombinant
10 DNA methodologies.

The IR traits include: Cry1F (U.S. Pat. Nos. 5,126,133; 5,188,960; 5,691,308; 6,096,708; 6,573,240; and 6,737,273), Cry1A(c) (U.S. Pat. Nos. 6,114,138; 5,710,020; 6,251,656; and 6,229,004), Cry1F and Cry1A(c) as a triple stack with either *dgt-28, dgt-31, dgt-32, or dgt-33*, Cry34Ab(1) (U.S. Pat. Nos. 7,323,556; 7,897,342;
15 7,888,495; 7,875,430; 7,932,033; 7,956,246; 6,340,593), Cry35 Ab(1) (U.S. Pat. No. 6,340,593; 7,323,556; 7,897,342; 7,888,495; 7,875,430; 7,932,033; 7,956,246), and/or Cry35Ab(1) and Cry 34Ab(1) as a triple stack with *dgt-28, dgt-31, dgt-32, and/or dgt-33*.

Benefits include the improved weed control offered by *dgt-28, dgt-31, dgt-32, or dgt-33*, and described in previous examples, linked with the ability to manage insect
20 pests and/or other agronomic stresses. Plants comprising such traits stacked with *dgt-28, dgt-31, dgt-32, and/or dgt-33* provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic issues. Combined IR and HT traits have application in most
25 agronomic and horticultural/ornamental crops and forestry.

The combination of *dgt-28, dgt-31, dgt-32, or dgt-33*, and the commensurate herbicide tolerance and insect resistance afforded by any of the number of Bt or non-Bt IR genes can be applied to the crop species listed herein. Use of any of various commercial herbicides listed herein in such crops is made possible by *dgt-28, dgt-31, dgt-32, or dgt-33* transformation and stacking with the corresponding HT trait or IR
30 trait, either by conventional breeding or genetic engineering. Specific application rates of herbicides representative of these chemistries are 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), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005).

Example 16: DGT Trait Stacked With an AAD Trait in Any Crop

5 By stacking a *dgt* trait with an *aad* trait (e.g., *aad-1* described in US Patent 7,838,733; or *aad-12* described in PCT International Patent Publication No. WO 2007/053482 A2), either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and the ability to manage weed shifts and herbicide resistance development are improved.

10 Transforming crops with *aad-1* allows a grower to selectively apply aryloxyalkanoate herbicides in monocot crops. Such monocot crops will have a higher margin of phenoxy auxin safety. In addition, phenoxy auxins can be selectively applied in dicot crops transformed with *aad-1*. Transforming crops with *aad-12* allows a grower to selectively apply pyridyloxy auxin and aryloxyalkanoate herbicides in
15 dicot crops to control weed species. By stacking *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* with the *aad-1* or *aad-12* traits, growers are provided a broader spectrum of herbicides for the management of weeds. Moreover, the use of herbicide combinations results in more flexibility for managing herbicide resistance within weed species.

The following weed control options are provided for a plant wherein a *dgt* trait
20 and an *aad* trait are stacked in any monocot or dicot crop species:

A. Glyphosate is applied at a standard postemergent application rate (420 to 2160 g ae/ha, for example, 560 to 1120 g ae/ha) for the control of most grass and broadleaf weed species. The *dgt* traits can provide tolerance at these application rates of glyphosate. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (e.g., *Commelina*
25 spp), 280-2240 g ae/ha (for example, 560-1120 g ae/ha) of 2,4-D is applied sequentially, tank mixed, or as a premix with glyphosate to provide additional control. Both *aad-1* and *aad-12* provide tolerance to 2,4-D. In addition, *aad-12* provides tolerance to pyridyloxy auxin herbicides such as triclopyr and fluroxypyr. The
30 pyridyloxy auxin herbicides are applied to control glyphosate resistant broadleaf weeds like *Conyza canadensis* and *Commelina* spp. For triclopyr, application rates typically range from 70-1120 g ae/ha, for example, 140-420 g ae/ha. For fluroxypyr, application rates typically range from 35-560 g ae/ha, for example, 70-280 ae/ha.

B. Glyphosate is applied at a standard postemergent application rate (420 to 2160 g ae/ha, for example, 560 to 1120 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica*, 10-200 g ae/ha (for example, 20-100 g ae/ha) quizalofop is applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control. *Aad-1* provides tolerance to quizalofop. Stacking *aad-1* in combination with *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* in crop species results in crops that are tolerant to the herbicides described above.

C. Glyphosate is efficacious in controlling grass species other than broadleaf weed species. *Aad-1* and *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* stacked traits allow for the application of grass-effective rates of glyphosate (105-840 g ae/ha, for example, 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, for example, 560-1120 g ae/ha) is then applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (for example, 20-100 g ae/ha and 20-35 g ae/ha), is used for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate also provides some benefit to the broadleaf weed control; however, primary control is from the 2,4-D.

D. Likewise, *aad-12* and *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* stacked traits allow for the application of grass-effective rates of glyphosate (105-840 g ae/ha, for example, 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, for example, 560-1120 g ae/ha) is then applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. Triclopyr and fluroxypyr used at rates mentioned above also are acceptable components in the treatment regimen. The low rate of glyphosate also provides some benefit to the broadleaf weed control; however, primary control is from the 2,4-D, triclopyr, or fluroxypyr.

Use of one or more commercial aryloxy auxin herbicides alone or in combination (sequentially or independently) is facilitated by *aad-12* transformation into crops. Likewise the use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicides is facilitated by *aad-1*. Stacking either of these traits with *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* allows for more robust management of weed species. The specific rates of other herbicides representative of these chemistries are 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), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005).

5

Example 17: DGT Trait Stacked with *AHAS* Trait in Any Crop

Traits encoding imidazolinone herbicide tolerance (*AHAS*) are currently present in a number of crops planted in North America including, but not limited to, corn, rice, sunflower, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar
10 beet) have been under development. 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 facilitated through imidazolinone tolerance traits like *AHAS*. Imidazolinone tolerant HTC's to date have the advantage of being non-transgenic. This
15 chemistry class also has significant soil residual activity, thus being able to provide weed control that extends 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
20 acetolactate synthase, ALS) to which many weeds have developed resistance (Heap I (2004). The international survey of herbicide resistant weeds, available at www.weedscience.com).

Dgt-28, *dgt-31*, *dgt-32*, or *dgt-33* is stacked with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event,
25 and weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development are improved.

The following weed control options are provided for a plant wherein a *dgt* trait and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

A. Imazethapyr is applied at a standard postemergent application rate (35 to
30 280 g ae/ha, for example, 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)

are controlled by tank mixing glyphosate at 420 to 2160 g ae/ha, for example, 560 to 1120 g ae/ha.

- ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea* spp. are controlled by tank mixing glyphosate at 420 to 2160 g ae/ha, for example, 560 to 1120 g ae/ha.
- iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. are controlled by tank mixing glyphosate at 420 to 2160 g ae/ha, for example, 560 to 1120 g ae/ha.
- iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) are controlled by tank mixing glyphosate at 420 to 2160 g ae/ha, for example, 560 to 1120 g ae/ha.

Use of any of various commercial imidazolinone herbicides or glyphosate herbicide, alone or in multiple combinations, is facilitated by *dgt-28* transformation with *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33*, and stacking with any imidazolinone tolerance trait, either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries are 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), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005).

Example 18: Soybean Transformation

Transgenic soybean (*Glycine max*) containing a stably integrated *dgt-28* transgene was generated through *Agrobacterium*-mediated transformation of soybean cotyledonary node explants. A disarmed *Agrobacterium* strain carrying a binary vector containing a functional *dgt-28* was used to initiate transformation.

Agrobacterium-mediated transformation was carried out using a modified half-cotyledonary node procedure of Zeng *et al.* (Zeng P., Vadnais D.A., Zhang Z., Polacco J.C., (2004), *Plant Cell Rep.*, 22(7): 478-482). Briefly, soybean seeds (cv. Maverick) were germinated on basal media and cotyledonary nodes are isolated and infected with *Agrobacterium*. Shoot initiation, shoot elongation, and rooting media are supplemented with cefotaxime, timentin and vancomycin for removal of *Agrobacterium*. Selection via a herbicide was employed to inhibit the growth of

non-transformed shoots. Selected shoots were transferred to rooting medium for root development and then transferred to soil mix for acclimatization of plantlets.

Terminal leaflets of selected plantlets were treated topically (leaf paint technique) with a herbicide to screen for putative transformants. The screened
5 plantlets were transferred to the greenhouse, allowed to acclimate and then leaf-painted with a herbicide to reconfirm tolerance. These putative transformed T_0 plants were sampled and molecular analyses were used to confirm the presence of the herbicidal selectable marker, and the *dgt-28* transgene. T_0 plants were allowed to self fertilize in the greenhouse to produce T_1 seed.

10 A second soybean transformation method can be used to produce additional transgenic soybean plants. A disarmed *Agrobacterium* strain carrying a binary vector containing a functional *dgt-28* is used to initiate transformation.

Agrobacterium-mediated transformation was carried out using a modified half-seed procedure of Paz *et al.*, (Paz M., Martinez J., Kalvig A., Fonger T., and
15 Wang K., (2005) *Plant Cell Rep.*, 25: 206-213). Briefly, mature soybean seeds were sterilized overnight with chlorine gas and imbibed with sterile H_2O twenty hours before *Agrobacterium*-mediated plant transformation. Seeds were cut in half by a longitudinal cut along the hilum to separate the seed and remove the seed coat. The embryonic axis was excised and any axial shoots/buds were removed from the
20 cotyledonary node. The resulting half seed explants were infected with *Agrobacterium*. Shoot initiation, shoot elongation, and rooting media were supplemented with cefotaxime, timentin and vancomycin for removal of *Agrobacterium*. Herbicidal selection was employed to inhibit the growth of non-transformed shoots. Selected shoots were transferred to rooting medium for
25 root development and then transferred to soil mix for acclimatization of plantlets.

Putative transformed T_0 plants were sampled and molecular analyses was used to confirm the presence of the selectable marker and the *dgt-28* transgene. Several events were identified as containing the transgenes. These T_0 plants were advanced for further analysis and allowed to self fertilize in the greenhouse to give
30 rise to T_1 seed.

Confirmation of heritability of *dgt-28* to the T_1 generation. Heritability of the DGT-28 protein into T_1 generation was assessed in one of two ways. The first method included planting T_1 seed into Metro-mix media and applying 411 g ae/ha

IGNITE™ 280 SL on germinated plants at the 1st trifoliolate growth stage. The second method consisted of homogenizing seed for a total of 8 replicates using a ball bearing and a genogrinder. ELISA strip tests to detect for the PAT protein were then used to detect heritable events as the selectable marker was on the same plasmid as *dgt-28*. For either method if a single plant was tolerant to glufosinate or was detected with the PAT ELISA strip test, the event demonstrated heritability to the T₁ generation.

A total of five constructs were screened for heritability as previously described. The plasmids contained *dgt-28* linked with TraP4, TraP8 and TraP23. The events across constructs demonstrated 68% heritability of the PAT::DGT-28 protein to the T₁ generation.

Postemergence herbicide tolerance in *dgt-28* transformed T₁ soybean. Seeds from T₁ events that were determined to be heritable by the previously described screening methods were planted in Metro-mix media under greenhouse conditions. Plants were grown until the 1st trifoliolate was fully expanded and treated with 411 g ae/ha IGNITE™ 280 SL for selection of the *pat* gene as previously described. Resistant plants from each event were given unique identifiers and sampled for zygosity analyses of the *dgt-28* gene. Zygosity data were used to assign 2 hemizygous and 2 homozygous replicates to each rate of glyphosate applied allowing for a total of 4 replicates per treatment when enough plants existed. These plants were compared against wildtype Petite havana tobacco. All plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed from a range of 560-4480 g ae/ha DURANGO™ dimethylamine salt (DMA). All applications were formulated in water with the addition of 2% w/v ammonium sulfate (AMS). Plants were evaluated at 7 and 14 days after treatment. Plants were assigned an injury rating with respect to overall visual stunting, chlorosis, and necrosis. The T₁ generation is segregating, so some variable response is expected due to difference in zygosity.

Table 24. Spray results demonstrate at 14 DAT (days after treatment) robust tolerance up to 4480 g ae/ha glyphosate of at least one *dgt-28* event per construct characterized. Representative single copy events of the constructs all provided tolerance up to 4480 g ae/ha compared to the Maverick negative control.

pDAB107543 (TraP4::dgt-28)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	0	4	0	33.8	7.5	25-40
1120 g ae/ha	2	2	0	25.0	11.5	15-35
2240 g ae/ha	2	2	0	17.5	2.9	15-20
4480 g ae/ha	0	2	2	33.8	13.1	20-45
pDAB107545 (TraP8::dgt-28)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	1.5	1.0	0-2
1120 g ae/ha	4	0	0	2.8	1.5	2-5
2240 g ae/ha	4	0	0	5.0	2.4	2-8
4480 g ae/ha	4	0	0	9.5	1.9	8-12
pDAB107548 (TraP4::dgt-28)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	1.8	2.4	0-5
1120 g ae/ha	4	0	0	2.8	1.5	2-5
2240 g ae/ha	4	0	0	3.5	1.7	2-5
4480 g ae/ha	4	0	0	8.8	3.0	5-12
pDAB107553 (TraP23::dgt-28)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	5.0	0.0	5
1120 g ae/ha	4	0	0	9.0	1.2	8-10
2240 g ae/ha	4	0	0	10.5	1.0	10-12
4480 g ae/ha	4	0	0	16.5	1.7	15-18

Maverick (neg. control)	% Injury			% Injury		
	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
Application Rate						
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	0	0	4	82.5	12.6	70-100
1120 g ae/ha	0	0	4	100.0	0.0	100
2240 g ae/ha	0	0	4	100.0	0.0	100
4480 g ae/ha	0	0	4	100.0	0.0	100

dgt-28 protection against elevated glyphosate rates in the T₂ generation. A 45 plant progeny test was conducted on two to five T₂ lines of *dgt-28* per construct. Homozygous lines were chosen based on zygosity analyses completed in the previous generation. The seeds were planted as previously described. Plants were then sprayed with 411 g ae/ha IGNITE 280 SL for the selection of the *pat* selectable marker as previously described. After 3 DAT, resistant and sensitive plants were counted.

For constructs containing TraP4 linked with *dgt-28* (pDAB107543 and pDAB107548), nine out of twelve lines tested did not segregate, thereby confirming homogeneous lines in the T₂ generation. Lines containing TraP8 linked with *dgt-28* (pDAB107545) demonstrated two out of the four lines with no segregants and demonstrating Mendelian inheritance through at least two generation of *dgt-28* in soybean. Tissue samples were taken from resistant plants and the DGT-28 protein was quantified by standard ELISA methods. Data demonstrated a range of mean DGT-28 protein from 32.8 – 107.5 ng/cm² for non-segregating T₂ lines tested. Lines from the construct pDAB107553 (TraP23::*dgt-28*) were not previously selected with glufosinate, and the dose response of glyphosate was utilized as both to test homogeneity and tolerance to elevated rates of glyphosate. Replicates from the lines from construct pDAB107553 were tolerant to rates ranging from 560-4480 g ae/ha glyphosate, and were therefore confirmed to be a homogeneous population and heritable to at least two generations.

Rates of DURANGO DMA ranging from 560-4480 g ae/ha glyphosate were applied to 2-3 trifoliolate soybean as previously described. Visual injury data 14 DAT confirmed the tolerance results that were demonstrated in the T₁ generation.

Table 25. The data demonstrate robust tolerance of the *dgt-28* tobacco up to 3360 g ae/ha glyphosate through two generations, compared to the non-transformed control.

pDAB107543 (TraP4::<i>dgt-28</i>)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	8.0	0.0	8
1120 g ae/ha	4	0	0	14.3	1.5	12-15
2240 g ae/ha	4	0	0	18.0	0.0	18
4480 g ae/ha	0	4	0	24.5	3.3	20-28
pDAB107545 (TraP8::<i>dgt-28</i>)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	2.8	1.5	2-5
2240 g ae/ha	4	0	0	5.0	0.0	5
4480 g ae/ha	4	0	0	10.0	0.0	10
pDAB107548 (TraP4::<i>dgt-28</i>)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	0.0	0.0	0
4480 g ae/ha	4	0	0	10.0	0.0	10

pDAB107553 (TraP23::dgt-28)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	-	0.0	0.0
560 g ae/ha	4	0	0	-	10.0	0.0
1120 g ae/ha	4	0	0	-	10.0	4.4
2240 g ae/ha	4	0	0	-	13.0	2.4
4480 g ae/ha	3	1	0	-	15.5	4.1
Maverick (neg. control)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	0	0	4	77.5	15.0	70-100
1120 g ae/ha	0	0	4	97.5	2.9	95-100
2240 g ae/ha	0	0	4	100.0	0.0	100
4480 g ae/ha	0	0	4	100.0	0.0	100

Example 19: Transformation of Rice with *dgt-28*

In an exemplary method, transgenic rice (*Oryza sativa*) containing a stably integrated *dgt-28* transgene is generated through *Agrobacterium*-mediated transformation of sterilized rice seed. A disarmed *Agrobacterium* strain carrying a binary vector containing a functional *dgt-28* is used to initiate transformation.

Culture media are adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytigel (Sigma-Aldrich, St. Louis, MO). Embryogenic calli are cultured in 100 x 20 mm petri dishes containing 30 ml semi-solid medium. Rice plantlets are grown on 50 ml medium in MAGENTA boxes. Cell suspensions are maintained in 125 ml conical flasks containing 35 mL liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures occur in the dark at 25–26°C, and plant regeneration and whole-plant culture occur in illuminated room with a 16-h photoperiod (Zhang *et al.* 1996).

Induction and maintenance of embryogenic callus is performed on a modified NB basal medium as described previously (Li *et al.* 1993), wherein the media is adapted to contain 500 mg/L glutamine. Suspension cultures are initiated

and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/L sucrose in place of maltose. Osmotic medium (NBO) consisting of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Herbicide resistant callus is selected on NB medium supplemented with the appropriate herbicide selective agent for 3–4 weeks. Pre-regeneration is performed on medium (PRH50) consisting of NB medium with 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/l α -naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and selective herbicide for 1 week. Regeneration of plantlets follow the culturing on regeneration medium (RNH50) comprising NB medium containing 2,4-D, 0.5 mg/l NAA, and selective herbicide until putatively transgenic shoots are regenerated. Shoots are transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and selective herbicide.

Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 are sterilized as described in Zhang *et al.* 1996. Embryogenic tissues are induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, is removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions are then maintained as described in Zhang 1996. Suspension-derived embryogenic tissues are 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 twenty hours after bombardment, tissues are transferred from NBO medium onto NBH50 selection medium, ensuring that the bombarded surface is facing upward, and incubated in the dark for 14–17 days. Newly formed callus is then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8–12 days, relatively compact, opaque callus is visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which become more compact and opaque is then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod. Regenerating shoots are transferred to MAGENTA boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and are treated as one independent plant line. A plant is scored as positive for the *dgt-28* gene if it produces thick, white roots and grows vigorously on ½ MSH50 medium. Once plantlets reach the top of the MAGENTA

boxes, they are transferred to soil in a 6-cm pot under 100% humidity for a week, and then are moved to a growth chamber with a 14-h light period at 30°C and in the dark at 21°C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds are collected and dried at 37°C for one week prior to storage at 4°C.

5 T₀ analysis of dgt-28 rice. Transplanted rice transformants obtained via *Agrobacterium* transformation were transplanted into media and acclimated to greenhouse conditions. All plants were sampled for PCR detection of *dgt-28* and results demonstrate twenty-two PCR positive events for pDAB110827 (TraP8::*dgt-28*) and a minimum of sixteen PCR positive events for pDAB110828
10 (TraP23::*dgt-28*). Southern analysis for *dgt-28* of the PCR positive events demonstrated simple (1-2 copy) events for both constructs. Protein expression of selected T₀ events demonstrated DGT-28 protein expression ranges from below levels of detection to 130 ng/cm². Selected T₀ events from construct pDAB110828 were treated with 2240 g ae/ha DURANGO DMA™ as previously described and
15 assessed 7 and 14 days after treatment. Data demonstrated robust tolerance to the rate of glyphosate applied. All PCR positive plants were allowed to produced T₁ seed for further characterization.

Dgt-28 heritability in rice. A 100 plant progeny test was conducted on four T₁ lines of *dgt-28* from construct pDAB110827 containing the chloroplast transit
20 peptide TraP8. The seeds were planted into pots filled with media. All plants were then sprayed with 560 g ae/ha DURANGO DMA™ for the selection of the *dgt-28* gene as previously described. After 7 DAT, resistant and sensitive plants were counted. Two out of the four lines tested for each construct segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis.
25 *Dgt-28* is a heritable glyphosate resistance gene in multiple species.

Postemergence herbicide tolerance in *dgt-28* transformed T₁ rice. T₁ resistant plants from each event used in the progeny testing were given unique identifiers and sampled for zygosity analyses of the *dgt-28* gene. Zygosity data were used to assign 2 hemizygous and 2 homozygous replicates to each rate of glyphosate
30 applied allowing for a total of 4 replicates per treatment. These plants were compared against wildtype kitaake rice. All plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed from a range of 560-2240 g ae/ha DURANGO DMA™. All applications were formulated in water with the addition

of 2% w/v ammonium sulfate (AMS). Plants were evaluated at 7 and 14 days after treatment. Plants were assigned an injury rating with respect to overall visual stunting, chlorosis, and necrosis. The T₁ generation is segregating, so some variable response is expected due to difference in zygoty.

- 5 Spray results demonstrate at 7 DAT (days after treatment) minimal vegetative injury to elevated rates of glyphosate were detected (data not shown).

Table 26. Visual injury data at 14 DAT demonstrates less than 15% mean visual injury up to 2240 g ae/ha glyphosate.

TraP8::dgt-28 Event 1	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	0.0	0.0	0
1120 g ae/ha	4	0	0	0.0	0.0	0
2240 g ae/ha	4	0	0	0.0	0.0	0
TraP8::dgt-28 Event 2	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	3.8	4.8	0-10
1120 g ae/ha	4	0	0	12.0	3.6	8-15
2240 g ae/ha	4	0	0	15.0	6.0	8-20
Non-transformed control	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	0	0	4	81.3	2.5	80-85
1120 g ae/ha	0	0	4	95.0	5.8	90-100
2240 g ae/ha	0	0	4	96.3	4.8	90-100

- 10 Protein detection of DGT-28 was assessed for replicates from all four T₁ lines tested from pDAB110827. Data demonstrated DGT-28 mean protein ranges from 20-82 ng/cm² and 21-209 ng/cm² for hemizygous and homozygous replicates

respectively. These results demonstrated stable protein expression to the T₁ generation and tolerance of *dgt-28* rice up to 2240 g ae/ha glyphosate following an application of 560 g ae/ha glyphosate used for selection.

Example 20: Transformation of Turf Grass with *dgt-28*

5 *Agrobacterium tumefaciens*-mediated genetic transformation of the *dgt-28* transgene in creeping bentgrass is achieved through embryogenic callus initiated from seeds (cv. Penn-A-4). See “Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation” (Luo *et. al.*, 2004).

10 Callus cells are infected with an *A. tumefaciens* strain harboring a super-binary vector that contains an herbicide-resistant transgene (e.g., *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33*) driven by a monocot specific promoter. The overall stable transformation efficiency ranges from 18% to 45%. Southern blot and genetic analysis confirm transgene integration within the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T₁ generation. All
15 independent transformation events carry one to three copies of the transgene, and a majority (60–65%) contain only a single copy of the transgene with no apparent rearrangements.

20 Mature seeds are dehusked with sand paper and surface sterilized in 10% (v/v) Clorox™ bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds are placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-*o*-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytigel. The pH of the medium is adjusted to 5.7 before autoclaving at 120°C for
25 20 min).

The culture plates containing prepared seed explants are kept in the dark at room temperature for 6 weeks. Embryogenic calli are visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

30 One day before *Agrobacterium* mediated-infection, the embryogenic callus is divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 μM acetosyringone. A 10-μl aliquot of *Agrobacterium* suspension (OD=1.0 at 660 nm) which harbors the *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* transgene is then

applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25°C. The callus is then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

5 Selection of transgenic plants occurs when the callus is moved to callus-induction medium containing 250 mg/l cefotaxime and a herbicide. The callus material is maintained on this medium for 8 weeks with a selection subculture interval of 3 weeks. The selection process is performed at room temperature in the dark.

10 For plant regeneration, the herbicide-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytigel) supplemented with cefotaxime, and a herbicide for selection. These calli are kept in the dark at room temperature for 1 week and then moved into the light for 2–3 weeks to develop shoots.

15 Developed shoots are separated and transferred to hormone-free regeneration medium containing a herbicide and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3–5 weeks) are then transferred to soil and grown either in the greenhouse or in the field.

20 Transgenic plants are maintained out of doors in a containment nursery (3-6 months) until the winter solstice in December. The vernalized plants are then transferred to the greenhouse and kept at 25°C under a 16/8 h photoperiod and surrounded by non-transgenic control plants that physically isolate the transgenic plants from other pollen sources. The transgenic plants begin flowering 3-4 weeks
25 after being moved back into the greenhouse. These plants are out-crossed with the pollen from the surrounding control plants. The seeds collected from each individual transgenic plant are germinated in soil at 25°C, and T₁ plants are grown in the greenhouse for further analysis.

30 Other grasses are transformed with *dgt-28* according to the described protocol, including Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Brachiaria, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra commutate*), Crabgrass, Creeping bent (*Agrostis*

stolonifera), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/sheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*),
5 Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra rubra*), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysiagrass.

10

Example 21: Transformation of *Brassica* spp. with DGT Trait

A *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* gene conferring resistance to glyphosate is used to transform *Brassica napus* var. Nexera™ 710 with *Agrobacterium*-mediated transformation.

15 *Brassica napus* seeds are surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds are then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25 °C, and a photoperiod of 16 hrs light/8 hrs dark.

20 Hypocotyl segments (3-5 mm) are excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments are then transferred into a petri plate and treated with an *Agrobacterium tumefaciens* strain containing a construct comprising *dgt-28*. The *Agrobacterium tumefaciens* is grown overnight at
25 28 °C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

After a 30 min treatment of the hypocotyl segments with *Agrobacterium*, these segments are placed back on the callus induction medium for 3 days. Following co-cultivation, the segments are placed in K1D1TC (callus induction
30 medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week of recovery. Alternately, the segments are placed directly on selection medium K1D1H1 (above medium with a herbicide). Carbenicillin and Timentin are the

antibiotics used to kill the *Agrobacterium*. The selection agent allows for the growth of the transformed cells.

Callus samples from isolated independent events are tested by PCR. Samples that test positive for the presence of *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* are confirmed and advanced to media for regeneration. The callused hypocotyl segments are then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, selective herbicide, Carbenicillin and Timentin) shoot regeneration medium. After 3 weeks shoots begin regeneration. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, selective herbicide, Carbenicillin and Timentin) for another 3 weeks.

Shoots are excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, selective herbicide, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants establish a root system, the plants are transplanted into soil. The plants are acclimated under controlled environmental conditions in a Conviron™ for 1-2 weeks before transfer to the greenhouse.

The transformed T₀ plants are self-pollinated in the greenhouse to obtain T₁ seed. The T₀ plants and T₁ progeny are sprayed with a range of glyphosate herbicide concentrations to establish the level of protection by the *dgt-28*, *dgt-31*, *dgt-32*, or *dgt-33* gene.

Example 22: Transformation of Tobacco with *dgt-28*

Tobacco (cv. Petit Havana) leaf pieces were transformed using *Agrobacterium tumefaciens* containing the *dgt-28* transgene. Single colonies containing the plasmid which contains the *dgt-28* transgene were inoculated into 4 mL of YEP medium containing spectinomycin (50 µg/mL) and streptomycin (125 µg/mL) and incubated overnight at 28°C on a shaker at 190 rpm. The 4 mL seed culture was subsequently used to inoculate a 25 mL culture of the same medium in a 125 mL baffled Erlenmeyer flask. This culture was incubated at 28°C shaking at

190 rpm until it reached an OD₆₀₀ of ~1.2. Ten mL of *Agrobacterium* suspension were then placed into sterile 60 x 20 mm Petri™ dishes.

Freshly cut leaf pieces (0.5 cm²) from plants aseptically grown on MS medium (Phytotechnology Labs, Shawnee Mission, KS,) with 30 g/L sucrose in
5 PhytaTrays™ (Sigma, St. Louis, MO) were soaked in 10 mL of overnight culture of *Agrobacterium* for a few minutes, blotted dry on sterile filter paper and then placed onto the same medium with the addition of 1 mg/L indoleacetic acid and 1 mg/L 6-benzylamino purine. Three days later, leaf pieces co-cultivated with *Agrobacterium* harboring the *dgt-28* transgene were transferred to the same medium
10 with 5 mg/L Basta™ and 250 mg/L cephotaxime.

After 3 weeks, individual T₀ plantlets were transferred to MS medium with 10 mg/L Basta™ and 250 mg/L cephotaxime an additional 3 weeks prior to transplanting to soil and transfer to the greenhouse. Selected T₀ plants (as identified using molecular analysis protocols described above) were allowed to self-pollinate
15 and seed was collected from capsules when they were completely dried down. T₁ seedlings were screened for zygosity and reporter gene expression (as described below) and selected plants containing the *dgt-28* transgene were identified.

Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag
20 (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30°C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (27°C day, 24°C night, 16 hour
25 day, minimum natural + supplemental light = 1200 µE/m²s¹).

Prior to propagation, T₀ plants were sampled for DNA analysis to determine the insert *dgt-28* copy number by real-time PCR. Fresh tissue was placed into tubes and lyophilized at 4 °C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry
30 grinding using a Kelco bead mill. The standard DNeasy™ DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek™) with known standards to obtain the concentration in ng/µl. A total of

100 ng of total DNA was used as template. The PCR reaction was 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
5 were analyzed by electrophoresis on a 1% agarose gel stained with EtBr and confirmed by Southern blots.

Five to nine PCR positive events with 1-3 copies of *dgt-28* gene from 3 constructs containing a different chloroplast transit peptide sequence (TraP4, TraP8 and TraP23) were regenerated and moved to the greenhouse.

10 All PCR positive plants were sampled for quantification of the DGT-28 protein by standard ELISA. DGT-28 protein was detected in all PCR positive plants and a trend for an increase in protein concentration was noted with increasing copy number of *dgt-28*.

aad-12 (v1) heritability in tobacco. A 100 plant progeny test was conducted
15 on five T₁ lines of *dgt-28* per construct. Constructs contained one of the following chloroplast transit peptide sequences: TraP4, TraP8 or TraP23. The seeds were stratified, sown, and transplanted with respect much like that of the *Arabidopsis* procedure exemplified above, with the exception that null plants were not removed by in initial selection prior to transplanting. All plants were then sprayed with 280 g
20 ae/ha IGNITE 280 SL for the selection of the *pat* selectable marker as previously described. After 3 DAT, resistant and sensitive plants were counted.

Four out of the five lines tested for each construct segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. *Dgt-28* is a heritable glyphosate resistance gene in multiple species.

25 Postemergence herbicide tolerance in *dgt-28* transformed T₁ tobacco. T₁ resistant plants from each event used in the progeny testing were given unique identifiers and sampled for zygosity analyses of the *dgt-28* gene. Zygosity data were used to assign 2 hemizygous and 2 homozygous replicates to each rate of glyphosate applied allowing for a total of 4 replicates per treatment. These plants were
30 compared against wildtype Petite havana tobacco. All plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed from a range of 560-4480 g ae/ha DURANGO DMA™. All applications were formulated in water with the addition of 2% w/v ammonium sulfate (AMS). Plants were evaluated at 7 and 14

days after treatment. Plants were assigned an injury rating with respect to overall visual stunting, chlorosis, and necrosis. The T₁ generation is segregating, so some variable response is expected due to difference in zygosity.

Spray results demonstrate at 7 DAT (days after treatment) minimal vegetative injury to elevated rates of glyphosate were detected (data not shown). Following 14 DAT, visual injury data demonstrates increased injury with single copy events of the construct containing TraP4 compared to single copy events from the constructs TraP8 and TraP23. **Table 27.**

Table 27. At a rate of 2240 g ae/ha glyphosate, an average injury of 37.5% was demonstrated with the event containing TraP4, where events containing TraP8 and TraP23 demonstrated an average injury of 9.3% and 9.5% respectively.

TraP4::dgt-28 (pDAB107543)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	2	2	0	18.0	8.1	10-25
1120 g ae/ha	1	3	0	24.5	4.9	18-30
2240 g ae/ha	0	3	1	37.5	6.5	30-45
4480 g ae/ha	0	2	2	42.5	2.9	40-45
TraP8::dgt-28 (pDAB107545)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	3.3	3.9	0-8
1120 g ae/ha	4	0	0	6.5	1.7	5-8
2240 g ae/ha	4	0	0	9.3	3.0	5-12
4480 g ae/ha	2	2	0	17.5	6.5	10-25
TraP23::dgt-28 (pDAB107553)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	4	0	0	10.0	1.6	8-12

1120 g ae/ha	4	0	0	8.8	3.0	5-12
2240 g ae/ha	4	0	0	9.5	4.2	5-15
4480 g ae/ha	4	0	0	15.8	1.5	15-18
<i>Petite havana</i>	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
560 g ae/ha	0	0	4	85.0	4.1	80-90
1120 g ae/ha	0	0	4	91.3	2.5	90-95
2240 g ae/ha	0	0	4	94.5	3.3	90-98
4480 g ae/ha	0	0	4	98.3	2.4	95-100

These results demonstrated tolerance of *dgt-28* up to 4480 g ae/ha glyphosate, as well as differences in tolerance provided by chloroplast transit peptide sequences linked to the *dgt-28* gene.

5 *Dgt-28* protection against elevated glyphosate rates in the T₂ generation. A 25 plant progeny test was conducted on two to three T₂ lines of *dgt-28* per construct. Homozygous lines were chosen based on zygosity analyses completed in the previous generation. The seeds were stratified, sown, and transplanted as previously described. All plants were then sprayed with 280 g ae/ha Ignite 280 SL for the
10 selection of the *pat* selectable marker as previously described. After 3 DAT, resistant and sensitive plants were counted. All lines tested for each construct did not segregate thereby confirming homogeneous lines in the T₂ generation and demonstrating Mendelian inheritance through at least two generation of *dgt-28* in tobacco.

15 Rates of DURANGO DMA™ ranging from 420-3360 g ae/ha glyphosate were applied to 2-3 leaf tobacco as previously described. Visual injury data 14 DAT confirmed the tolerance results that were demonstrated in the T₁ generation. Foliar results from a two copy lines from the construct containing TraP4 demonstrated similar tolerance to that of single copy TraP8 and TraP23 lines (data not shown).

20 **Table 28.** Single copy lines from the construct containing TraP4 with *dgt-28* demonstrated increased injury compared to lines from constructs containing TraP8 and TraP23 with *dgt-28*.

TraP4::dgt-28 (pDAB107543)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha	0	4	0	23.8	4.8	20-30
840 g ae/ha	0	4	0	30.0	4.1	25-35
1680 g ae/ha	0	4	0	35.0	5.8	30-40
3360 g ae/ha	0	4	0	31.3	2.5	30-35
TraP8::dgt-28 (pDAB107545)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha	4	0	0	0.0	0.0	0
840 g ae/ha	4	0	0	2.5	2.9	0-5
1680 g ae/ha	4	0	0	9.3	3.4	5-12
3360 g ae/ha	4	0	0	10.5	1.0	10-12
TraP23::dgt-28 (pDAB107553)	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha	4	0	0	0.0	0.0	0
840 g ae/ha	4	0	0	6.3	2.5	5-10
1680 g ae/ha	4	0	0	10.0	0.0	10
3360 g ae/ha	3	1	0	13.8	4.8	10-20
Petite havana	% Injury			% Injury		
Application Rate	<20%	20-40%	>40%	Ave	Std. Dev.	Range (%)
0 g ae/ha glyphosate	4	0	0	0.0	0.0	0
420 g ae/ha	0	0	4	95.0	0.0	95
840 g ae/ha	0	0	4	98.8	1.0	98-100
1680 g ae/ha	0	0	4	99.5	1.0	98-100
3360 g ae/ha	0	0	4	100	0.0	100

The data demonstrate robust tolerance of *dgt-28* tobacco up to 3360 g ae/ha glyphosate through two generations compared to the non-transformed control.

Selected plants from each event were sampled prior to glyphosate applications for analyses of the DGT-28 protein by standard DGT-28 ELISA. Data demonstrated DGT-28 mean protein expression of the simple (1-2 copy) lines across constructs ranging from 72.8-114.5 ng/cm². Data demonstrates *dgt-28* is expressing protein in the T₂ generation of transformed tobacco and tolerance data confirms functional DGT-28 protein.

Stacking of *dgt-28* to increase herbicide spectrum in tobacco (cv. Petit Havana). Homozygous *dgt-28* (pDAB107543 and pDAB107545) and *aad-12 v1* (pDAB3278) plants (*see* PCT/US2006/042133 for the latter) were both reciprocally crossed and F₁ seed was collected. The F₁ seed from two reciprocal crosses of each gene were stratified and treated 6 reps of each cross were treated with 1120 g ae/ha glyphosate (selective for the *dgt-28* gene), 1120 g ae/ha 2,4-D (selective for the *aad-12* gene), or a tank mixture of the two herbicides at the rates described. Plants were graded at 14 DAT. Spray results are shown in **Table 29**.

Table 29. Response of F₁ *aad-12* and *dgt-28*

	<i>aad-12</i> TraP4:: <i>dgt-28</i>	x	<i>aad-12</i> TraP8:: <i>dgt-28</i>	x	<i>Petite</i> <i>havana</i>
Application Rate	Tolerance				
1120 g ae/ha 2,4-D	++++		++++		-
1120 g ae/ha glyphosate	++		++		-
1120 g ae/ha 2,4-D + 1120 g ae/ha glyphosate	++		++		-

The results confirm that *dgt-28* can be successfully stacked with *aad-12 (v1)*, thus increasing the spectrum herbicides that may be applied to the crop of interest (glyphosate + phenoxyactetic acids for *dgt-28* and *aad-12*, respectively). In crop production where hard to control broadleaf weeds or resistant weed biotypes exist the stack can be used as a means of weed control and protection of the crop of interest. Additional input or output traits could also be stacked with the *dgt-28* gene.

Example 23: Resistance to Glyphosate in Wheat

Production of binary vectors encoding DGT-28. Binary vectors containing DGT-28 expression and PAT selection cassettes were designed and assembled using skills and techniques commonly known in the art. Each DGT-28 expression cassette
5 contained the promoter, 5' untranslated region and intron from the Ubiquitin (*Ubi*) gene from *Zea mays* (Toki *et al.* Plant Physiology 1992, 100 1503-07), followed by a coding sequence consisting of one of four transit peptides (TraP4, TraP8, TraP23 or TraP5) fused to the 5' end of a synthetic version of the 5-enolpyruvylshikimate-3-phosphate synthase gene (DGT-28), which had been
10 codon optimized for expression in plants. The DGT-28 expression cassette terminated with a 3' untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of a lipase gene (*Vpl*) from *Z. mays* (Paek *et al.* Mol Cells 1998 30;8(3) 336-42). The PAT selection cassette comprised of the promoter, 5' untranslated region and intron from the Actin (*Act1*) gene from *Oryza*
15 *sativa* (McElroy *et al.* The Plant Cell 1990 2(2) 163-171), followed by a synthetic version of the phosphinothricin acetyl transferase (PAT) gene isolated from *Streptomyces viridochromogenes*, which had been codon optimized for expression in plants. The PAT gene encodes a protein that confers resistance to inhibitors of glutamine synthetase comprising phosphinothricin, glufosinate, and bialaphos
20 (Wohlleben *et al.* Gene 1988, 70(1), 25-37). The selection cassette was terminated with the 3' UTR comprising the transcriptional terminator and polyadenylation sites from the 35s gene of cauliflower mosaic virus (CaMV) (Chenault *et al.* Plant Physiology 1993 101 (4), 1395-1396).

The selection cassette was synthesized by a commercial gene synthesis
25 vendor (GeneArt, Life Technologies) and cloned into a Gateway-enabled binary vector. The DGT-28 expression cassettes were sub-cloned into pDONR221. The resulting ENTRY clone was used in a LR Clonase II (Invitrogen, Life Technologies) reaction with the Gateway-enabled binary vector encoding the phosphinothricin acetyl transferase (PAT) expression cassette. Colonies of all assembled plasmids
30 were initially screened by restriction digestion of purified DNA using restriction endonucleases obtained from New England BioLabs (NEB; Ipswich, MA) and Promega (Promega Corporation, WI). Plasmid DNA preparations were performed using the QIAprep *Spin Miniprep* Kit (Qiagen, Hilden) or the Pure Yield Plasmid

Maxiprep System (Promega Corporation, WI), following the instructions of the suppliers. Plasmid DNA of selected clones was sequenced using ABI Sanger Sequencing and Big Dye Terminator v3.1 cycle sequencing protocol (Applied Biosystems, Life Technologies). Sequence data were assembled and analyzed using
5 the SEQUENCHER™ software (Gene Codes Corporation, Ann Arbor, MI).

The resulting four binary expression clones: pDAS000122 (TraP4-DGT28), pDAS000123 (TraP8-DGT28), pDAS000124 (TraP23-DGT28) and pDAS000125 (TraP5-DGT28) were each transformed into *Agrobacterium tumefaciens* strain EHA105.

10 Production of transgenic wheat events with *dgt-28* expression construct.

Transgenic wheat plants expressing one of the four DGT-28 expression constructs were generated by *Agrobacterium*-mediated transformation using the donor wheat line Bobwhite MPB26RH, following a protocol similar to Wu *et al.* Transgenic Research 2008, 17:425-436. Putative T₀ transgenic events were selected for
15 phosphinothricin (PPT) tolerance, the phenotype conferred by the PAT selectable marker, and transferred to soil. The T₀ plants were grown under glasshouse containment conditions and T₁ seed was produced. Overall, about 45 independent T₀ events were generated for each DGT-28 expression construct.

Glyphosate resistance in T₀ wheat *dgt-28* wheat events. T₀ events were
20 allowed to acclimate in the greenhouse and were grown until 2-4 new, normal looking leaves had emerged from the whorl (*i.e.*, plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 25°C under 12 hour of supplemental lighting in the greenhouse until maturity. An initial screen of glyphosate tolerance and Taqman analyses was completed on T₁ plants grown under
25 the same conditions as previously described. Data allowed for determination of heritable T₁ events to be further characterized. Six low copy (1-2 copy) and two multi-copy T₁ events were replanted under greenhouse conditions and grown until the 3 leaf stage. T₁ plants were sprayed with a commercial formulation of glyphosate (Durango DMA™) from a range of 420 – 3360 g ae/ha, which are
30 capable of significant injury to untransformed wheat lines. The addition of 2% w/v ammonium sulfate was included in the application. A lethal dose is defined as the rate that causes >75% injury to the Bob White MPB26RH non-transformed control. Herbicide was applied.

In this example, the glyphosate applications were utilized for both determining the segregation of the *dgt-28* gene in the T₁ generation as well as demonstrating tolerance to increasing levels of glyphosate. The response of the plants is presented in terms of a scale of visual injury 21 days after treatment (DAT).

5 Data are presented as a histogram of individuals exhibiting less than 25% visual injury (4), 25%-50% visual injury (3), 50%-75% visual injury (2) and greater than 75% injury (1). An arithmetic mean and standard deviation is presented for each construct used for wheat transformation. The scoring range of individual response is also indicated in the last column for each rate and transformation. Wild-type,

10 non-transformed wheat (c.v. Bob White MPB26RH) served as a glyphosate sensitive control. In the T₁ generation hemizygous and homozygous plants were available for testing for each event and therefore were included for each rate of glyphosate tested. Hemizygous plants will contain half of the dose of the gene as homozygous plants, therefore variability of response to glyphosate may be expected in the T₁ generation.

15 The results of the T₁ *dgt-28* wheat plants demonstrated that tolerance to glyphosate was achieved at rates up to 3360 g ae/ha with the chloroplast transit peptides TraP4, TraP5, TraP8 and TraP23. **Table 30.** Data are of a low copy T₁ event but are representative of the population for each construct.

Table 30. Response of low copy T₁ *dgt-28* wheat events to glyphosate 21 days after treatment.

TraP4:: <i>dgt-28</i>	% Injury				% Injury		
	<25%	25-50%	50-75%	>75%	Ave	Std. Dev.	Range (%)
Application Rate							
420 g ae/ha	5	0	0	0	4.00	0.00	4
840 g ae/ha	6	2	0	0	3.75	0.46	3-4
1680 g ae/ha	4	2	0	0	3.67	0.52	3-4
3360 g ae/ha	4	2	0	0	3.67	0.52	3-4

TraP8::<i>dgt-28</i>	% Injury				% Injury		
Application Rate	<25%	25-50%	50-75%	>75%	Ave	Std. Dev.	Range (%)
420 g ae/ha	5	3	0	0	3.63	0.52	3-4
840 g ae/ha	3	5	0	0	3.38	0.52	3-4
1680 g ae/ha	4	3	0	0	3.57	0.53	3-4
3360 g ae/ha	5	5	0	0	3.50	0.53	3-4
TraP23::<i>dgt-28</i>	% Injury				% Injury		
Application Rate	<25%	25-50%	50-75%	>75%	Ave	Std. Dev.	Range (%)
420 g ae/ha	9	2	0	0	3.82	0.40	3-4
840 g ae/ha	8	1	0	0	3.89	0.33	3-4
1680 g ae/ha	7	5	0	0	3.58	0.0	3-4
3360 g ae/ha	8	2	0	0	3.80	4.8	3-4
TraP5::<i>dgt-28</i>	% Injury				% Injury		
Application Rate	<25%	25-50%	50-75%	>75%	Ave	Std. Dev.	Range (%)
420 g ae/ha	5	2	0	0	3.71	0.49	3-4
840 g ae/ha	4	2	0	0	3.67	0.52	3-4
1680 g ae/ha	7	3	0	0	3.70	0.48	3-4
3360 g ae/ha	6	0	0	0	4.00	0.00	3-4
Bobwhite MPB26RH	% Injury				% Injury		
Application Rate	<25%	25-50%	50-75%	>75%	Ave	Std. Dev.	Range (%)
420 g ae/ha	0	1	1	10	1.25	0.62	1-3
840 g ae/ha	0	0	0	10	1.00	0.00	1
1680 g ae/ha	0	0	0	12	1.17	0.58	1-3
3360 g ae/ha	0	0	0	10	1.00	0.00	1

At 21 DAT, resistant and sensitive plants are counted to determine the percentage of lines that segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. **Table 31.** These data demonstrate

5 that *dgt-28* is inheritable as a robust glyphosate resistance gene in a monocot species.

Table 31. Percentage of T₁ *dgt-28* events by construct that demonstrated heritability in a mendelian fashion based off of a glyphosate selection at rates ranging from 420-3360 g ae/ha.

Construct ID	CTP:GOI	%T ₁ events tested that segregated at a single locus	%T ₁ events tested that segregated as 2 loci	No. T ₁ events tested
pDAS000122	TraP4:: <i>dgt-28</i>	62.5%	37.5%	8
pDAS000123	TraP8:: <i>dgt-28</i>	87.5%	12.5%	8
pDAS000124	TraP23:: <i>dgt-28</i>	12.5%	87.5%	8
pDAS000125	TraP5:: <i>dgt-28</i>	62.5%	0.0%	8

5 Molecular confirmation of T₀ transgenic plants for integration of T-DNAs encoding DGT-28. Genomic DNA was extracted from freeze-dried leaf material of all putative T₀ wheat plants. Freshly harvested leaf tissue was snap frozen in liquid nitrogen and freeze-dried for 24 h in a Labconco Freezone 4.5® (Labconco, Kansas City, MO) at -40°C and 133 x 10⁻³ mBar pressure. The lyophilized material was
10 subjected to DNA extraction using the DNeasy® Plant DNA Extraction Mini kit (Qiagen) following the manufacturer's instructions.

DNA from each T₀ plant was tested for the presence-absence of carryover *Agrobacterium tumefaciens* strain and for the number of integrated copies of the T-DNA encoding DGT-28. The presence-absence of *A. tumefaciens* strain was
15 performed using a duplex Taqman® qPCR assay to amplify the endogenous ubiquitin gene (forward and reverse primers and probe:

5' GCGAAGATCCAGGACAAGGA 3' (SEQ ID NO:85; Forward primer)

5' CTGCTTACCGGCAAAGATGAG 3' (SEQ ID NO:86; Reverse primer)

5' TTCCCCCGGACCAGCAGCGT 3' (SEQ ID NO:87; Probe)

20 from the wheat genome, and virC from pTiBo542:

5' CCGACGAGAAAGACCAGCAA 3' (SEQ ID NO:88; Forward primer)

5' CTTAAGTTGTGTCGATCGGGACTGT 3' (SEQ ID NO:89; Reverse primer)

5' TGAGCCTCTCGTCGCCGATCACAT 3' (SEQ ID NO:90; Probe).

25 The number of integrated T-DNA copies was estimated using a duplex Taqman® qPCR assay following the procedure of Livak and Schmittgen (Methods 2001 25:402-8). The assay amplified the endogenous single-copy puroindoline-b

(*Pinb*) gene in the D-genome of hexaploid wheat (Gautier *et al.* Plant Science 2000 153, 81–91):

5' ATTTTCCATTCACTTGGCCC 3' (SEQ ID NO:91; Forward primer)

5' TGCTATCTGGCTCAGCTGC 3' (SEQ ID NO:92; Reverse primer)

5' ATGGTGGAAGGGCGGTTGTGA 3' (SEQ ID NO:93; Probe)

and a region of the Actin (*Act1*) promoter present on the T-DNA:

5' CTCCCGCGCACCGATCTG 3' (SEQ ID NO:94; Forward primer)

5' CCCGCCCTCTCCTCTTTC 3' (SEQ ID NO:95; Reverse primer)

5' AAGCCGCCTCTCGCCACCCA 3' (SEQ ID NO:96; Probe).

Plants that did not amplify a product from *virC* and from which correct products were amplified with primers to the endogenous ubiquitin and rice actin promoter were classified as transgenic. The number of integrated T-DNA was estimated from $2\Delta\Delta c(T)$, according to Livak and Schmittgen (Methods 2001 25:402-8). Overall, about 95% of all T₀ plants had at least one integrated copy of the T-DNA. **Table 32.**

Table 32. Number of independent T₀ plants generated and estimated number of integrated T-DNA encoding DGT-28.

Vector	# Independent T ₀ plants tested	# Independent T ₀ events positive for transgene	# Multi-copy (≥4) T-DNA events	# Low-copy (≥3) T-DNA events
pDAS000122	45	43	15	28
pDAS000123	44	42	11	31
pDAS000124	45	44	21	23
pDAS000125	46	39	15	24

Development of PCR zygosity assays for tracking transgene inheritance.

The sequences flanking the T-DNA integration sites were identified by digestion of purified genomic DNA with eight restriction endonucleases, followed by ligation of double-stranded adapters specific to the overhangs created by the restriction endonucleases. Following adapter ligation, PCR was performed with a biotinylated primer to either the 3' or 5' end of the T-DNA encoding DGT-28 and a primer to each adapter. The PCR products were captured and purified on Ampure Solid Phase

Reversible Immobilization (SPRI) beads (Agencourt Bioscience Corporation, Beckman Coulter Company). A nested PCR was then performed and the amplification products were Sanger sequenced using BigDye® v3.1 chemistry (Applied Biosystems) on an ABI3730xl® automated capillary electrophoresis platform. Sequence analysis performed using Sequencher software (GeneCodes, Ann Arbor, MI) was used to generate (where possible) a consensus sequence. The resulting consensus sequence and singletons were used as BlastN queries against assembled genome survey sequence contigs for flow-sorted chromosome arms of wheat variety Chinese Spring (www.wheatgenome.org) to determine the chromosomes in which T-DNA integration had occurred and to enable the design of sub-genome-specific primers for the development of PCR zygosity assays.

Two PCR assays were developed for each transgenic event to enable transgene inheritance to be tracked in subsequent generations. The first assay (hereafter referred to as out-out PCR) was designed to amplify across the T-DNA integration site. Sub-genome-specific amplification in this assay was achieved using on-off PCR with primers designed to position the penultimate base (which contained a phosphorthioate linkage) over nucleotide sequence variation that distinguished the targeted locus from duplicated (both homoeologous and paralogous) copies of the locus elsewhere in the wheat genome. The second assay (hereafter referred to as in-out PCR) was designed to amplify from the T-DNA into the endogenous sequence. This assay utilised one of the primers from the out-out PCR assay and a primer designed to the 3' or 5' end of the T-DNAs encoding DGT-28. The PCR primers were designed to be between 18 and 27 nucleotides in length and to have a melting temperature of 60 to 65°C, optimal 63°C. Both out-out and in-out PCR assays were performed in a 25 µl reaction volume with 0.2mM dNTP, 1x Phusion PCR buffer (New England BioLabs), 1.5 mM MgCl₂, 0.5U Hotstart Phusion DNA polymerase (New England BioLabs), 25 ng purified genomic DNA and 0.4 µM of each primer. PCR cycling conditions were 98°C for 30s then (98°C for 10s, 65°C for 20s, 72°C for 60s) for 40 cycles. The zygosity of transgenic plants was assigned as shown in **Table 33**.

Table 33. Transgenic events for which PCR zygosity assays were developed and primer sequences used for out-out and in-out PCR.

Out-out PCR				
Construct	Event code	Primer 1 (5'→3')*	Primer 2 (5'→3')*	Size (bp)
pDAS0001 22	hh08-6678-2- 1	GGTTTGTGTAATCCCT CTGTTGG*T (SEQ ID NO:97)	GTGGTCATGACAGTAT GATAACAG*G (SEQ ID NO:98)	303
pDAS0001 22	hh08-6678-8- 1	GGGTCTGCCCAATGAA GCG*A (SEQ ID NO:99)	TCTCGCTTCTCTCATA ACACATCGT*G (SEQ ID NO:100)	217
pDAS0001 23	hh08-6729-5- 1	GACCTCTCTCACCCCTC CTCCT*C (SEQ ID NO:101)	CCAAATAATAAGTGAG AGAGGGGCA*T (SEQ ID NO:102)	286
pDAS0001 23	mp45-6739-1 4-1	TAGTTCCCCTGTCGTG TGCAA*A (SEQ ID NO:103)	CAACAGCAGCCTCACC AATCA*C (SEQ ID NO:104)	555
pDAS0001 23	mp45-6739-5 -1	CAAGAACGGTGCTCCT TTTTTAA*G (SEQ ID NO:105)	AGCCCTTCCTCTGCAT CCTT*A (SEQ ID NO:106)	440
pDAS0001 24	yl02-6762-8- 1	GGCTGTGTTGCACACA AATAGAG*A (SEQ ID NO:107)	CAGCAGCACGGTAGGT AGATTG*T (SEQ ID NO:108)	473
pDAS0001 24	gt19-6752-4- 1	CCGATAAGACGGCAAC TGATTAA*A (SEQ ID NO:109)	AGGCTGGCTTCTAGTG GAAGGA*G (SEQ ID NO:110)	215
pDAS0001 24	hh08-6761-1- 1	GGGTTTCCGGCTGGAG AC*G (SEQ ID NO:111)	CCAAAAGCAATTTTCG TTATAAGATGC*C (SEQ ID NO:112)	302
OlipDAS0 00124	yl02-6762-6- 1	CCAGATAATCTGTGGG CTCCT*G (SEQ ID NO:113)	GCAGCAGCTTGCCTTA AGC*A (SEQ ID NO:114)	161
pDAS0001 25	hh08-6780-10 -1	TGCTTGTTTCTGTTGT CATCATAGGT*T (SEQ ID NO:115)	CATTTGTTGGGTTTCC ACGTAC*G (SEQ ID NO:116)	145
pDAS0001 25	hh08-6780-8- 1	GAGCGCGGCTAAAGGT CAAAA*C (SEQ ID NO:117)	CCGATTTACATGGACT TGATGGAG*T (SEQ ID NO:118)	241
In-out PCR				
pDAS0001 22	hh08-6678-2- 1	GGTTTGTGTAATCCCT CTGTTGG*T (SEQ ID NO:119)	GCCGCCTCCAGTGAGT GTTGCTGCTTGTGTA* G (SEQ ID NO:120)	732
pDAS0001 22	hh08-6678-8- 1	GGGTCTGCCCAATGAA GCG*A (SEQ ID NO:121)	GCCGCCTCCATAATGT GTGAGTAGTTCCCAGA TAAG*G (SEQ ID NO:122)	297
pDAS0001 23	hh08-6729-5- 1	GCCGCCTCCAGTGAGT GTTGCTGCTTGTGTA* G (SEQ ID NO:123)	CCAAATAATAAGTGAG AGAGGGGCA*T (SEQ ID NO:124)	510

pDAS0001 23	mp45-6739-1 4-1	GCCGCCTCCATAATGT GTGAGTAGTTCCCAGA TAAG*G (SEQ ID NO:125)	CAACAGCAGCCTCACC AATCA*C (SEQ ID NO:126)	510
pDAS0001 23	mp45-6739-5 -1	GCCGCCTCCATAATGT GTGAGTAGTTCCCAGA TAAG*G (SEQ ID NO:127)	AGCCCTTCCTCTGCAT CCTT*A (SEQ ID NO:128)	580
pDAS0001 24	yl02-6762-8- 1	GCCGCCTCCATAATGT GTGAGTAGTTCCCAGA TAAG*G (SEQ ID NO:129)	CAGCAGCACGGTAGGT AGATTG*T (SEQ ID NO:130)	672
pDAS0001 24	gt19-6752-4- 1	GCCGCCTCCAGTGAGT GTTGCTGCTTGTGTA* G (SEQ ID NO:131)	AGGCTGGCTTCTAGTG GAAGGA*G (SEQ ID NO:132)	594
pDAS0001 24	hh08-6761-1- 1	GCCGCCTCCATAATGT GTGAGTAGTTCCCAGA TAAG*G (SEQ ID NO:133)	CCAAAAGCAATTTTCG TTATAAGATGC*C (SEQ ID NO:134)	528
pDAS0001 24	yl02-6762-6- 1	GCCGCCTCCAGTGAGT GTTGCTGCTTGTGTA* G (SEQ ID NO:135)	GCAGCAGCTTGCCTTA AGC*A (SEQ ID NO:136)	633
pDAS0001 25	hh08-6780-10 -1	GCCGCCTCCATAATGT GTGAGTAGTTCCCAGA TAAG*G (SEQ ID NO:137)	CATTTGTTGGGTTTCC ACGTAC*G (SEQ ID NO:138)	280
pDAS0001 25	hh08-6780-8- 1	GCCGCCTCCAGTGAGT GTTGCTGCTTGTGTA* G (SEQ ID NO:139)	CCGATTTACATGGACT TGATGGAG*T (SEQ ID NO:140)	680

*indicates phosphorothioate linkage

Phenotypic assessment of T₁ transgenic plants for glyphosate tolerance. To determine if transgenic events with DGT-28 expression constructs exhibited glyphosate tolerance, T₁ plants derived from individual events were phenotypically assessed under glasshouse containment conditions. Two phenotypic screens were performed. In the first (preliminary) screen, transgenic events (with sufficient T₁ seed for both phenotypic screens) were assessed for glufosinate and glyphosate tolerance to confirm DGT-28 expression and to establish the rank order for herbicide tolerance among events. In the second (detailed) screen, selected transgenic events were assessed for glyphosate tolerance at different spray dose rates to establish the level of herbicide tolerance conferred within events and between DGT-28 expression constructs.

Twelve T1 seed per selected event and three replicates (12 seeds each) of the untransformed donor wheat line Bobwhite MPB26RH were sown in 85 mm pots and grown to the 2-leaf stage under well-watered conditions at 25°C with supplementary lighting providing a 12 hour photoperiod. The pots were placed in a randomised design to allow environmental effects to be removed during data analysis. The transgenic events screened are listed in **Table 34**. At the 2-leaf stage, all T1 plants and the first replicate of 12 untransformed donor wheat plants were sprayed with glufosinate at a dose rate of 420 g ai/ha. The plants were visually inspected after four days and representative plants capturing the range of phenotypic responses were used to develop a scoring scale from 0 to 6. **Table 35**.

Table 34. Transgenic events tested in preliminary screen.

Entry	Vector	Event Code	Estimated number of integrated T-DNA encoding DGT-28*
1	pDAS000122	hh08-6678-6-1	Low-copy event
2	pDAS000122	mp45-6696-2-1	Low-copy event
3	pDAS000122	hh08-6718-2-1	Low-copy event
4	pDAS000122	km51-6686-1-1	Low-copy event
5	pDAS000122	mp45-6677-5-1	Low-copy event
6	pDAS000122	mp45-6696-4-1	Low-copy event
7	pDAS000122	mp45-6711-2-1	Low-copy event
8	pDAS000122	mp45-6711-4-1	Low-copy event
9	pDAS000122	hh08-6678-7-1	Low-copy event
10	pDAS000122	mp45-6711-7-1	Low-copy event
11	pDAS000122	mp45-6711-3-1	Low-copy event
12	pDAS000122	hh08-6678-2-1	Low-copy event
13	pDAS000122	mp45-6711-5-1	Low-copy event
14	pDAS000122	mp45-6711-6-1	Low-copy event
15	pDAS000122	mp45-6696-1-1	Low-copy event
16	pDAS000122	hh08-6678-8-1	Low-copy event
17	pDAS000122	gt19-6680-3-1	Multi-copy event
18	pDAS000122	mp45-6711-10-1	Multi-copy event
19	pDAS000122	mp45-6711-31-1	Multi-copy event
20	pDAS000122	yl02-6709-1-1	Multi-copy event
21	pDAS000122	mp45-6711-11-1	Multi-copy event
22	pDAS000123	hh08-6729-6-1	Low-copy event
23	pDAS000123	mp45-6739-4-1	Low-copy event
24	pDAS000123	gt19-6733-7-1	Low-copy event
25	pDAS000123	mp45-6739-7-1	Low-copy event
26	pDAS000123	gt19-6733-9-1	Low-copy event
27	pDAS000123	gt19-6733-2-1	Low-copy event

Entry	Vector	Event Code	Estimated number of integrated T-DNA encoding DGT-28*
28	pDAS000123	yl02-6735-5-1	Low-copy event
29	pDAS000123	yl02-6735-1-1	Low-copy event
30	pDAS000123	hh08-6729-8-1	Low-copy event
31	pDAS000123	gt19-6733-5-1	Low-copy event
32	pDAS000123	mp45-6739-14-1	Low-copy event
33	pDAS000123	mp45-6739-2-1	Low-copy event
34	pDAS000123	hh08-6729-5-1	Low-copy event
35	pDAS000123	mp45-6739-5-1	Low-copy event
36	pDAS000123	hh08-6729-7-1	Low-copy event
37	pDAS000123	hh08-6729-9-1	Low-copy event
38	pDAS000123	gt19-6733-10-1	Low-copy event
39	pDAS000123	gt19-6733-8-1	Low-copy event
40	pDAS000123	hh08-6729-3-1	Multi-copy event
41	pDAS000123	mp45-6739-16-1	Multi-copy event
42	pDAS000123	gt19-6733-6-1	Multi-copy event
43	pDAS000123	di01-6745-1-1	Multi-copy event
44	pDAS000123	gt19-6733-1-1	Multi-copy event
45	pDAS000123	mp45-6739-1-1	Multi-copy event
46	pDAS000124	mp45-6756-4-1	Low-copy event
47	pDAS000124	yl02-6762-3-1	Low-copy event
48	pDAS000124	yl02-6762-11-1	Low-copy event
49	pDAS000124	gt19-6752-10-1	Low-copy event
50	pDAS000124	gt19-6752-14-1	Low-copy event
51	pDAS000124	yl02-6762-4-1	Low-copy event
52	pDAS000124	mp45-6756-2-1	Low-copy event
53	pDAS000124	mp45-6756-1-1	Low-copy event
54	pDAS000124	yl02-6762-8-1	Low-copy event
55	pDAS000124	yl02-6762-6-1	Low-copy event
56	pDAS000124	gt19-6752-4-1	Low-copy event
57	pDAS000124	gt19-6752-23-1	Low-copy event
58	pDAS000124	hh08-6761-1-1	Low-copy event
59	pDAS000124	hh08-6761-3-1	Low-copy event
60	pDAS000124	yl02-6762-1-1	Low-copy event
61	pDAS000124	yl02-6762-7-1	Low-copy event
62	pDAS000124	gt19-6752-7-1	Low-copy event
63	pDAS000124	yl02-6762-12-1	Multi-copy event
64	pDAS000124	gt19-6752-6-1	Multi-copy event
65	pDAS000124	gt19-6752-22-1	Multi-copy event
66	pDAS000124	gt19-6752-24-1	Multi-copy event
67	pDAS000124	gt19-6752-18-1	Multi-copy event
68	pDAS000124	yl02-6762-5-1	Multi-copy event
69	pDAS000125	hh08-6780-9-1	Low-copy event
70	pDAS000125	yl02-6781-8-1	Low-copy event
71	pDAS000125	hh08-6780-1-1	Low-copy event

Entry	Vector	Event Code	Estimated number of integrated T-DNA encoding DGT-28*
72	pDAS000125	hh08-6785-3-1	Low-copy event
73	pDAS000125	hh08-6780-7-1	Low-copy event
74	pDAS000125	hh08-6780-4-1	Low-copy event
75	pDAS000125	gt19-6777-2-1	Low-copy event
76	pDAS000125	hh08-6785-4-1	Low-copy event
77	pDAS000125	yl02-6781-4-1	Low-copy event
78	pDAS000125	hh08-6780-16-1	Low-copy event
79	pDAS000125	hh08-6780-8-1	Low-copy event
80	pDAS000125	hh08-6780-10-1	Low-copy event
81	pDAS000125	hh08-6780-11-1	Low-copy event
82	pDAS000125	hh08-6780-12-1	Low-copy event
83	pDAS000125	hh08-6780-6-1	Low-copy event
84	pDAS000125	gt19-6777-5-1	Low-copy event
85	pDAS000125	hh08-6785-7-1	Low-copy event
86	pDAS000125	hh08-6780-13-1	Low-copy event
87	pDAS000125	hh08-6785-1-1	Low-copy event
88	pDAS000125	hh08-6785-8-1	Multi-copy event
89	pDAS000125	yl02-6781-1-1	Multi-copy event
90	pDAS000125	hh08-6780-3-1	Multi-copy event
91	pDAS000125	yl02-6781-7-1	Multi-copy event
92	pDAS000125	hh08-6780-15-1	Multi-copy event

*Based on duplex Taqman® qPCR assay. Low- and multi-copy indicates ≤ 3 and ≥ 4 integrated T-DNA, respectively.

Table 35. Scoring scale used to record phenotypic response to glufosinate at 4 days after spraying.

Score	Description
0	Delayed germination or poor plant establishment; exclude from subsequent analyses
1	>75% leaves necrotic; chlorotic/wilted/dead shoot
2	25-75% leaves necrotic; shoot/leaves mostly chlorotic
3	10-25% leaves necrotic; <50% leaves chlorotic; moderate wilting; minor chlorotic shoot
4	<10% leaves necrotic; minor wilting; minor chlorosis
5	Necrotic leaf tips; remaining plant healthy
6	Healthy plant

Each plant in the trial was then scored relative to the scoring scale, with the scorer “blinded” with regard to plant genotype to eliminate scoring bias. Five days after glufosinate scoring, all T1 plants and the first and second replicates of

untransformed donor wheat plants were sprayed with glyphosate at a dose rate of 420 g ai/ha. The remaining third replicate of untransformed donor wheat line (total 12 plants) was not sprayed. The plants were visually inspected at 7, 14 and 21 days after spraying. A scoring scale capturing the range of phenotypic responses was developed for each time point and used to score the entire trial. At each time point, the scorer “blinded” with regard to the plant genotype. The scoring scale at 7 days after spraying ranged from 0 to 7 (**Table 36**), and from 1 to 4 at 14 and 21 days after spraying (**Table 37**). Plant length, tiller number and morphological abnormalities were also recorded for each plant at 14 days after glyphosate spraying. Plants with delayed germination or poor establishment were excluded from subsequent analyses.

Table 36. Scoring scale used to record phenotypic response to glyphosate at 7 days after spraying.

Score	Description
0	Plant dead
1	>75% leaves necrotic; chlorotic/ wilted/dead shoot
2	50-75% leaves necrotic; severe chlorosis and wilting
3	25-50% leaves necrotic; <50% chlorotic leaves; moderate wilting
4	10-25% leaves necrotic; <25% leaves chlorotic; minor wilting
5	<10% leaves necrotic; minor chlorosis
6	Necrotic leaf tips; remaining plant healthy
7	Healthy plant

Table 37. Scoring scale used to record phenotypic response to glyphosate at 14 and 21 days after spraying.

Score	Description
1	Plant dead
2	50-75% leaves necrotic; severe chlorosis and wilting; plant dying
3	<25% leaves necrotic; <25% leaves chlorotic; minor wilting; signs of growth
4	Healthy plant

Analysis of glufosinate response failed to reveal a clear phenotypic difference between untransformed donor wheat plants that were sprayed and untransformed donor plants that were not sprayed (data not shown). As a consequence, the tolerance of the transgenic events to glufosinate could not be reliably assessed. In contrast, analysis of glyphosate response at 21 days after spraying revealed a clear phenotypic difference between the sprayed and unsprayed

untransformed donor plants. **Table 38.** Hence, analyses for glyphosate tolerance among the transgenic events was based on response scores collected at 21 days after spraying. A transgenic event was considered to exhibit glyphosate tolerance when 4 or more of the 12 T1 plants for that event had a response score greater than or equal to 3. This criteria was based on the expectation that each event would segregate 1:2:1 (homozygous present: hemizygous : homozygous absent) for the transgene in the T1 generation and to enable events with weak DGT28 expression to be identified. The transgenic events were rank ordered for observed glyphosate tolerance using an arbitrary aggregate score calculated from individual tolerant plants. The aggregate score was calculated from the response scores at 14 and 21 days and plant length, tiller number and morphological abnormalities recorded at 14 days after spraying.

Table 38. Phenotypic response of untransformed donor wheat plants to herbicide treatment at 21 days after spraying.

	Glufosinate sprayed	Glyphosate sprayed	Survival rate
Replicate 1	Yes	Yes	10 of 12 dead/dying
Replicate 2	No	Yes	10 of 12 dead/dying
Replicate 3	No	No	12 of 12 healthy

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Overall, 67 of the 92 transgenic events screened showed evidence for glyphosate tolerance. **Table 39.** Six transgenic events estimated to have ≤ 3 integrated copies of the transgene and two transgenic events estimated to have 4 or more integrated transgene were selected for each DGT-28 expression vectors for inclusion in the second (detailed) phenotypic screen.

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Table 39. Rank ordered phenotypic response of transgenic events to glyphosate treatment.

Construct	Event code	Estimated T-DNA copy number	% glyphosate tolerant plants	Event phenotype	Standardised aggregate score*	Selected for detailed screen
pDAS000122	mp45-6711-7-1	Low-copy	66.7%	Tolerant	8.0	Yes
pDAS000124	gt19-6752-4-1	Low-copy	91.7%	Tolerant	7.3	Yes
pDAS000122	gt19-6680-3-1	Multi-copy	100.0%	Tolerant	7.2	Yes
pDAS000123	gt19-6733-1-1	Multi-copy	50.0%	Tolerant	7.2	No
pDAS000122	mp45-6711-4-1	Low-copy	66.7%	Tolerant	6.2	Yes
pDAS000125	hh08-6780-8-1	Low-copy	58.3%	Tolerant	6.1	Yes
pDAS000123	mp45-6739-16-1	Multi-copy	75.0%	Tolerant	6.1	Yes

Construct	Event code	Estimated T-DNA copy number	% glyphosate tolerant plants	Event phenotype	Standardised aggregate score*	Selected for detailed screen
pDAS000125	hh08-6780-10-1	Low-copy	75.0%	Tolerant	6.1	Yes
pDAS000125	hh08-6785-8-1	Multi-copy	50.0%	Tolerant	5.9	Yes
pDAS000125	hh08-6785-7-1	Low-copy	50.0%	Tolerant	5.8	Yes
pDAS000124	gt19-6752-22-1	Multi-copy	91.7%	Tolerant	5.8	Yes
pDAS000123	di01-6745-1-1	Multi-copy	100.0%	Tolerant	5.7	Yes
pDAS000122	hh08-6678-2-1	Low-copy	75.0%	Tolerant	5.5	Yes
pDAS000122	hh08-6678-7-1	Low-copy	58.3%	Tolerant	5.3	Yes
pDAS000125	hh08-6780-6-1	Low-copy	66.7%	Tolerant	5.2	Yes
pDAS000123	gt19-6733-6-1	Multi-copy	83.3%	Tolerant	5.0	No
pDAS000125	hh08-6780-11-1	Low-copy	66.7%	Tolerant	5.0	Yes
pDAS000125	hh08-6780-7-1	Low-copy	66.7%	Tolerant	3.7	Yes
pDAS000124	yl02-6762-8-1	Low-copy	83.3%	Tolerant	3.5	Yes
pDAS000124	yl02-6762-6-1	Low-copy	83.3%	Tolerant	3.3	Yes
pDAS000122	mp45-6711-2-1	Low-copy	66.7%	Tolerant	3.2	Yes
pDAS000122	mp45-6711-11-1	Multi-copy	100.0%	Tolerant	3.0	Yes
pDAS000122	mp45-6677-5-1	Low-copy	33.3%	Tolerant	2.7	No
pDAS000125	hh08-6785-4-1	Low-copy	58.3%	Tolerant	2.5	No
pDAS000123	yl02-6735-1-1	Low-copy	83.3%	Tolerant	2.1	Yes
pDAS000122	mp45-6711-3-1	Low-copy	66.7%	Tolerant	1.7	No
pDAS000125	yl02-6781-1-1	Multi-copy	41.7%	Tolerant	1.7	No
pDAS000124	hh08-6761-1-1	Low-copy	100.0%	Tolerant	1.6	Yes
pDAS000125	hh08-6780-16-1	Low-copy	91.7%	Tolerant	1.4	No
pDAS000123	hh08-6729-8-1	Low-copy	83.3%	Tolerant	1.1	Yes
pDAS000125	hh08-6780-1-1	Low-copy	91.7%	Tolerant	0.9	No
pDAS000123	hh08-6729-5-1	Low-copy	83.3%	Tolerant	0.7	Yes
pDAS000124	mp45-6756-1-1	Low-copy	66.7%	Tolerant	0.7	Yes
pDAS000123	mp45-6739-14-1	Low-copy	91.7%	Tolerant	0.5	Yes
pDAS000125	gt19-6777-2-1	Low-copy	75.0%	Tolerant	0.3	No
pDAS000124	yl02-6762-5-1	Multi-copy	91.7%	Tolerant	0.2	No
pDAS000125	hh08-6780-3-1	Multi-copy	66.7%	Tolerant	0.0	Yes
pDAS000122	mp45-6696-1-1	Low-copy	83.3%	Tolerant	-0.1	No
pDAS000122	hh08-6678-8-1	Low-copy	58.3%	Tolerant	-0.1	Yes
pDAS000125	hh08-6780-12-1	Low-copy	75.0%	Tolerant	-0.3	No
pDAS000125	yl02-6781-4-1	Low-copy	66.7%	Tolerant	-0.4	No
pDAS000124	gt19-6752-6-1	Multi-copy	50.0%	Tolerant	-0.9	No
pDAS000122	mp45-6696-4-1	Low-copy	66.7%	Tolerant	-0.9	No
pDAS000125	hh08-6780-9-1	Low-copy	66.7%	Tolerant	-1.1	No
pDAS000123	mp45-6739-5-1	Low-copy	83.3%	Tolerant	-1.1	Yes
pDAS000124	yl02-6762-1-1	Low-copy	66.7%	Tolerant	-1.1	No
pDAS000123	yl02-6735-5-1	Low-copy	83.3%	Tolerant	-1.3	No
pDAS000124	yl02-6762-7-1	Low-copy	91.7%	Tolerant	-1.6	Yes
pDAS000124	gt19-6752-24-1	Multi-copy	50.0%	Tolerant	-1.6	No
pDAS000123	mp45-6739-7-1	Low-copy	41.7%	Tolerant	-1.7	No
pDAS000124	gt19-6752-18-1	Multi-copy	100.0%	Tolerant	-1.7	Yes
pDAS000123	gt19-6733-2-1	Low-copy	66.7%	Tolerant	-1.8	No
pDAS000124	gt19-6752-10-1	Low-copy	33.3%	Tolerant	-2.0	No
pDAS000123	mp45-6739-4-1	Low-copy	66.7%	Tolerant	-2.0	Yes
pDAS000125	gt19-6777-5-1	Low-copy	50.0%	Tolerant	-2.4	No
pDAS000124	gt19-6752-14-1	Low-copy	58.3%	Tolerant	-2.5	No
pDAS000122	yl02-6709-1-1	Multi-copy	50.0%	Tolerant	-2.5	No
pDAS000125	yl02-6781-8-1	Low-copy	58.3%	Tolerant	-2.7	No
pDAS000124	gt19-6752-23-1	Low-copy	83.3%	Tolerant	-2.7	No

Construct	Event code	Estimated T-DNA copy number	% glyphosate tolerant plants	Event phenotype	Standardised aggregate score*	Selected for detailed screen
pDAS000124	mp45-6756-2-1	Low-copy	75.0%	Tolerant	-2.7	No
pDAS000124	yl02-6762-3-1	Low-copy	66.7%	Tolerant	-3.4	No
pDAS000123	gt19-6733-8-1	Low-copy	41.7%	Tolerant	-3.4	No
pDAS000123	hh08-6729-7-1	Low-copy	50.0%	Tolerant	-3.6	No
pDAS000125	hh08-6785-3-1	Low-copy	66.7%	Tolerant	-4.2	No
pDAS000123	mp45-6739-1-1	Multi-copy	33.3%	Tolerant	-4.4	No
pDAS000122	mp45-6711-31-1	Multi-copy	41.7%	Tolerant	-4.5	No
pDAS000125	hh08-6780-15-1	Multi-copy	66.7%	Tolerant	-4.8	No
pDAS000123	gt19-6733-10-1	Low-copy	33.3%	Tolerant	-5.1	No
pDAS000125	hh08-6780-13-1	Low-copy	100.0%	Tolerant	-5.5	No
pDAS000122	mp45-6711-5-1	Low-copy	75.0%	Tolerant	-5.7	No
pDAS000122	mp45-6711-10-1	Multi-copy	58.3%	Tolerant	-10.4	No
pDAS000123	gt19-6733-5-1	Low-copy	0.0%	Susceptible	n/a	No
pDAS000124	gt19-6752-7-1	Low-copy	0.0%	Susceptible	n/a	No
pDAS000122	hh08-6718-2-1	Low-copy	0.0%	Susceptible	n/a	No
pDAS000123	hh08-6729-9-1	Low-copy	0.0%	Susceptible	n/a	No
pDAS000124	hh08-6761-3-1	Low-copy	0.0%	Susceptible	n/a	No
pDAS000125	hh08-6785-1-1	Low-copy	0.0%	Susceptible	n/a	No
pDAS000122	mp45-6696-2-1	Low-copy	0.0%	Susceptible	n/a	No
pDAS000123	mp45-6739-2-1	Low-copy	0.0%	Susceptible	n/a	No
pDAS000122	km51-6686-1-1	Low-copy	8.3%	Susceptible	n/a	No
pDAS000122	mp45-6711-6-1	Low-copy	8.3%	Susceptible	n/a	No
pDAS000124	yl02-6762-12-1	Multi-copy	8.3%	Susceptible	n/a	No
pDAS000123	gt19-6733-7-1	Low-copy	16.7%	Susceptible	n/a	No
pDAS000123	hh08-6729-3-1	Multi-copy	16.7%	Susceptible	n/a	No
pDAS000124	mp45-6756-4-1	Low-copy	16.7%	Susceptible	n/a	No
pDAS000125	hh08-6780-4-1	Low-copy	16.7%	Susceptible	n/a	No
pDAS000122	hh08-6678-6-1	Low-copy	25.0%	Susceptible	n/a	No
pDAS000123	gt19-6733-9-1	Low-copy	25.0%	Susceptible	n/a	No
pDAS000123	hh08-6729-6-1	Low-copy	25.0%	Susceptible	n/a	No
pDAS000124	yl02-6762-11-1	Low-copy	25.0%	Susceptible	n/a	No
pDAS000124	yl02-6762-4-1	Low-copy	25.0%	Susceptible	n/a	No
pDAS000125	yl02-6781-7-1	Multi-copy	25.0%	Susceptible	n/a	No

*A positive score indicates higher glyphosate tolerance. The standardised aggregate score for the untreated untransformed donor wheat plants was 12.2.

Detailed phenotypic screen. Four replicates of 12 T1 seeds per selected event and eight replicates (12 seeds each) of the untransformed donor wheat line Bobwhite MPB26RH were sown in 85 mm pots and grown to the 2-leaf stage under well-watered conditions at 25°C with supplementary lighting providing a 12 hour photoperiod. The pots were placed in a randomised design to allow environmental effects to be removed during data analysis. The transgenic events screened are listed in **Table 40**. At the 2-leaf stage, plant length and number of leaves was recorded for each plant before spraying with glyphosate. The first, second, third and fourth replicate of T1 plants for each selected event and the untransformed donor wheat

line were sprayed at a dose rate of 420, 840, 1680 and 3360 g ai/ha, respectively. The fifth, sixth, seventh and eighth replicate of untransformed donor wheat line (total 48 plants) were not sprayed. At 7, 14 and 21 days after spraying, the plants were scored for plant length, number of leaves and phenotypic response to glyphosate using the scoring scale in **Table 37**. Any morphological abnormalities were also recorded. For scoring, the scorer was “blinded” with regard to plant genotype and spray dose rate to prevent scoring bias. Plants with delayed germination and poor establishment (criteria: plant length <6 cm) at the pre-spray scoring were excluded from subsequent analyses.

Table 40. Transgenic events tested in detailed phenotypic screen.

Entry	Vector	Event Code	Estimated number of integrated T-DNA encoding DGT-28*
1	pDAS000122	mp45-6677-5-1	Low-copy event
2	pDAS000122	mp45-6711-7-1	Low-copy event
3	pDAS000122	mp45-6711-4-1	Low-copy event
4	pDAS000122	hh08-6678-2-1	Low-copy event
5	pDAS000122	hh08-6678-7-1	Low-copy event
6	pDAS000122	hh08-6678-8-1	Low-copy event
7	pDAS000122	mp45-6711-2-1	Low-copy event
8	pDAS000122	gt19-6680-3-1	Multi-copy event
9	pDAS000122	mp45-6711-11-1	Multi-copy event
10	pDAS000123	yl02-6735-1-1	Low-copy event
11	pDAS000123	hh08-6729-8-1	Low-copy event
12	pDAS000123	hh08-6729-5-1	Low-copy event
13	pDAS000123	mp45-6739-14-1	Low-copy event
14	pDAS000123	mp45-6739-5-1	Low-copy event
15	pDAS000123	mp45-6739-7-1	Low-copy event
16	pDAS000123	mp45-6739-4-1	Low-copy event
17	pDAS000123	mp45-6739-16-1	Multi-copy event
18	pDAS000123	di01-6745-1-1	Multi-copy event
19	pDAS000124	gt19-6752-4-1	Low-copy event
20	pDAS000124	yl02-6762-8-1	Low-copy event
21	pDAS000124	yl02-6762-6-1	Low-copy event
22	pDAS000124	hh08-6761-1-1	Low-copy event
23	pDAS000124	mp45-6756-1-1	Low-copy event
24	pDAS000124	yl02-6762-7-1	Low-copy event
25	pDAS000124	gt19-6752-22-1	Multi-copy event
26	pDAS000124	gt19-6752-18-1	Multi-copy event
27	pDAS000125	hh08-6780-8-1	Low-copy event
28	pDAS000125	hh08-6780-10-1	Low-copy event
29	pDAS000125	hh08-6785-7-1	Low-copy event

Entry	Vector	Event Code	Estimated number of integrated T-DNA encoding DGT-28*
30	pDAS000125	hh08-6780-6-1	Low-copy event
31	pDAS000125	hh08-6780-11-1	Low-copy event
32	pDAS000125	hh08-6780-7-1	Low-copy event

*Based on duplex Taqman® qPCR assay. Low- and multi-copy indicates ≤ 3 and ≥ 4 integrated T-DNA, respectively.

Analysis of glyphosate response at 7, 14 and 21 days after spraying revealed a clear-cut phenotypic difference between the sprayed and unsprayed untransformed donor wheat plants. This differentiation was maximal at 21 days and was observed across all glyphosate dose rates. **Table 41.** To assess the tolerance of the transgenic events to glyphosate at each spray dose rate, null T1 plants (i.e., plants not carrying the transgene) were excluded from subsequent analyses. T1 plants with a response score of less than three at 21 days after spraying were considered to have the null genotype. Analysis of variance (ANOVA) based on tolerant phenotypes revealed a significant effect for DGT-28 expression construct, transgenic event and glyphosate spray dose. **Table 42.** However, multiple comparison tests failed to unveil meaningful biological interpretation for the origin of these differences due to the limited range of response scores (i.e., 1 to 4; **Table 40**) used to record the phenotype of individual plants. In general, the eight independent transgenic events tested for each DGT-28 expression construct showed similar tolerance to glyphosate at each spray dose rate, indicating that all four DGT-28 transgenes conferred a dominant phenotype and that a single copy was sufficient to confer glyphosate tolerance. Each of the DGT-28 expression constructs revealed effective tolerance to at least 3360 g ai/ha glyphosate.

Table 41. Phenotypic response of untransformed donor wheat plants to different glyphosate treatments at 21 days after spraying.

	Dose Rate (g ai/ha)	No. plants at pre-spray scoring	No. surviving plants at 14 days after spraying	No. surviving plants at 21 days after spraying
Replicate 1	420	10	0	0
Replicate 2	840	10	3	0
Replicate 3	1680	11	0	0

	Dose Rate (g ai/ha)	No. plants at pre-spray scoring	No. surviving plants at 14 days after spraying	No. surviving plants at 21 days after spraying
Replicate 4	3360	10	1	0
Replicate 5	0	9	8	8
Replicate 6	0	8	8	8
Replicate 7	0	12	12	12
Replicate 8	0	12	12	12

Table 42. Analysis of variance (ANOVA) based on glyphosate tolerant plants.

	Df ¹	Sum of Squares	Mean Square	F-value	Pr(>F) ²
Replicate	11	1.29	0.12	0.728	0.71181
Vector	4	139.54	34.88	216.025	2.00E-16***
Event_Code	29	178.52	6.16	38.122	2.00E-16***
Spray_Dose	3	2.14	0.71	4.417	0.00427**

¹Degrees of freedom; ²Statistically significant at 0.001 (***) and 0.01 (**),

5 respectively.

Molecular confirmation of T-DNA presence in glyphosate tolerant T1 plants.

The PCR zygosity assays developed in Example 2 were used to confirm the presence of T-DNA encoding DGT-28 in the glyphosate tolerant T1 plants saved for T2 seed production (see Example 6). Overall, PCR zygosity tests were performed for 104 T1 plants, of which 89% were confirmed to contain at least one copy of the transgene.

Table 43. These results confirmed that the observed glyphosate tolerance was conferred by the presence of T-DNA encoding DGT-28.

Table 43. Observed transgene segregation among T1 plants.

Construct	Event Code	Homozygous for presence of transgene	Hemizygous for presence of transgene	Homozygous for absence of transgene	Escapes plants observed
pDAS000122	hh08-6678-2-1	0	7	2	Yes
pDAS000122	hh08-6678-8-1	2	7	0	No
pDAS000123	hh08-6729-5-1	7	4	0	No
pDAS000123	mp45-6739-14-1	1	8	0	No
pDAS000123	mp45-6739-5-1	0	9	0	No
pDAS000124	gt19-6752-4-1	5 (homo or hemi)		3	Yes
pDAS000124	hh08-6761-1-1	3	2	3	Yes

Construct	Event Code	Homozygous for presence of transgene	Hemizygous for presence of transgene	Homozygous for absence of transgene	Escapes plants observed
pDAS000124	yl02-6762-6-1	7 (homo or hemi)		2	Yes
pDAS000124	yl02-6762-8-1	3	6	0	No
pDAS000125	hh08-6780-10-1	6	4	0	No
pDAS000125	hh08-6780-8-1	2	7	1	Yes

Generation of T2 seed for glyphosate tolerant transgenic events. About eight glyphosate tolerant T1 plants were saved from the phenotypic screens for the 32 transgenic events that were selected for inclusion in the detailed phenotypic screen (Table 40). The plants were transferred to 200 mm pots and grown under well-watered conditions at 25°C with supplementary lighting providing a 12 hour photoperiod. The spikes on each plant were individually bagged prior to anthesis to prevent out-crossing.

While aspects of this invention have been described in certain embodiments, they can be further modified within the spirit and scope of this disclosure. This application is therefore intended to cover any variations, uses, or adaptations of embodiments of the invention using its general principles. Further, this application is intended to cover such departures from the present disclosure as come within known or customary practice in the art to which these embodiments pertain and which fall within the limits of the appended claims.

CLAIMS

What is claimed is:

- 5 1. An isolated nucleic acid molecule selected from the group consisting of:
a nucleic acid molecule comprising a nucleotide sequence having at least 80% sequence identity to the nucleotide sequence of SEQ ID NO:2 or SEQ ID NO:3;
10 a nucleic acid molecule that encodes a polypeptide having at least 90% sequence identity with the amino acid sequence of SEQ ID NO:1; and
a heterologous nucleic acid comprising a nucleotide sequence that hybridizes to another nucleic acid having the nucleotide sequence of SEQ ID NO:2 or SEQ ID NO:3 under high-stringency conditions.
- 15 2. The nucleic acid molecule of claim 1, wherein the nucleic acid comprises a synthetic sequence that has been designed for expression in a plant.
3. A vector comprising the nucleic acid molecule of claim 1.
- 20 4. The vector of claim 3, further comprising a nucleic acid encoding a heterologous polypeptide.
5. The vector of claim 3, wherein the nucleic acid is operably linked to a
25 promoter.
6. A host cell that contains the nucleic acid molecule of claim 1 as a heterologous nucleic acid operably linked to a promoter.
- 30 7. The vector of claim 5, wherein the promoter is the AtUbi10 promoter.
8. The host cell of claim 6, wherein the host cell is a plant cell.

9. A transgenic plant, plant part, plant organ, plant seed, or plant cell that comprises the nucleic acid of claim 1.

10. The transgenic plant, plant part, plant organ, plant seed, or plant cell of claim 9, wherein the plant, plant part, plant organ, plant seed, and/or plant cell is tolerant to glyphosate, when compared to a wild-type plant of the same species.

11. The transgenic plant, plant part, plant organ, plant seed, or plant cell of claim 9, wherein the nucleic acid does not encode LGNAAT (SEQ ID NO:141) and does not encode ALLMXAPLT, wherein X is selected from the group consisting of alanine, serine, and threonine (SEQ ID NO:142).

12. The plant, plant part, plant organ, plant seed, or plant cell of claim 9, wherein the nucleic acid encodes a polypeptide having at least 90% sequence identity with the amino acid sequence of SEQ ID NO:1, that when aligned with SEQ ID NO:1, comprises an alanine at the position corresponding to position 84 of SEQ ID NO:1 and/or a threonine at the position corresponding to position 172 of SEQ ID NO:1.

13. A tissue culture of regenerable cells produced from the plant, plant part, plant organ, plant seed, and/or plant cell of claim 9.

14. Protoplasts produced from the transgenic plant, plant part, plant organ, plant seed, or plant cell of claim 9.

15. The tissue culture of claim 13, wherein the regenerable cells are produced from a tissue type selected from the group consisting of leaves, pollen, embryos, cotyledons, hypocotyls, meristematic cells, roots, root tips, anthers, flowers, stems and pods.

16. A plant regenerated from the tissue culture of claim 13, wherein the plant is resistant to glyphosate.

17. The plant, plant part, plant organ, plant seed, or plant cell of claim 9, wherein the genome comprises a nucleic acid molecule having at least 95% sequence identity to SEQ ID NO:2 or SEQ ID NO:3.

5 18. A method of generating a plant, plant part, plant organ, plant seed, or plant cell resistant to glyphosate, the method comprising:
transforming the plant, plant part, plant organ, plant seed, or plant cell with the nucleic acid molecule of claim 1; and
expressing a polypeptide having at least 90% sequence identity with SEQ ID NO:1.

10

19. The method according to claim 18, wherein the transformed plant, plant part, plant organ, plant seed, or plant cell is resistant to glyphosate.

20. The method according to claim 18, wherein the polypeptide does not
15 comprise LGNAAT (SEQ ID NO:141) and/or does not comprise ALLMXAPLT, wherein X is selected from the group consisting of alanine, serine, and threonine (SEQ ID NO:142).

21. The method according to claim 18, wherein the polypeptide, when
20 aligned with SEQ ID NO:1, comprises an alanine at the position corresponding to position 84 of SEQ ID NO:1 and/or a threonine at the position corresponding to position 172 of SEQ ID NO:1.

22. The method according to claim 18, wherein the nucleic acid molecule
25 has at least 95% sequence identity to SEQ ID NO:2 or SEQ ID NO:3.

23. The method according to claim 18, wherein the nucleic acid molecule comprises SEQ ID NO:2 or SEQ ID NO:3.

30 24. A method for controlling weeds in an area under cultivation containing herbicide-resistant plants, the method comprising:
planting a plant or a plant seed comprising the nucleic acid molecule of claim 1 in the area under cultivation; and

applying to the area under cultivation a sufficient amount of herbicide to control weeds in the area under cultivation without significantly affecting the plant or plant seed.

5 25. The method according to claim 24, wherein the herbicide is glyphosate.

26. The method according to claim 24, wherein the plant or plant seed comprising the nucleic acid molecule of claim 1 comprises a second nucleic acid encoding a heterologous polypeptide.

10

27. The method according to claim 24, wherein the second nucleic acid comprises *aad-1* or *aad-12*.

28. A method for conferring resistance to an herbicide in a plant, the
15 method comprising:

transforming the plant with a DNA construct, said construct comprising a promoter operably linked with the nucleic acid molecule of claim 1;

regenerating a transformed plant; and

expressing the nucleic acid molecule so as to produce a polypeptide having at least

20

90% sequence identity with SEQ ID NO:1.

29. The method according to claim 28, wherein the herbicide is glyphosate.

30. The method according to claim 28, wherein the DNA construct
25 comprises a second nucleic acid molecule encoding a heterologous polypeptide expressible in the plant.

31. The method according to claim 30, wherein the heterologous polypeptide comprises *aad-1* or *aad-12*.

30

32. A plant having stably integrated into its genome the nucleic acid of claim 1 as a heterologous nucleic acid, wherein the nucleic acid is operably linked to a promoter.

33. The plant of claim 32, wherein the plant is a soybean plant.

34. The plant of claim 32, wherein the plant is a corn plant.

5

35. The plant of claim 32, wherein the plant is selected from the group consisting of wheat, corn, soybean, tobacco, brachiaria, rice, millet, barley, tomato, apple, pear, strawberry, orange, alfalfa, cotton, carrot, potato, sugar beets, yam, lettuce, spinach, petunia, rose, chrysanthemum, turf grass, pine, fir, spruce, heavy metal
10 accumulating plants, sunflower, safflower, rapeseed, and *Arabidopsis*.

36. The plant of claim 32, wherein the plant is a species selected from the group consisting of genera *Asparagus*, *Avena*, *Brachiaria*, *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Cucurbita*, *Daucus*, *Erigeron*, *Glycine*, *Gossypium*, *Hordeum*, *Helianthus*,
15 *Lactuca*, *Lolium*, *Lycopersicon*, *Malus*, *Manihot*, *Nicotiana*, *Orychophragmus*, *Oryza*, *Persea*, *Phaseolus*, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Secale*, *Solanum*, *Sorghum*, *Triticum*, *Vitis*, *Vigna*, and *Zea*.

37. The host cell of claim 8, wherein the plant cell is not regenerable to
20 produce a plant.

38. The plant cell of claim 9, wherein the plant cell is not regenerable to produce a plant.

25 39. The method claim 18, wherein a plant cell that is not regenerable to produce a plant is transformed.

SEQ ID NO: 67	DGT-33	--DRNRVGRPD-GPA-PDAD-----VYCRDGATTARFLPTLVAALASCTYRFDASAOQMRRRPLAPI--TRALTALGVDLRH	*
SEQ ID NO: 68	DGT-32	--DRNRVOGRPA-GPA-TTAA-----VYCRDGATTARFLPTLVAALASCTYRFDASAOQMRRRPLAPI--TRALTALGVDLRH	*
SEQ ID NO: 145	DGT-31	(60) --DRNHIEGRPS-GPAADAA-----VHCRDGATTARFLPTLAAAASEFYRFDASAOQMRRRPLAPI--TEALRTIGVDLRH	
SEQ ID NO: 69	DGT-28	(60) --DTQVVDGRPQ-GPAVAEAL-----VYCRDGATTARFLPTLAAAG-HGTYRFDASAOQMRRRPLPI--SRALRDLIGVDLRH	
SEQ ID NO: 70	grg-23	(59) --DDWAVEGLGQ--APNLDA-----WCEDAGIVARFLPEFVAAG-QCKTIEDGSEQLRRRPLRPVVDGIRHLGARVSS	
SEQ ID NO: 71	CP4 Agro	(75) --DTWVVDGVNGGLI-PPAP-----IDFGNAATGCRITMGIVGVYDFDSTIITDASLTKRPMGRV--INPLREMGVQVKS	
SEQ ID NO: 72	DGT-3	(74) VNNRAVVEGCGGIFBASLDSKSDIELYIGNAGTAMRPLTAAVTAACGNASVYLDGVPRMRERPEIGDIVVGLKQLGADVEC	
SEQ ID NO: 73	DGT-1	(81) TTKQALVEGCGGLFPTSKSKDEINLFIGNAGTAMRPLTAAVVAAGCNASVYLDGVPRMRERPEIGDIVVGLKQLGADVDC	
SEQ ID NO: 74	DGT-7	(82) VAKRAVVGCGGRFFVEKDAQEEVKLFIGNAGTAMRPLTAAVVAAGCNASVYLDGVPRMRERPEIGDIVVGLQQLGADADC	
SEQ ID NO: 75	aroA	(69) --DTRCEIINGCGFLHADG--ALELFIIGNAGTAMRPLAAALCLSN--DIVLTGEPRMRERPEIGHIVDALRLGCAKITY	

FIG. 1

DRWHIEGRPSGPAADAEVHCRDGAATTARFLPTLAAAAASGTYRFDASAQMRRRPLAPLTEALRTLGVDLRHD

SEQ ID NO: 76	DGT-3	(1)	MAQSSRICHGVQNP	CVIIISNL	SKSNQNKSPFSV	SLKTHQPRASSWGLKSGTMLNGSVIRP	KVTA	SVTSEKASEIVLQ	80				
SEQ ID NO: 77	DGT-1	(1)	-----	-----	-----	-----	MASVAAA	EKPSTSPETVILE					
SEQ ID NO: 78	DGT-7	(1)	-----	-----	-----	-----	MATSVAA	PAAPSGAEVVLQ					
DGT-3	(81)		PIREISGLIKLP	SGKSLSNRILLIAAL	SEGTTVVDNLLINSE	DIHYMLDAIKKLG	ENVERISVNNRAV	VEGCGGIFPASLD	160				
DGT-1	(20)		PIKDFSGTTLP	SGKSLSNRILLIAAL	SEGTTVVDNLLYSE	DIHYMLGALITL	GERVEDDKTKQA	VEGCGGIFPSTKE					
DGT-7	(21)		PIREISGAYQL	PGSKSLSNRILLIS	ALSEGTTVVDNLLINSE	DIHYMLDALEAL	GLSVEADKVA	KRAVVVGGGGRFVEKO					
DGT-3	(161)		SKSDIEIMLGN	ACTAMRPLTA	AVTAAGGNASYVLDG	VPRMR	RP	IGDLVGLKOLGAD	VECTLG	240			
DGT-1	(100)		SKDEINLFLGN	ACTAMRPLTA	AVTAAGGNASYVLDG	VPRMR	RP	IGDLVGLKOLGAD	VDCTLG				
DGT-7	(101)		QDEENKLELGN	ACTAMRPLTA	AVTAAGGNATYVLDG	VPRMR	RP	IGDLVGLQOLGAD	ADCTLG				
DGT-3	(241)		GKVKLSGSISS	QYLTALIMAA	PLAIGDVEIEI	DKLISVPY	VENTLKL	MERFGVSA	EHSDSWDRF	320			
DGT-1	(180)		GKVKLSGSISS	QYLTALIMAA	PLAIGDVEIEI	DKLISVPY	VENTLKL	MERFGVSV	EHSGNWDRL				
DGT-7	(181)		GKVKLSGSISS	QYLTALIMAA	PLAIGDVEIEI	DKLISVPY	VENTLKL	MERFGVTA	EHSDSWDRF	400			
DGT-3	(321)		VEGDASSASYEL	AGAAITG	ETVIVEGGCT	TSLOGDV	KFAEVL	EKMGCKV	SWTENSV	TVIGPSR	DAFGM	HLRAVD	VNNMK
DGT-1	(260)		VEGDASSASYEL	AGAAITG	ETVIVNGGCT	TSLOGDV	KFAEVL	EKMGCKV	SWTENSV	TVIGPSR	DAFGM	HLRAVD	VNNMK
DGT-7	(261)		VEGDASSASYEL	AGAAITG	ETVIVEGGCT	TSLOGDV	KFAEVL	EKMGCKV	SWTENSV	TVIGPSR	DAFGM	HLRAVD	VNNMK
DGT-3	(401)		MPDVAMTLAV	VALFADG	PPTTIRDVAS	NRVKETER	MTAICTE	ILKLGAT	VEEGSDY	CVITPPA	KPAE	IDTYDD	HRMAMA
DGT-1	(340)		MPDVAMTLAV	VALFANG	PPTAIRDVAS	NRVKETER	MTAICTE	ILKLGAT	VEEGSDY	CVITPPA	KPAE	IDTYDD	HRMAMA
DGT-7	(341)		MPDVAMTLAV	VALFADG	PPTAIRDVAS	NRVKETER	MTAICTE	ILKLGAT	VEEGSDY	CVITPPA	KPAE	IDTYDD	HRMAMA
DGT-3	(481)		FSLAACADVP	VTIKDPG	CTRKT	FPDYEQV	IESITKH						
DGT-1	(420)		FSLAACGDP	VTIKDPG	CTRKT	FPDYFV	IERITKH						
DGT-7	(421)		FSLAACAEV	PVTIIPDPG	CTRKT	FPNYFV	ISFVKN						

FIG. 2

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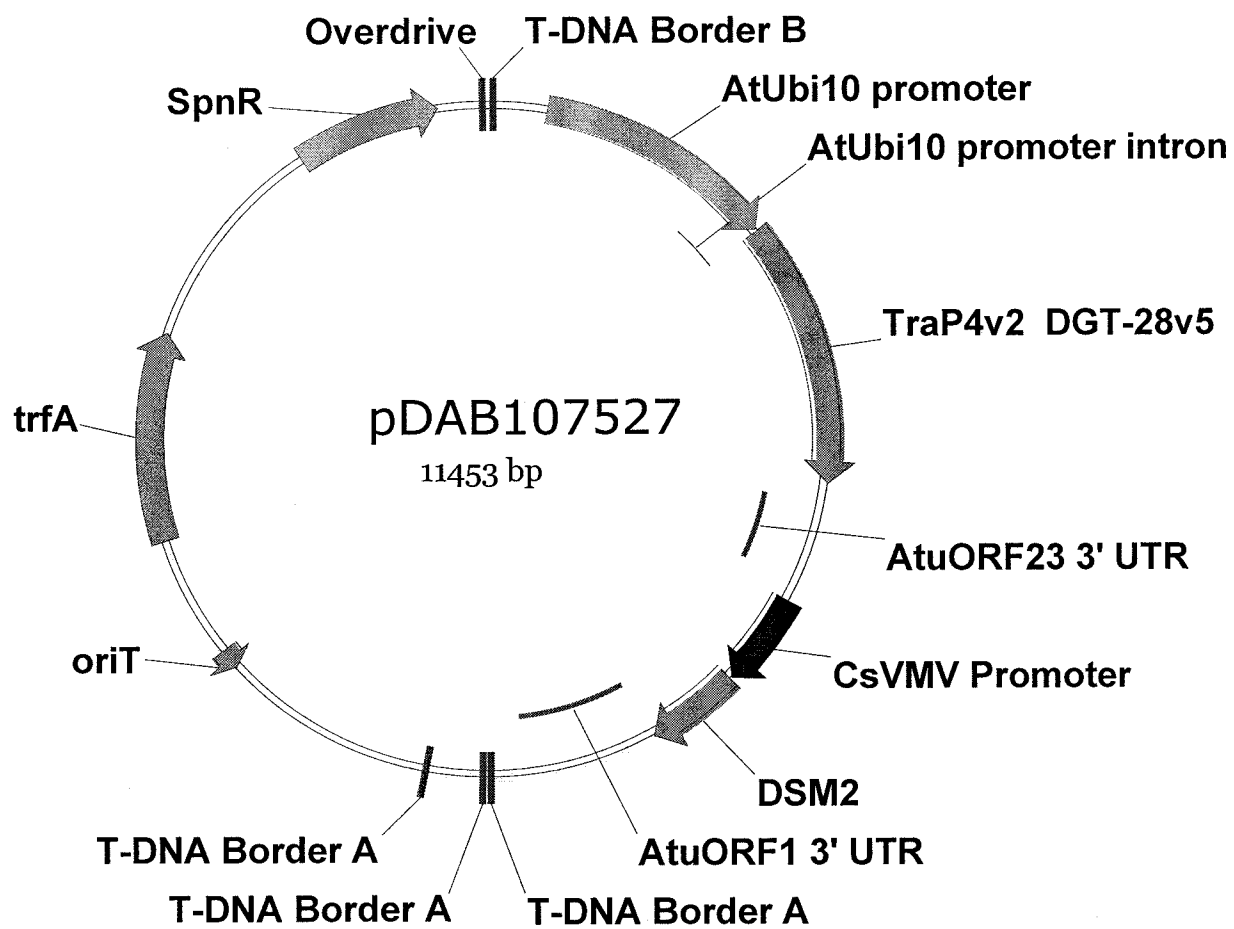


FIG.3

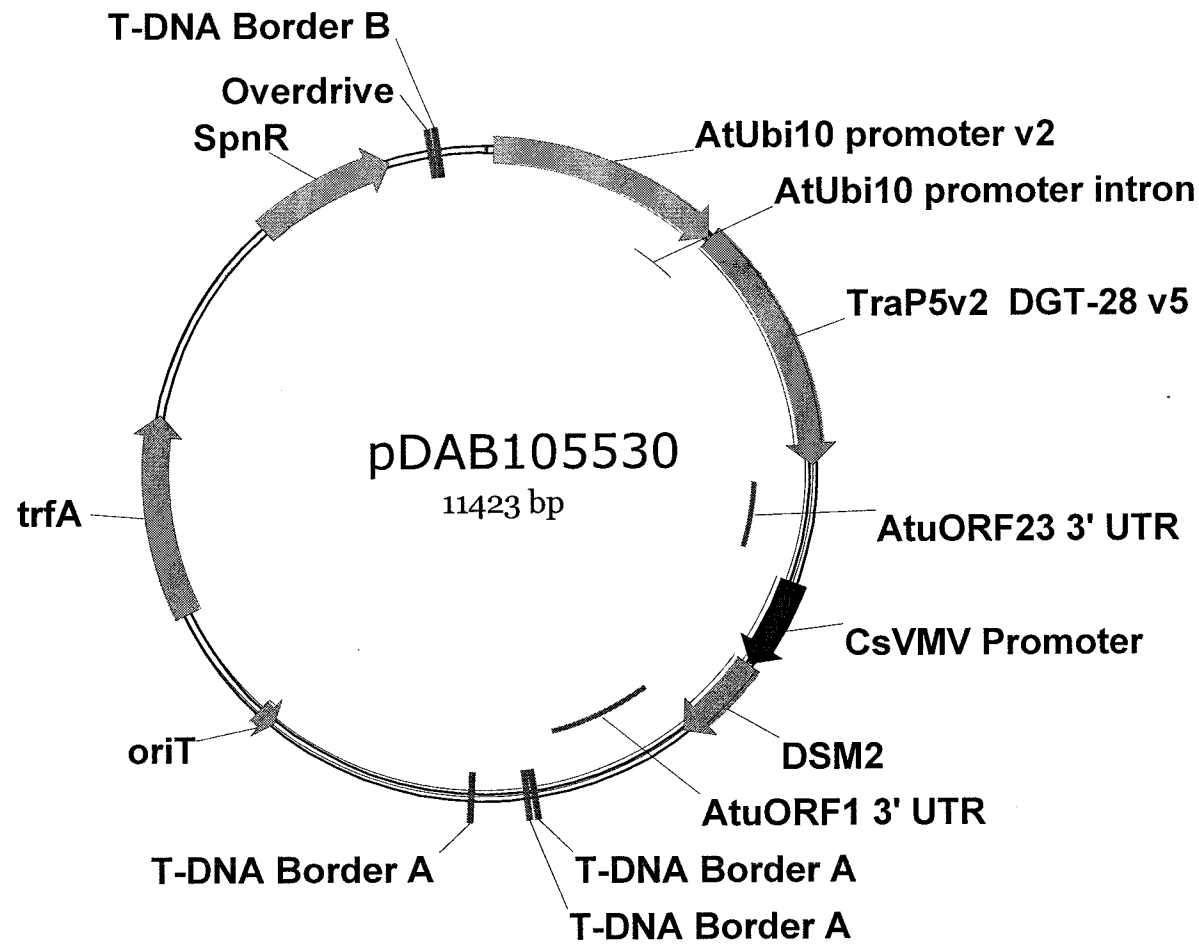


FIG.4

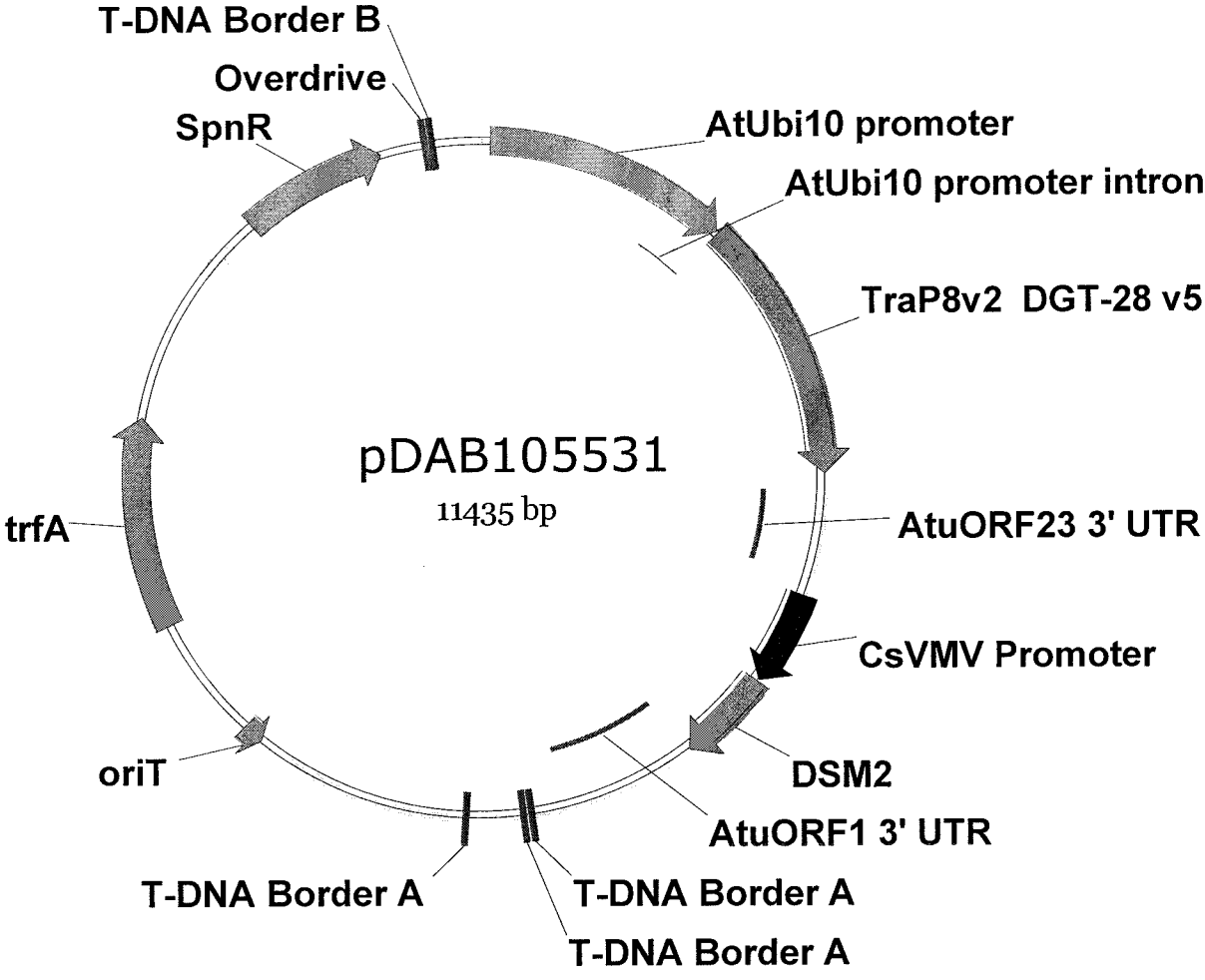


FIG.5

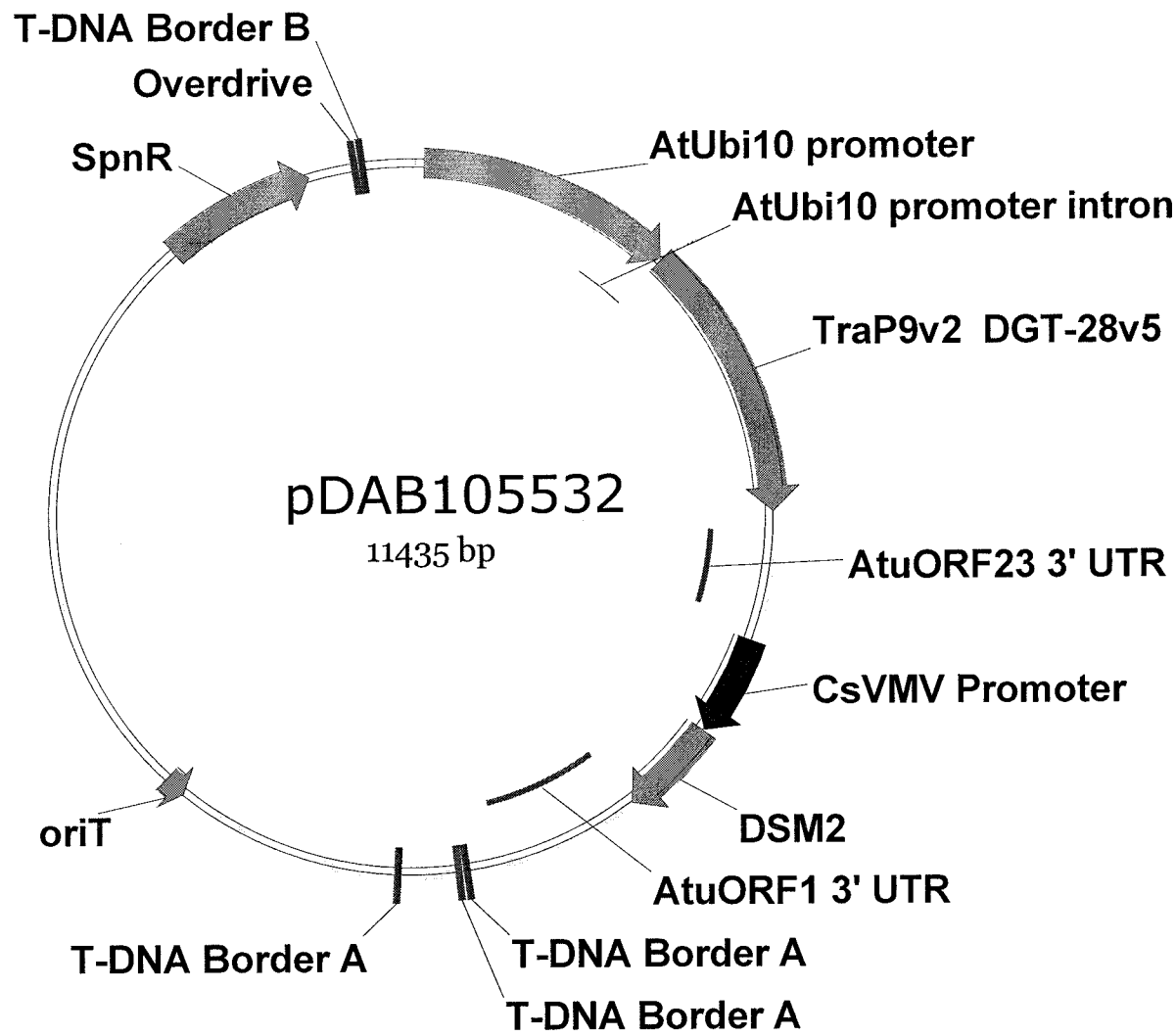


FIG.6

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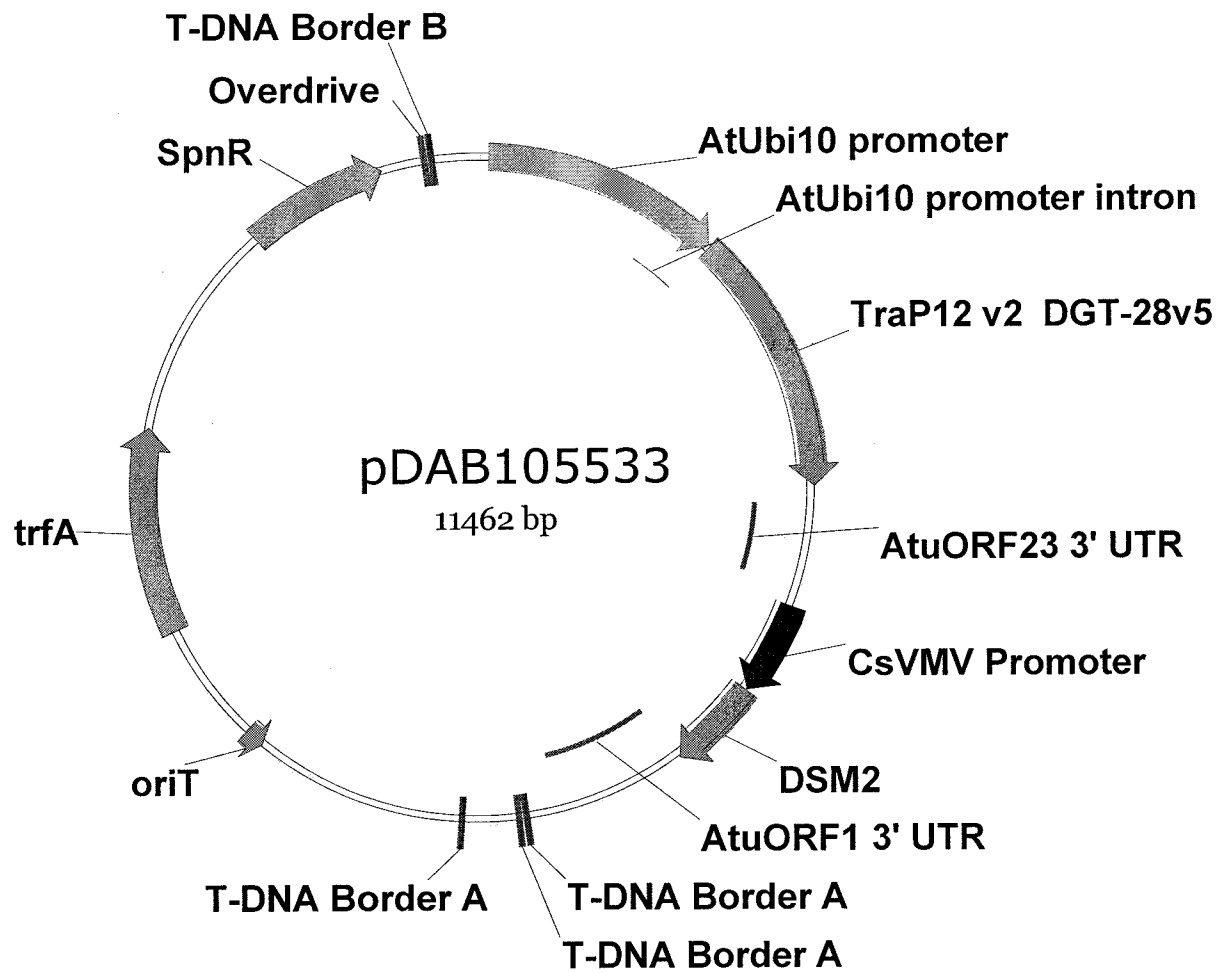


FIG.7

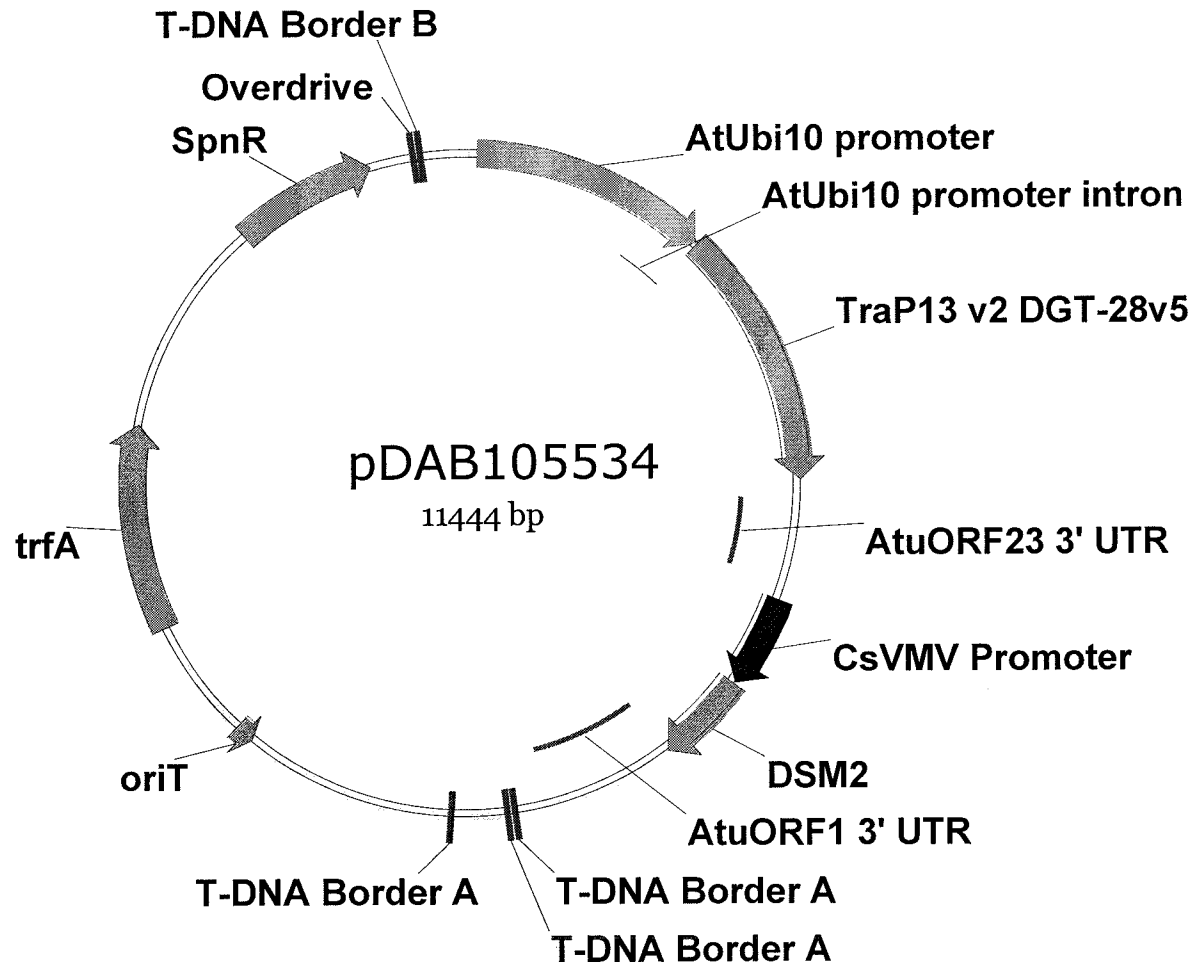


FIG.8

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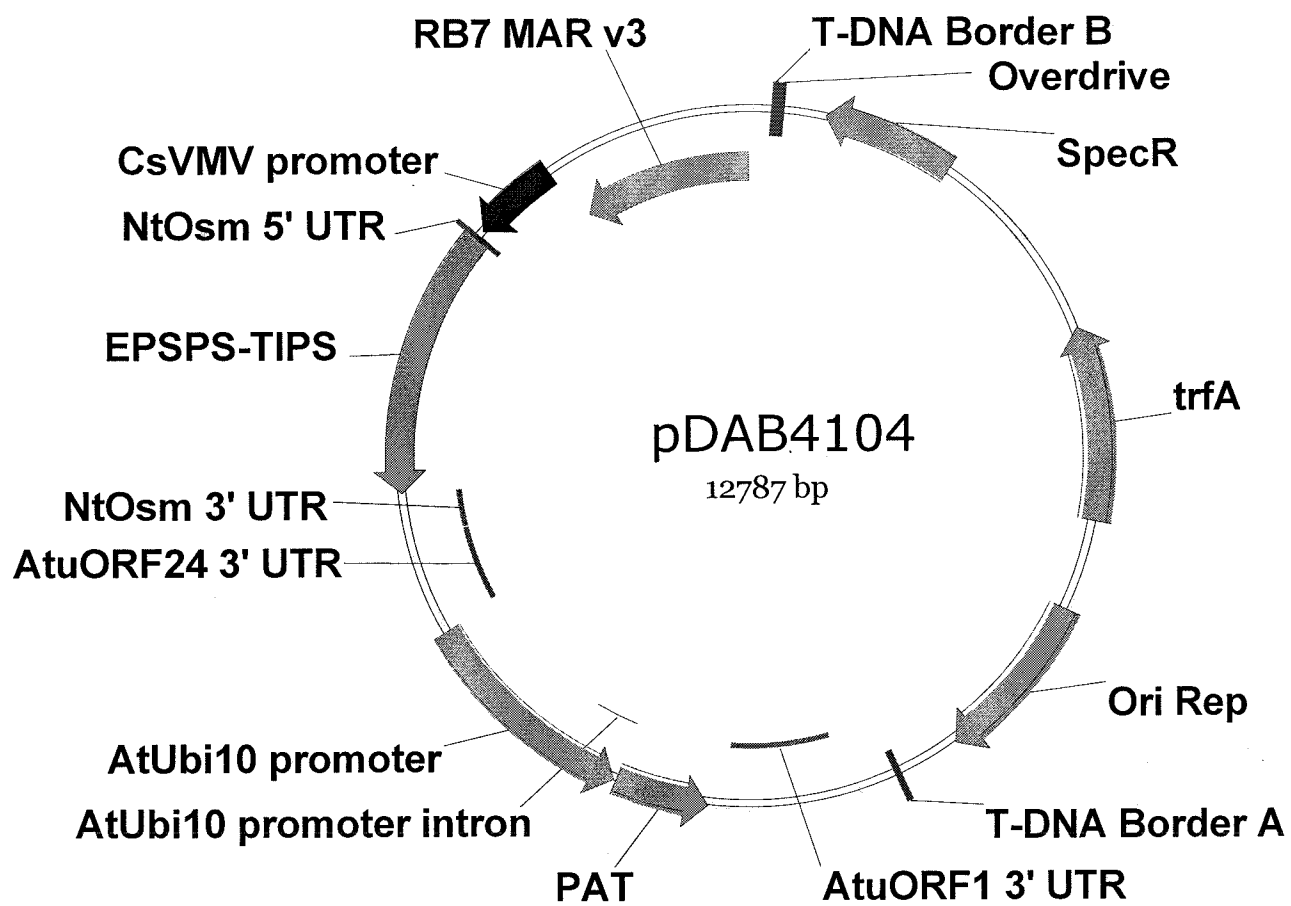


FIG.9

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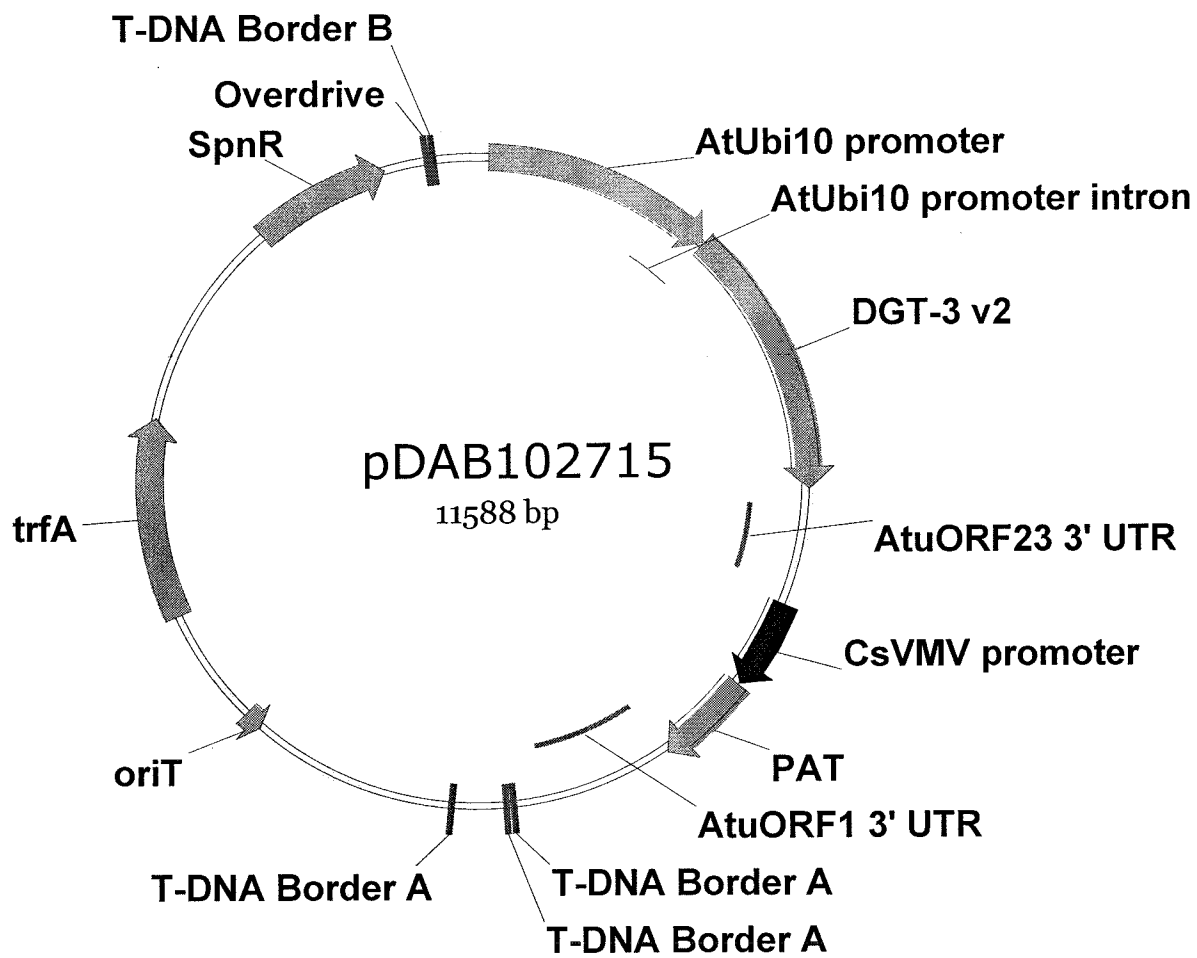


FIG. 10

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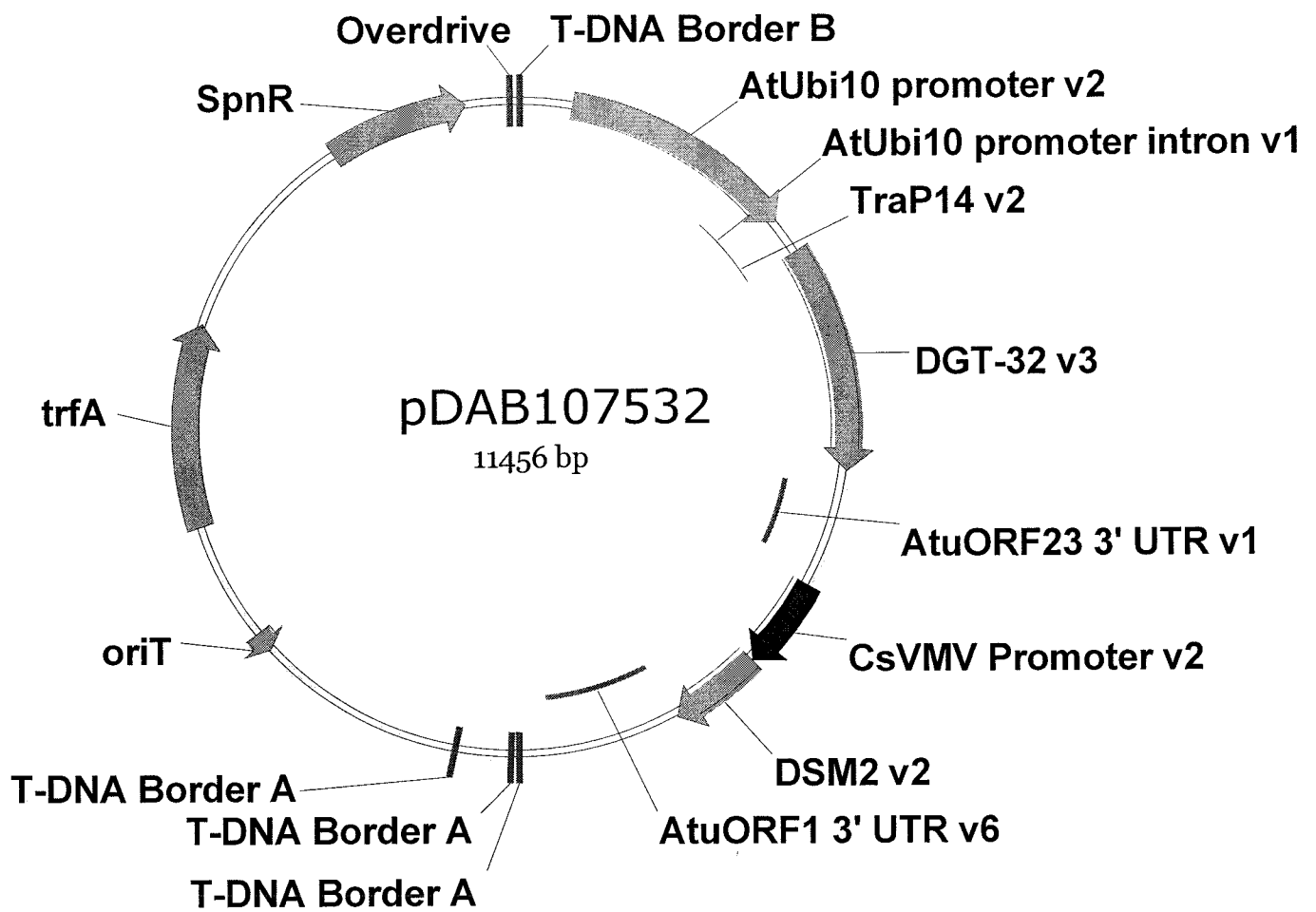


FIG. 11

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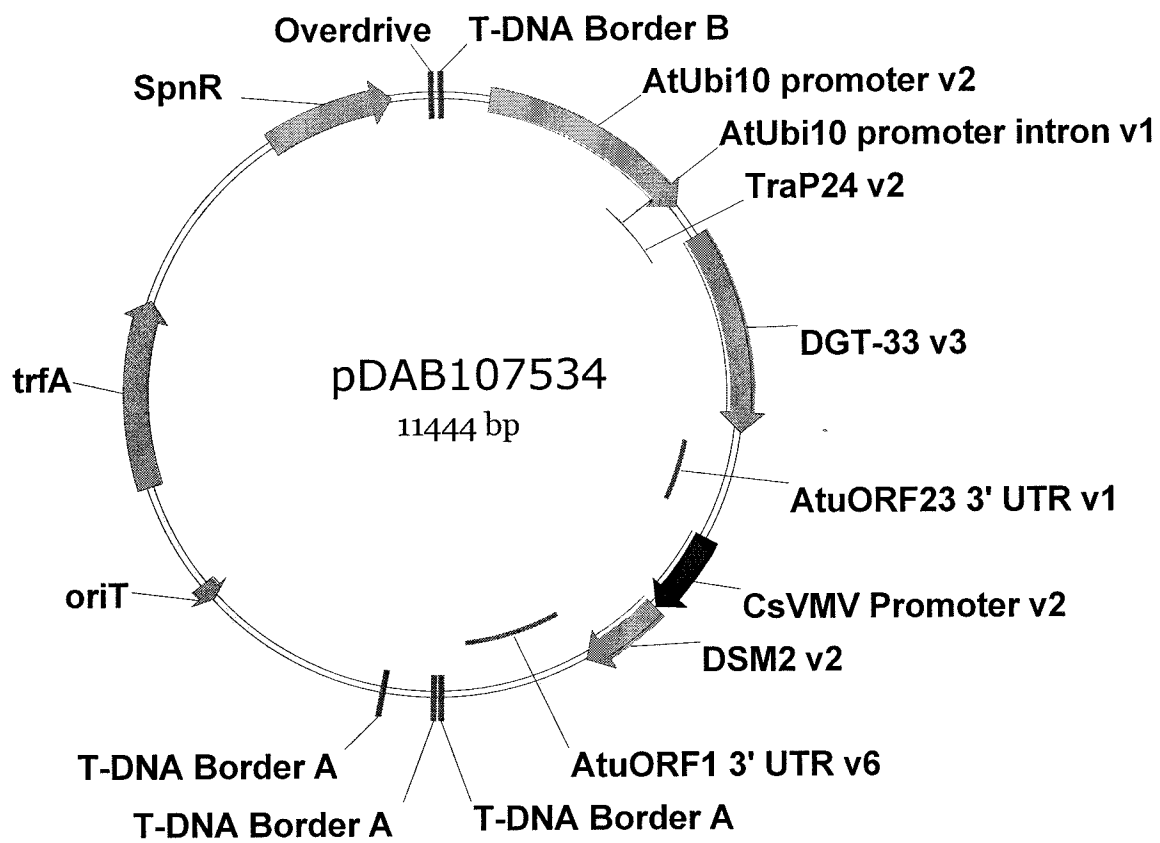


FIG. 12

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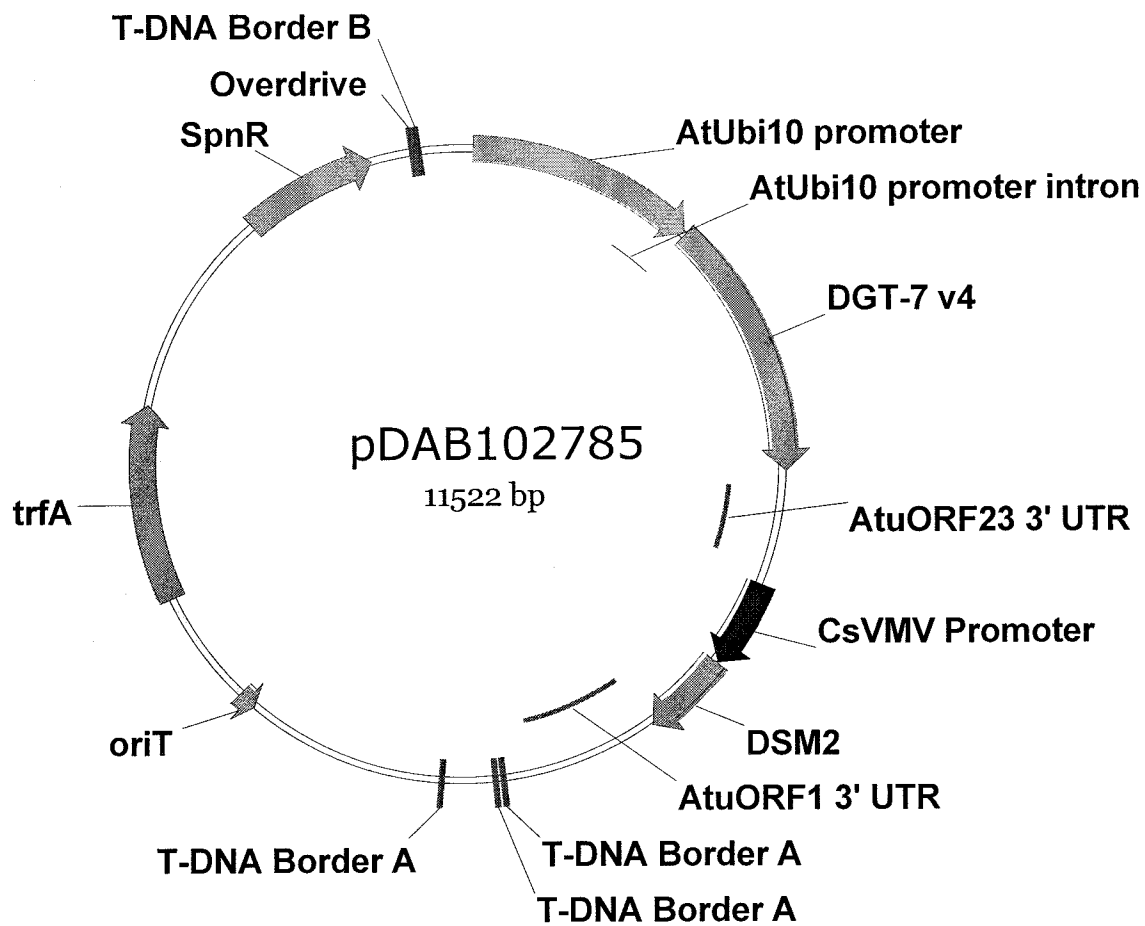


FIG. 13

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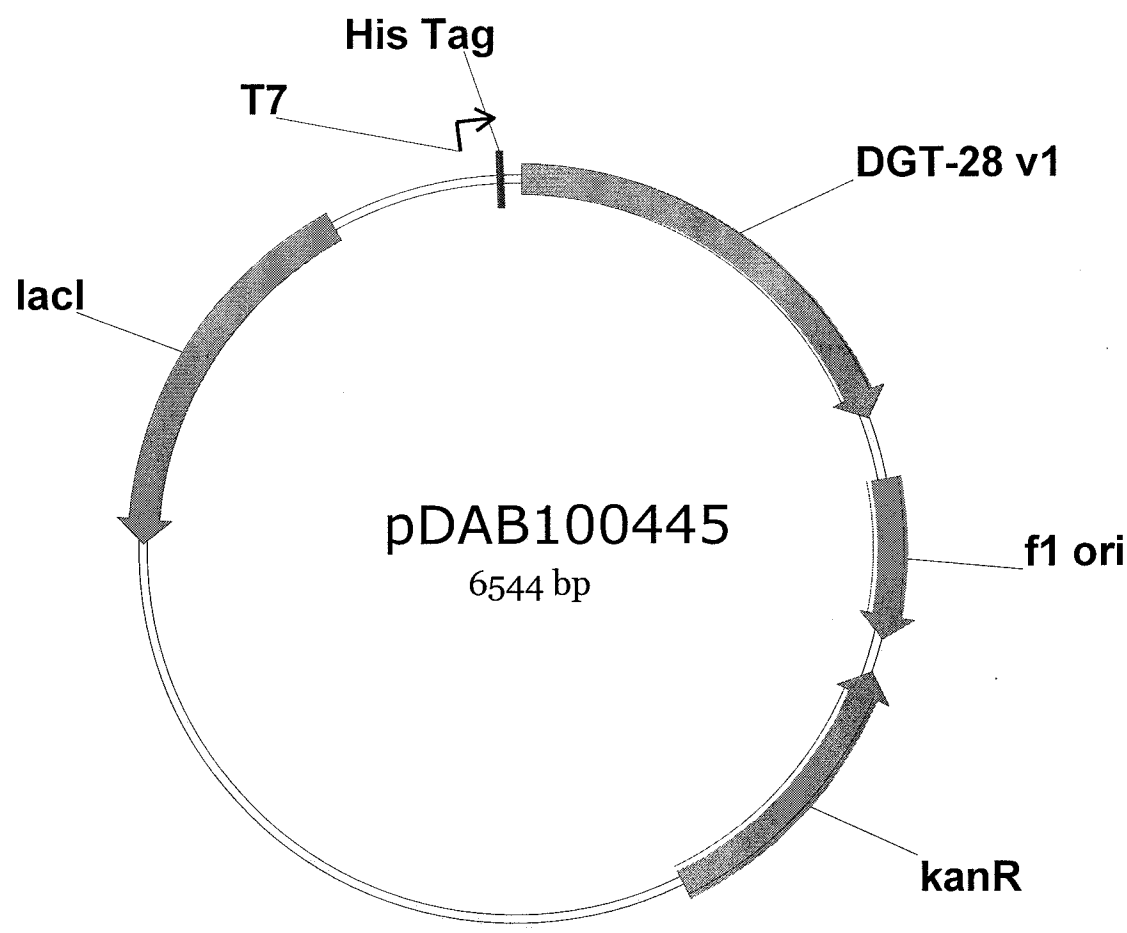


FIG. 14

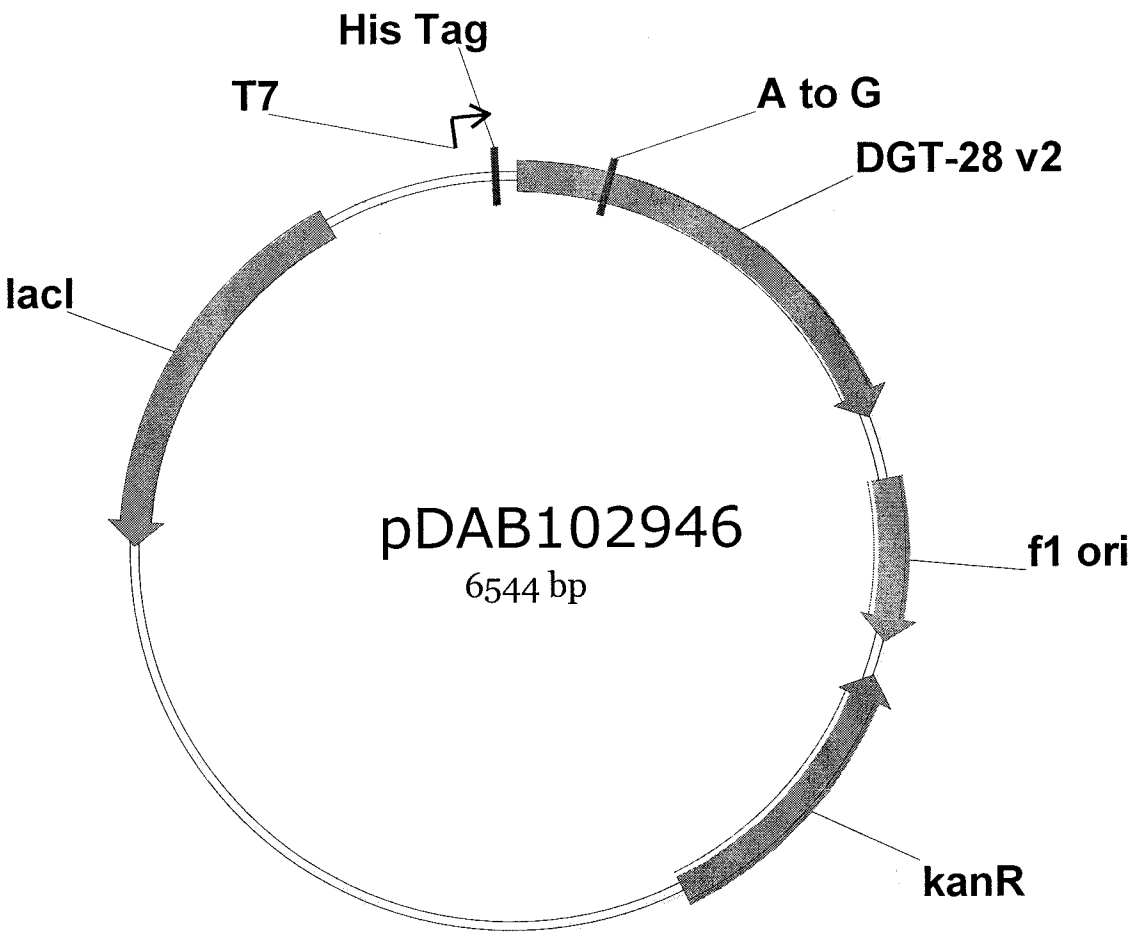


FIG. 15

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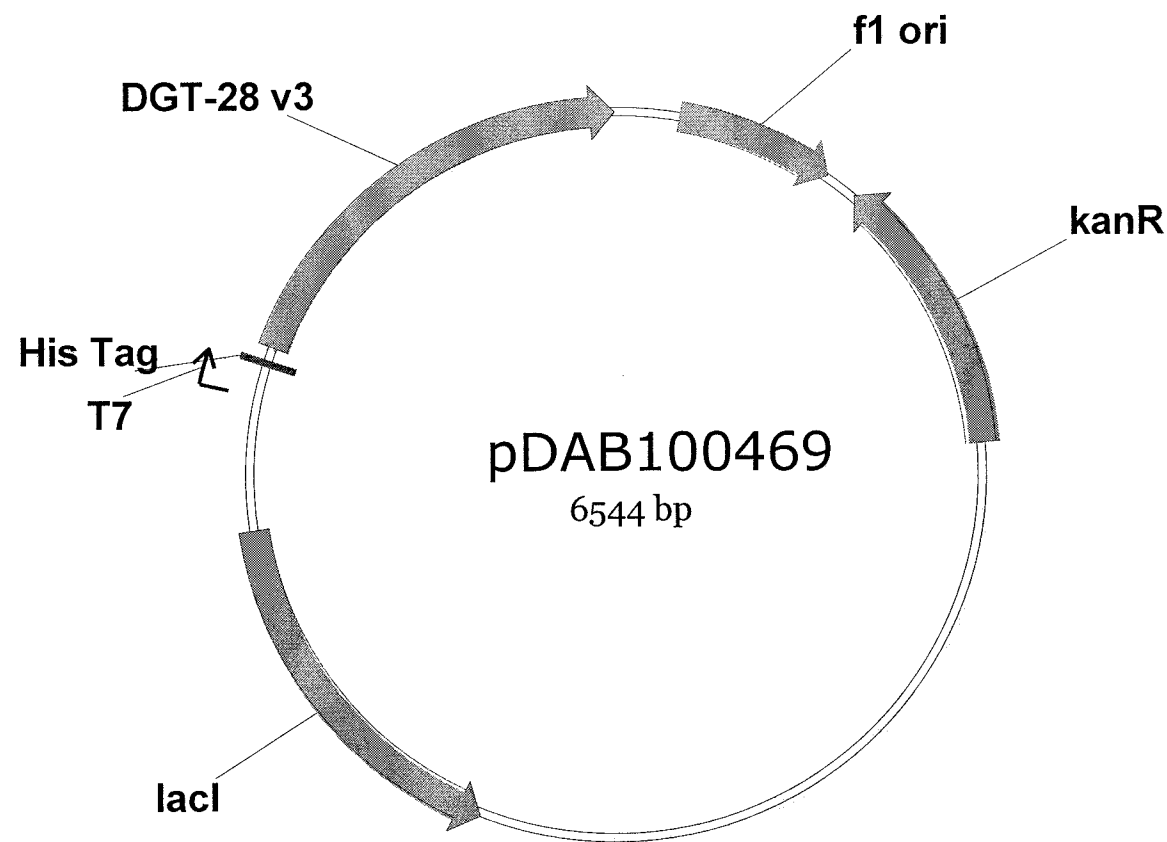


FIG. 16

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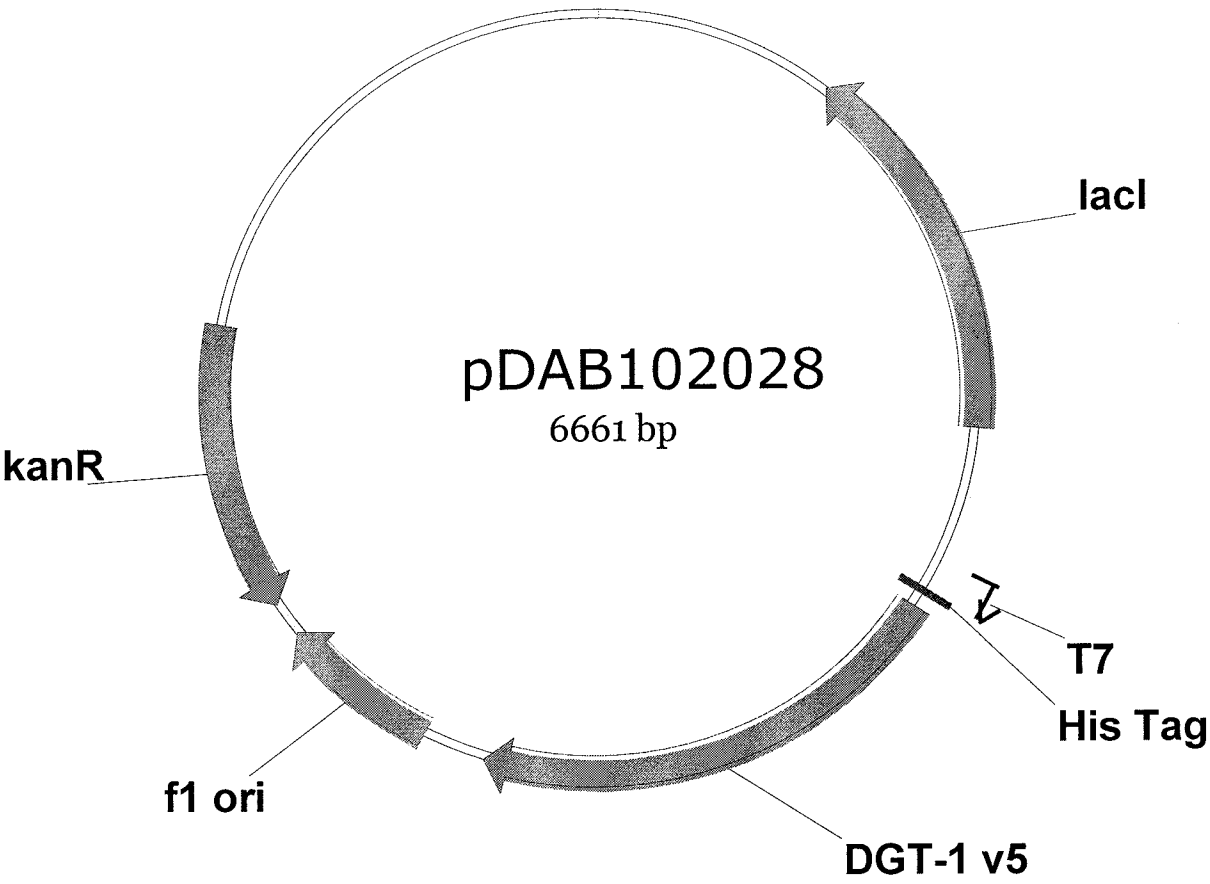


FIG. 17

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FIG. 18

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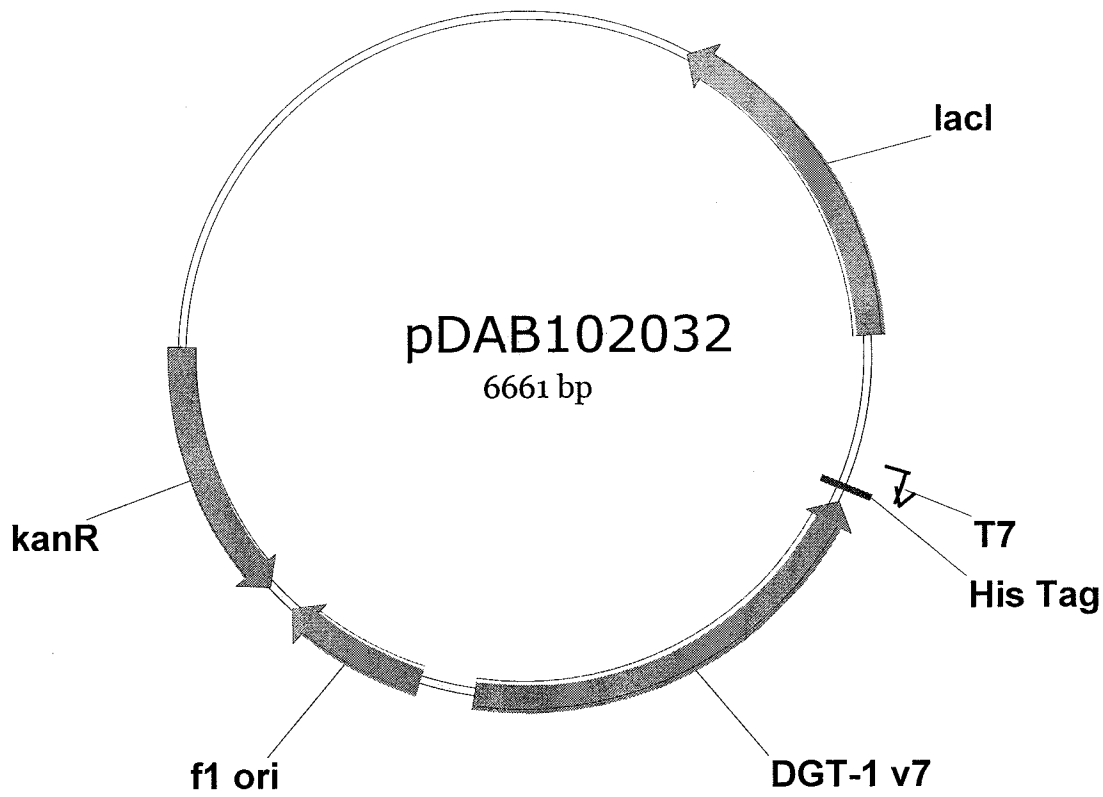


FIG. 19

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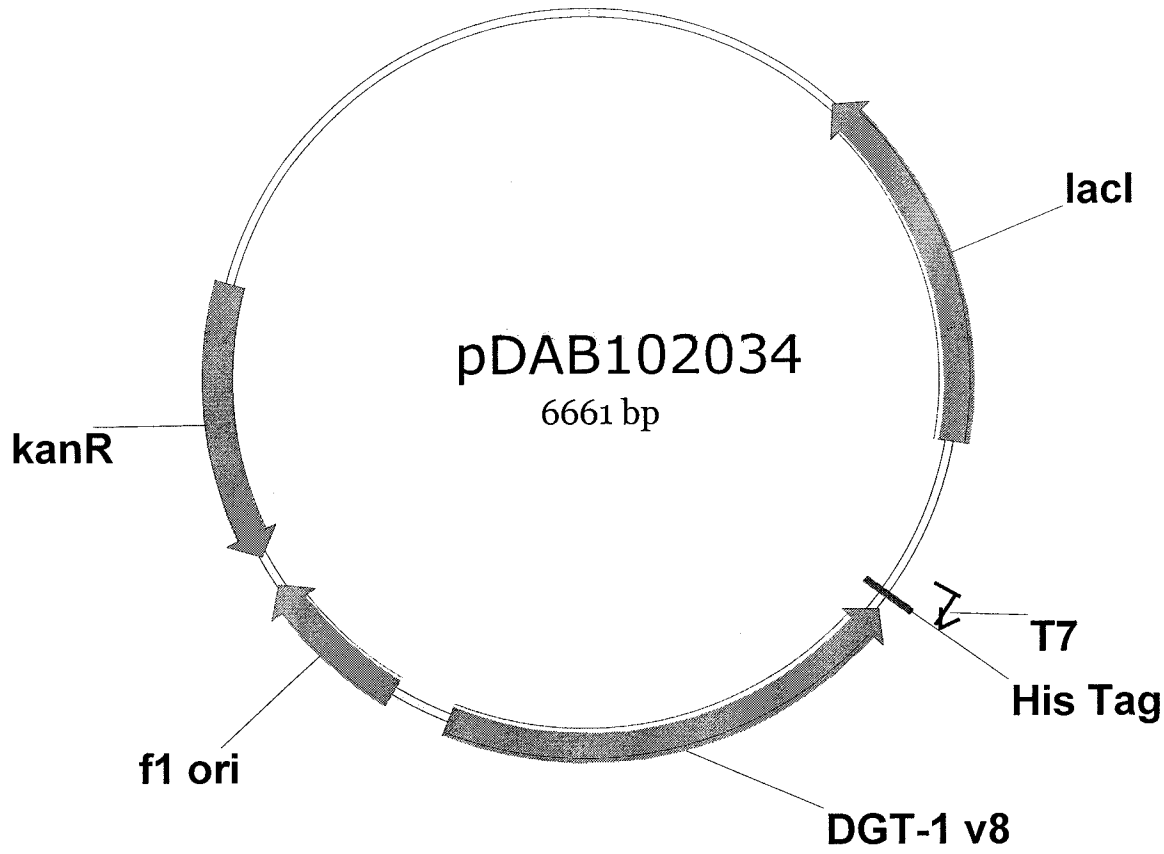


FIG. 20

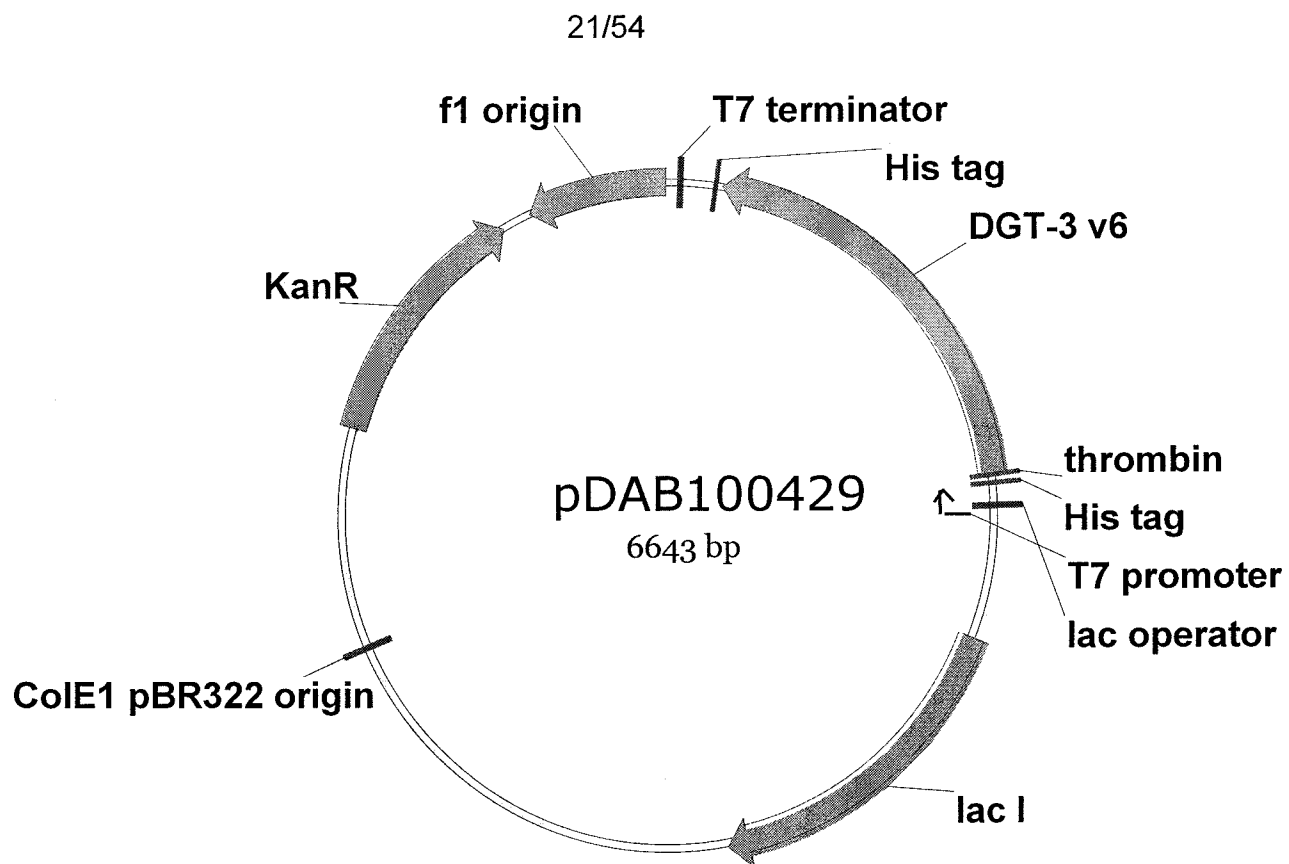


FIG. 21



FIG. 22

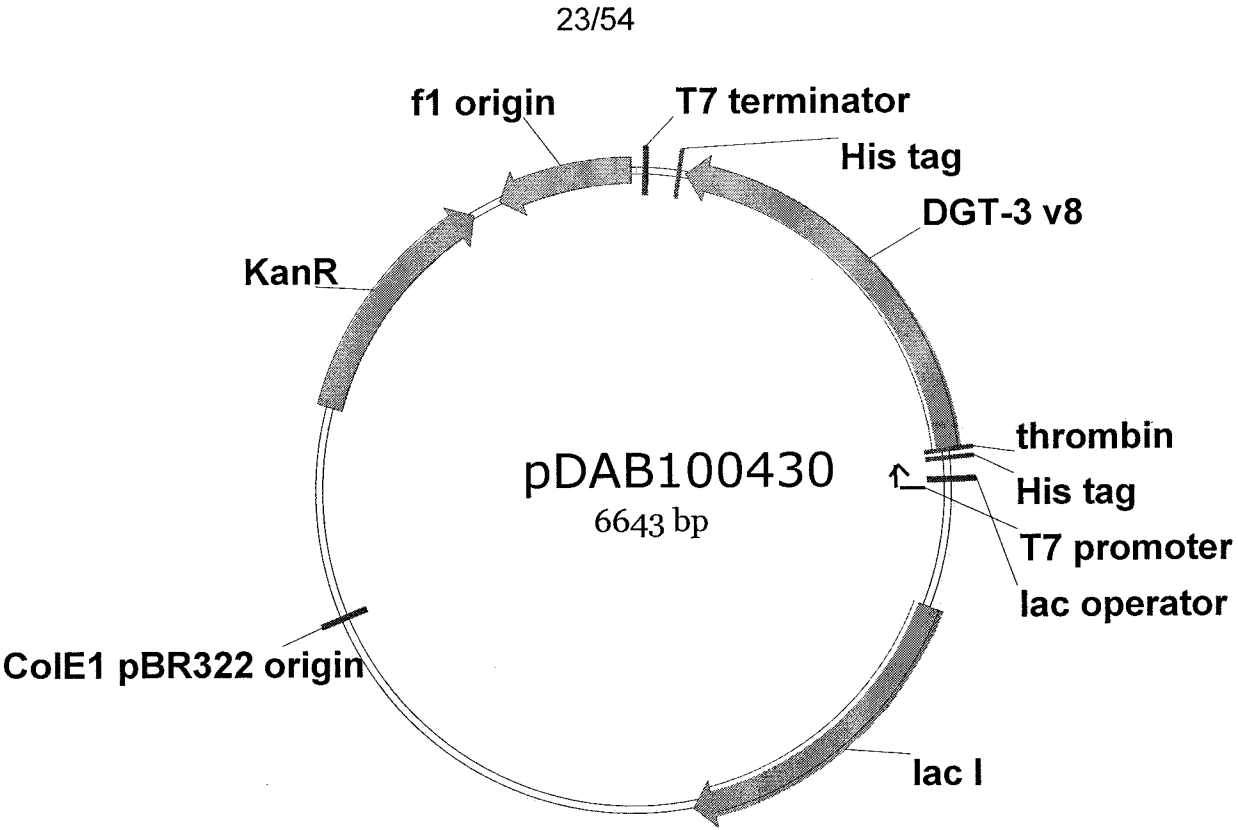


FIG. 23

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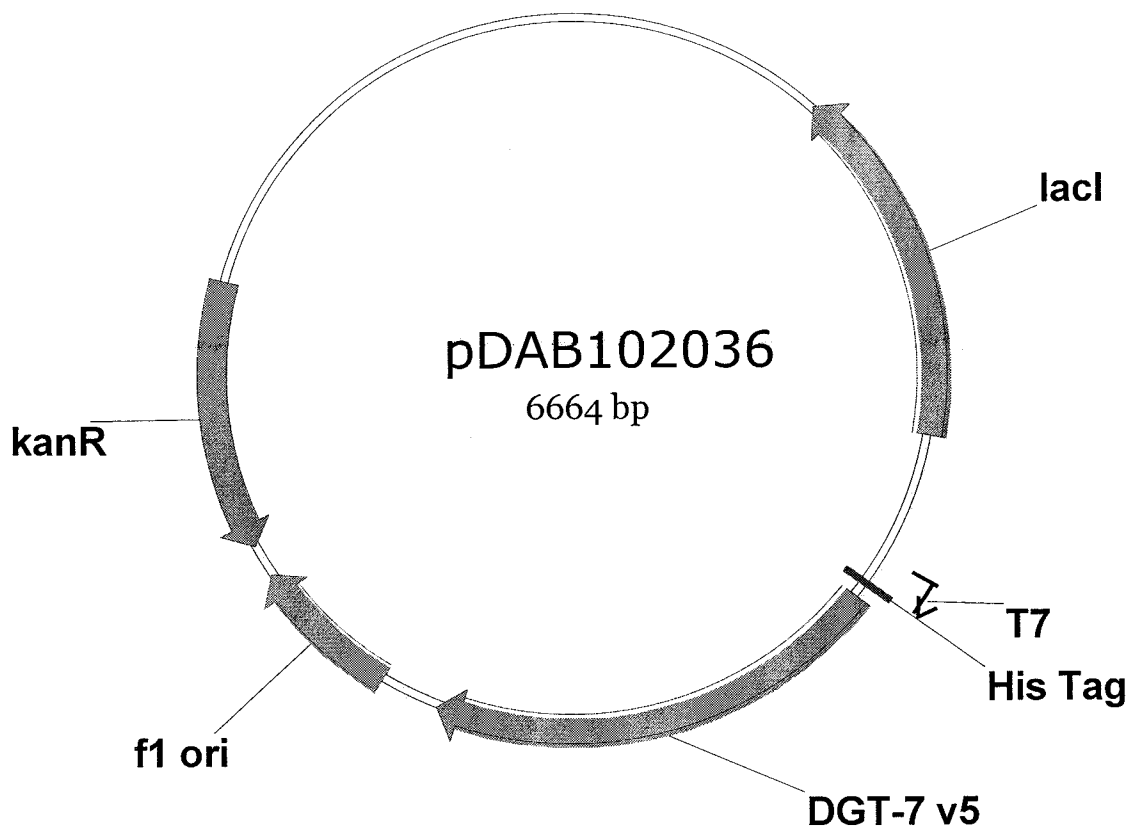


FIG. 24

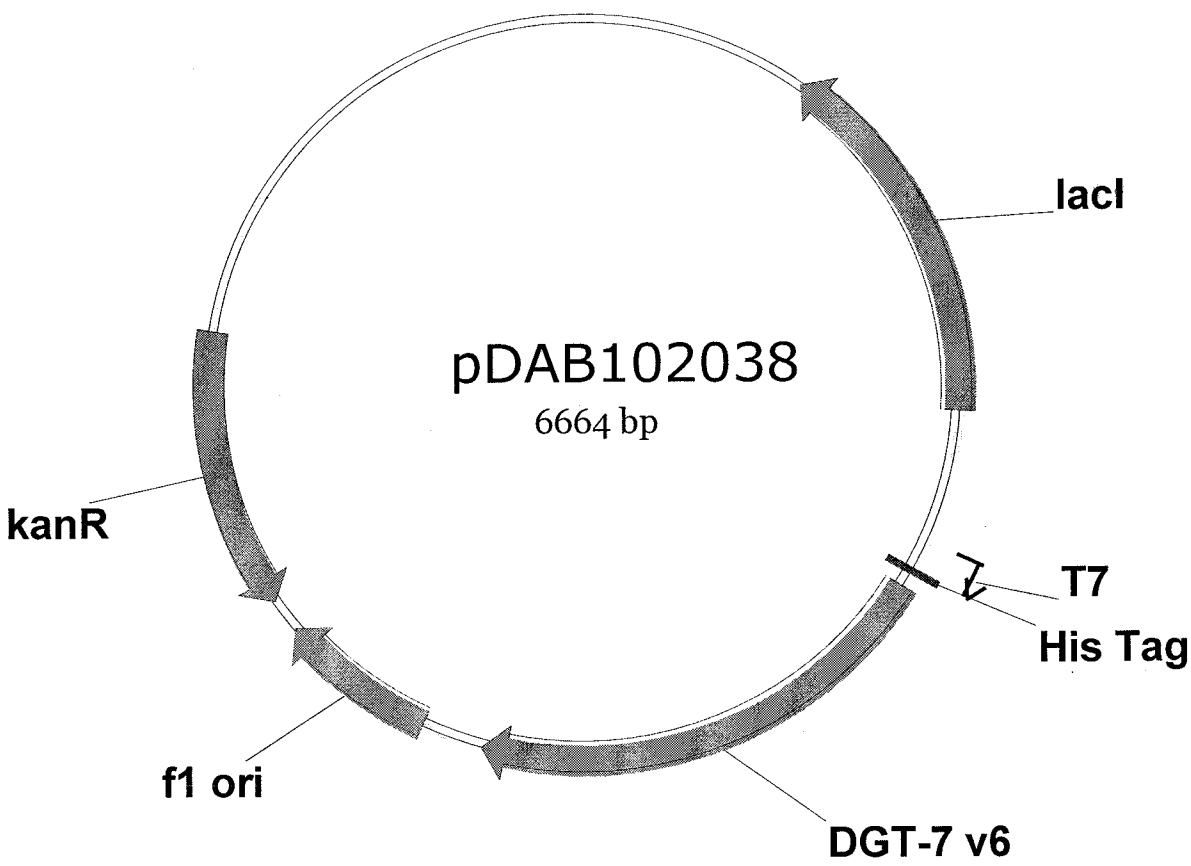


FIG. 25

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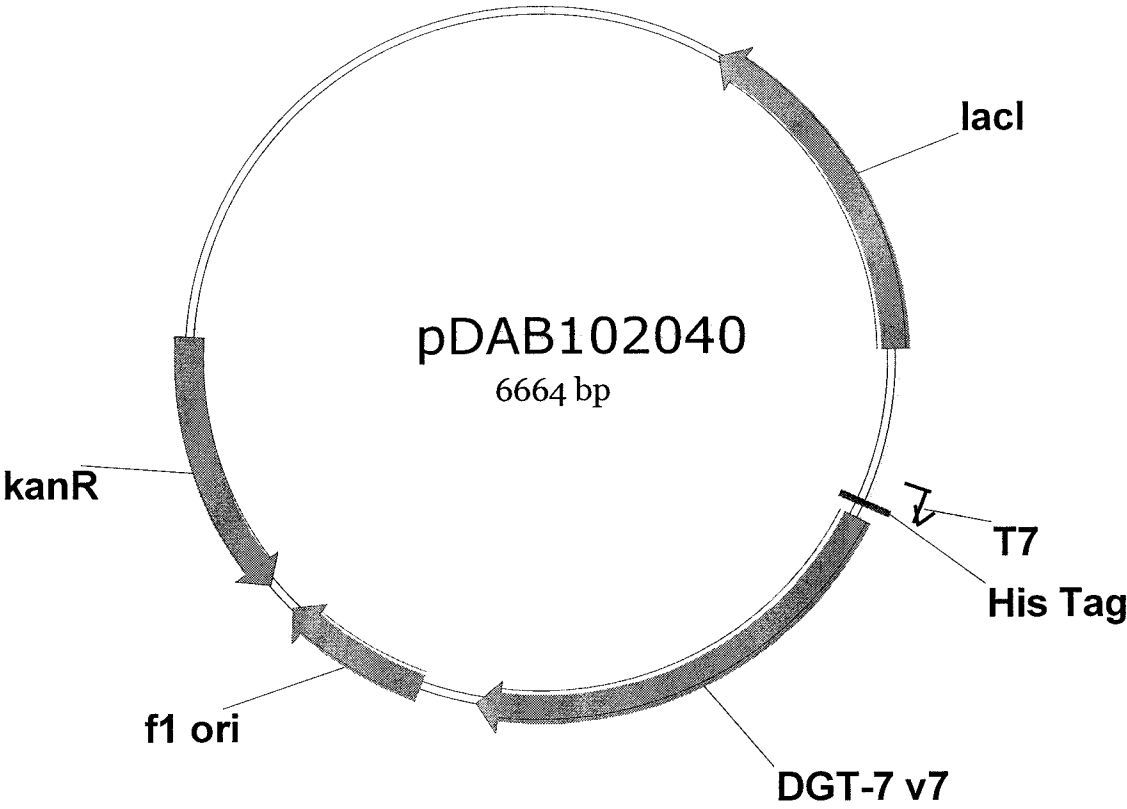


FIG. 26

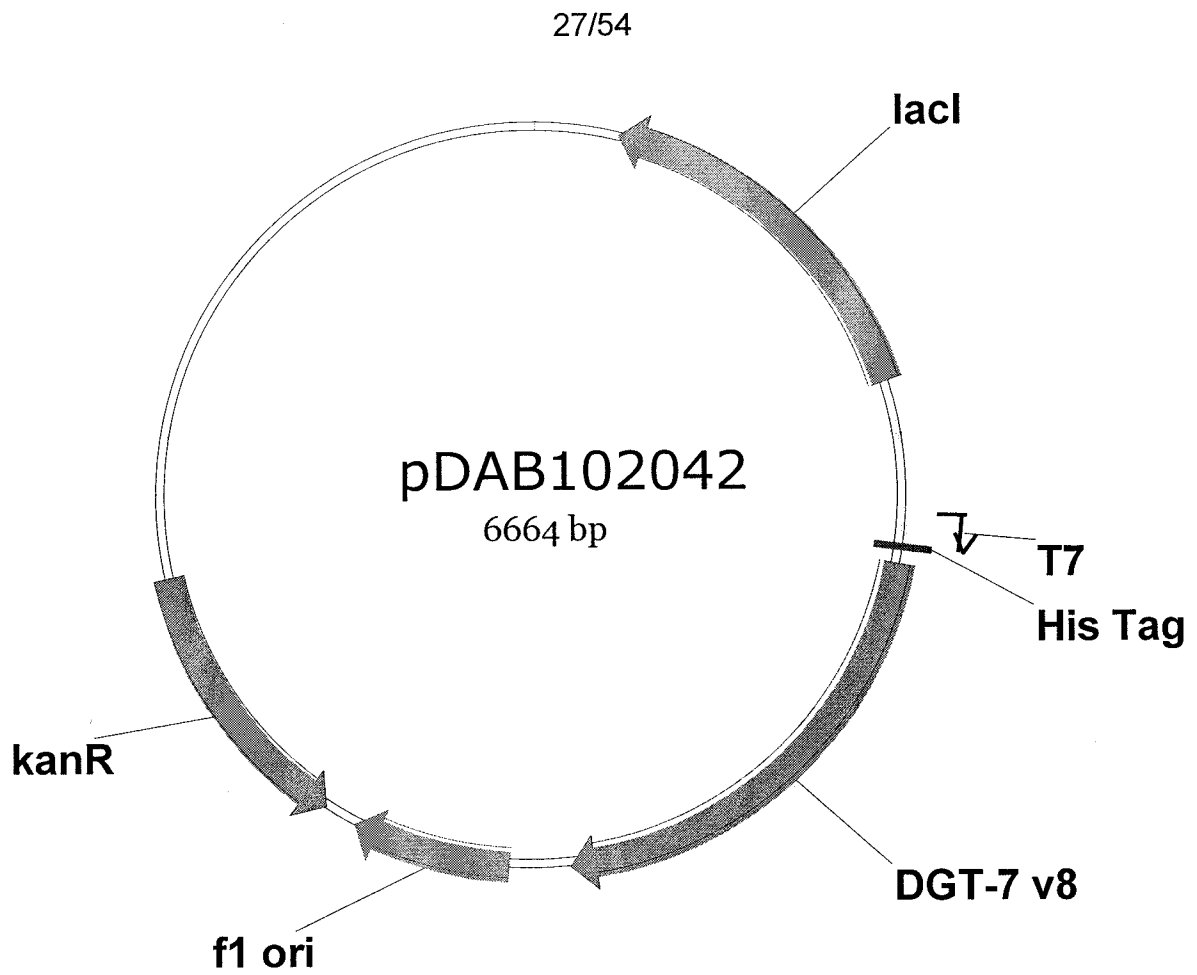


FIG. 27

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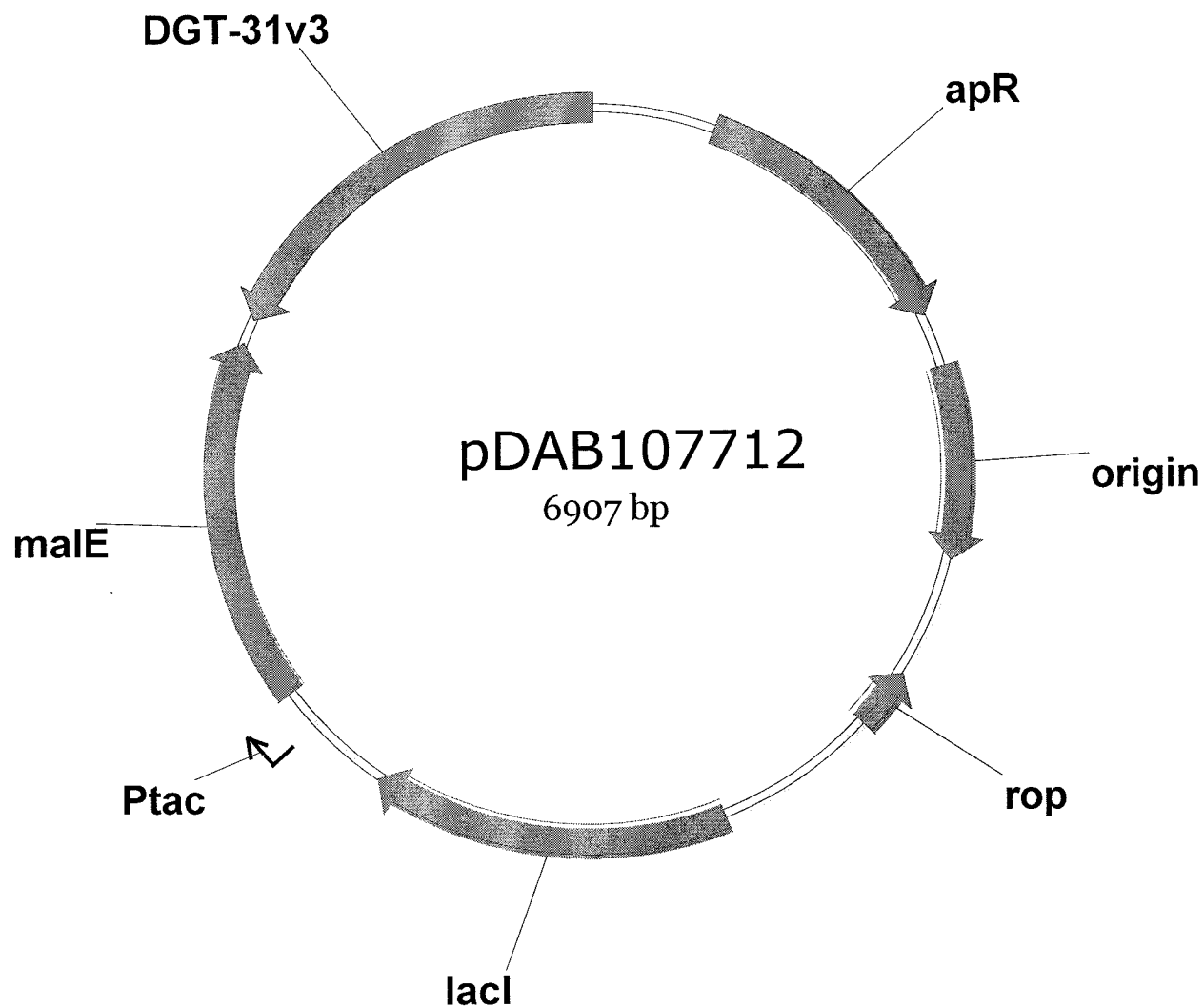


FIG. 28

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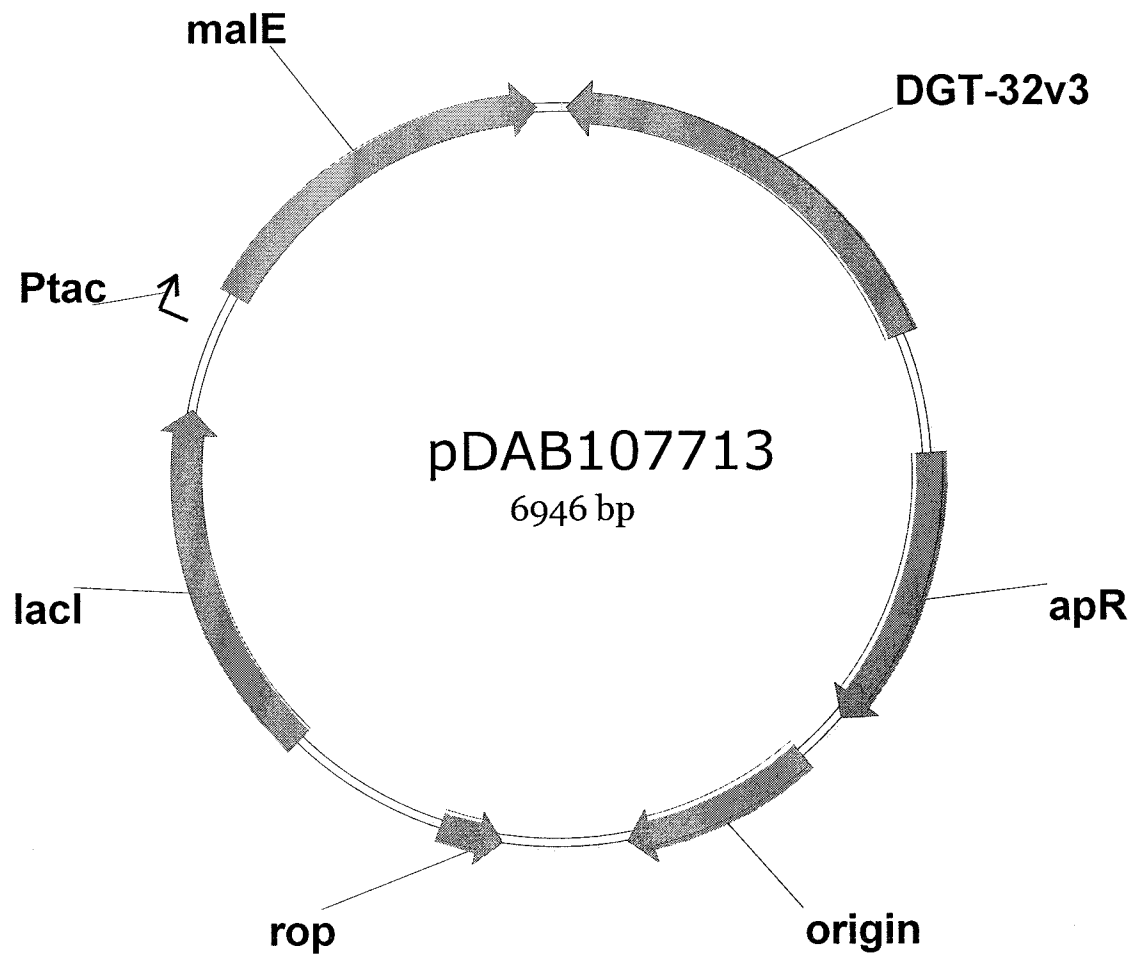


FIG. 29

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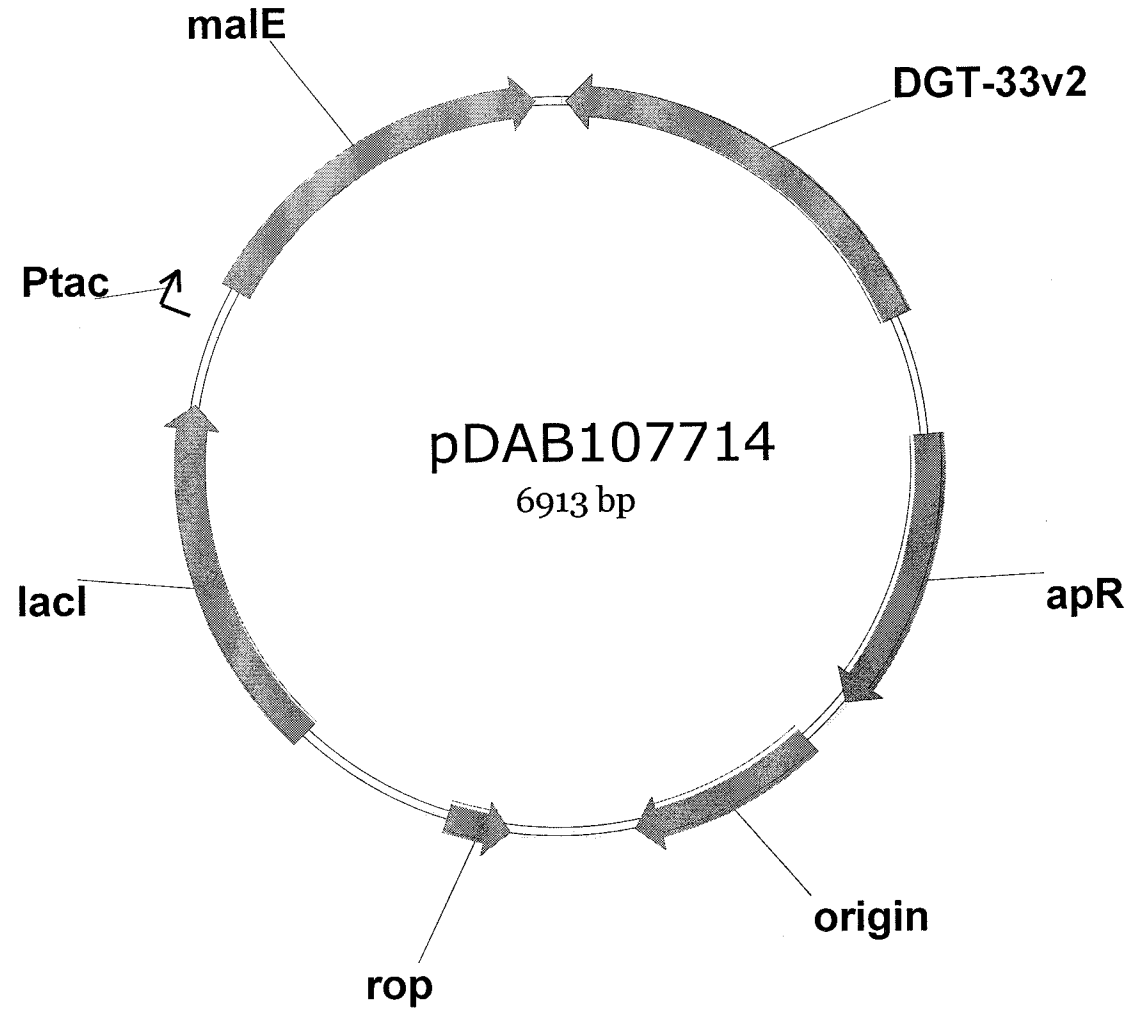


FIG. 30

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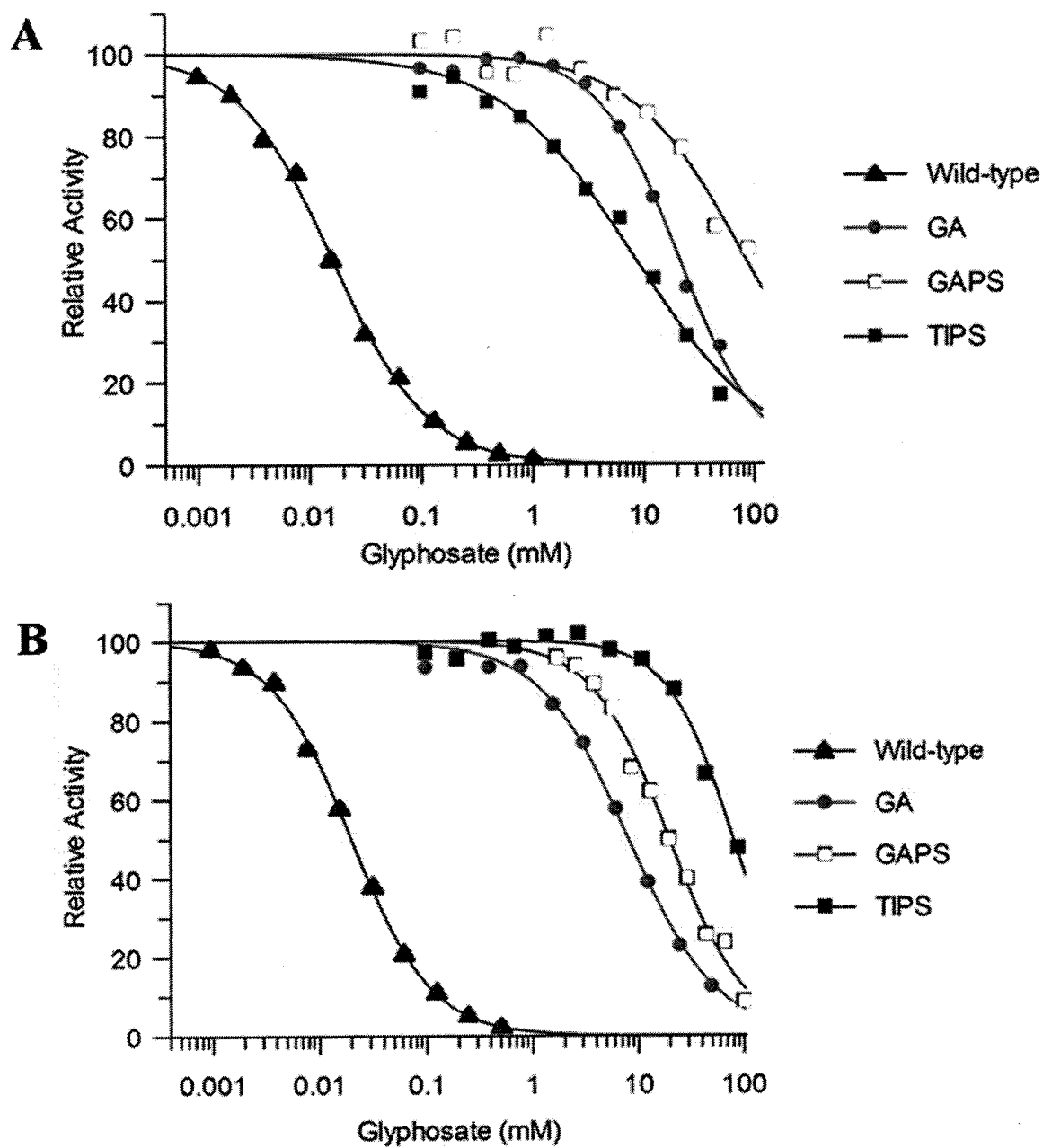


FIG. 31

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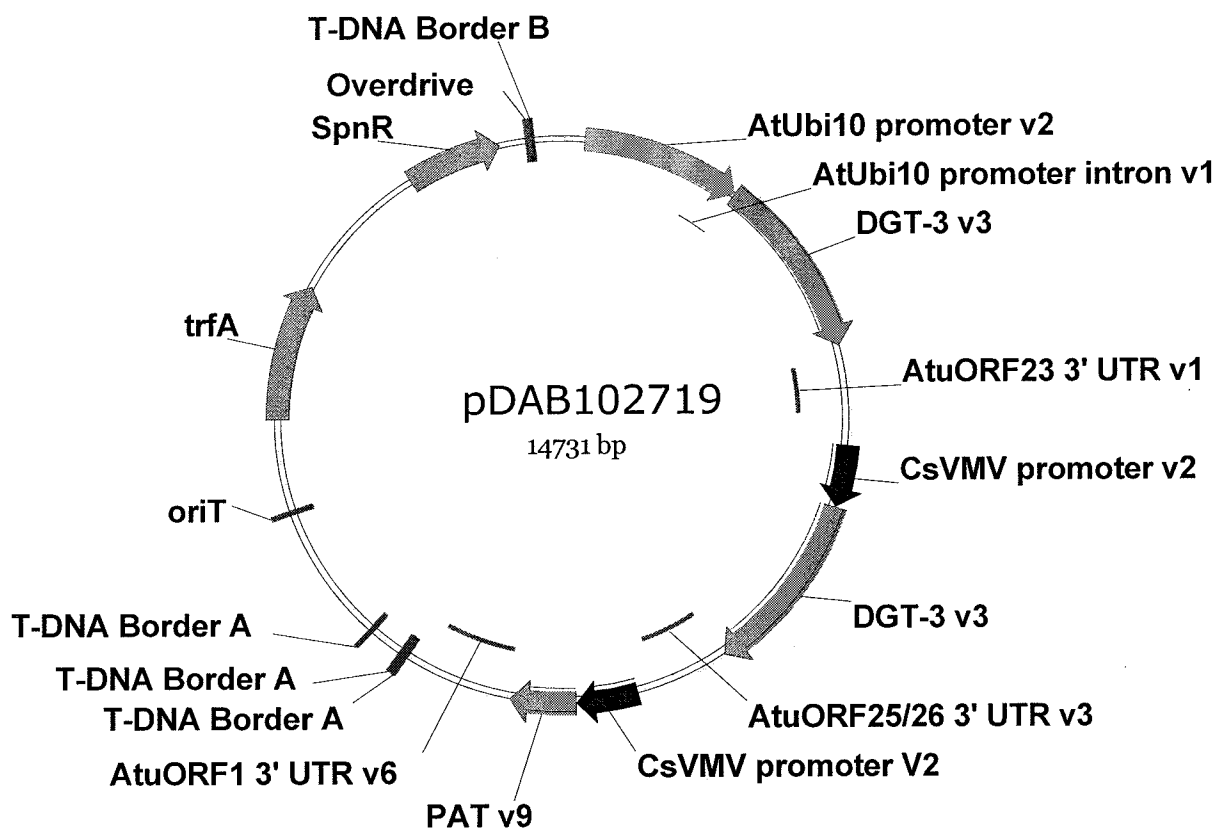


FIG. 32

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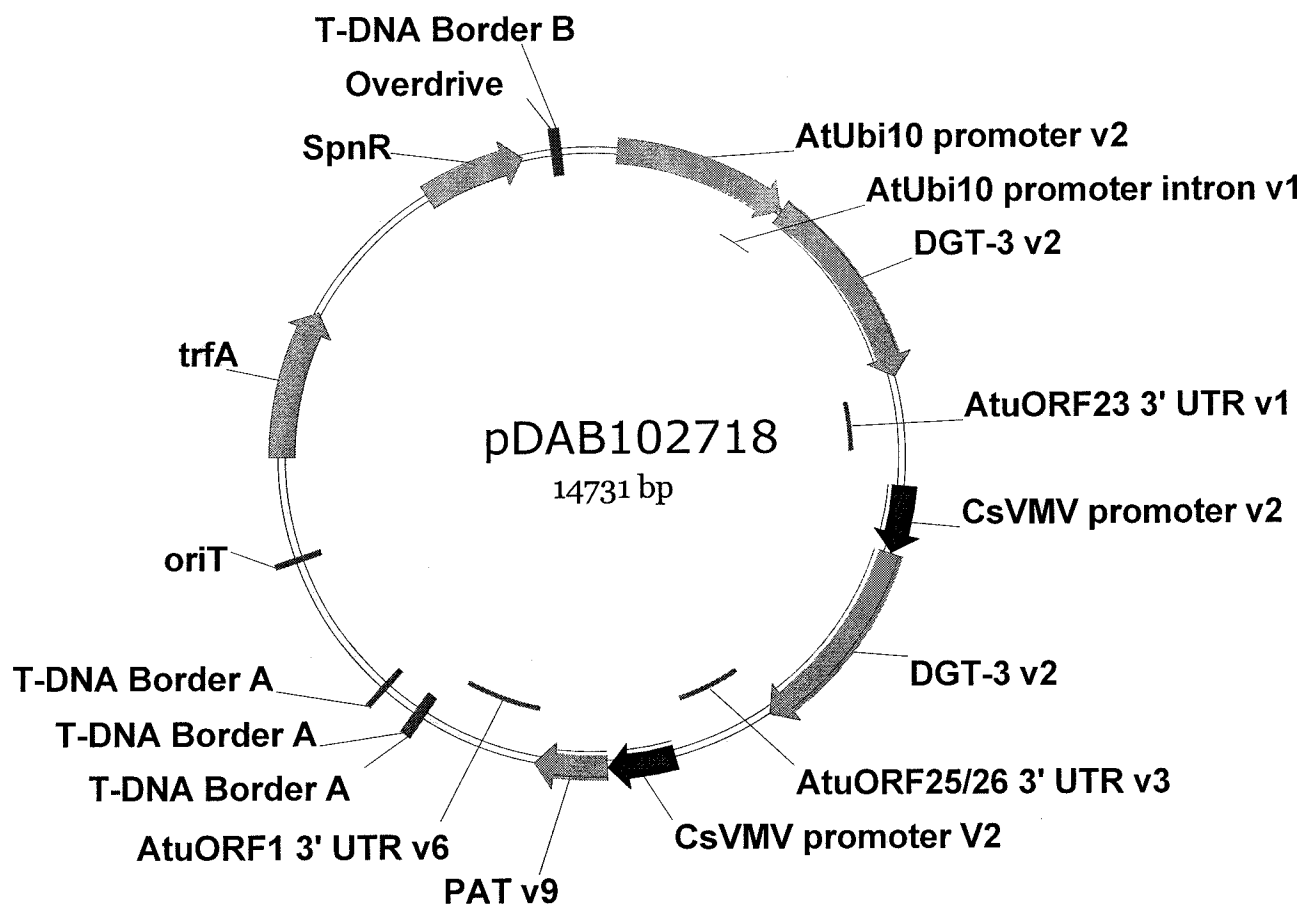


FIG. 33

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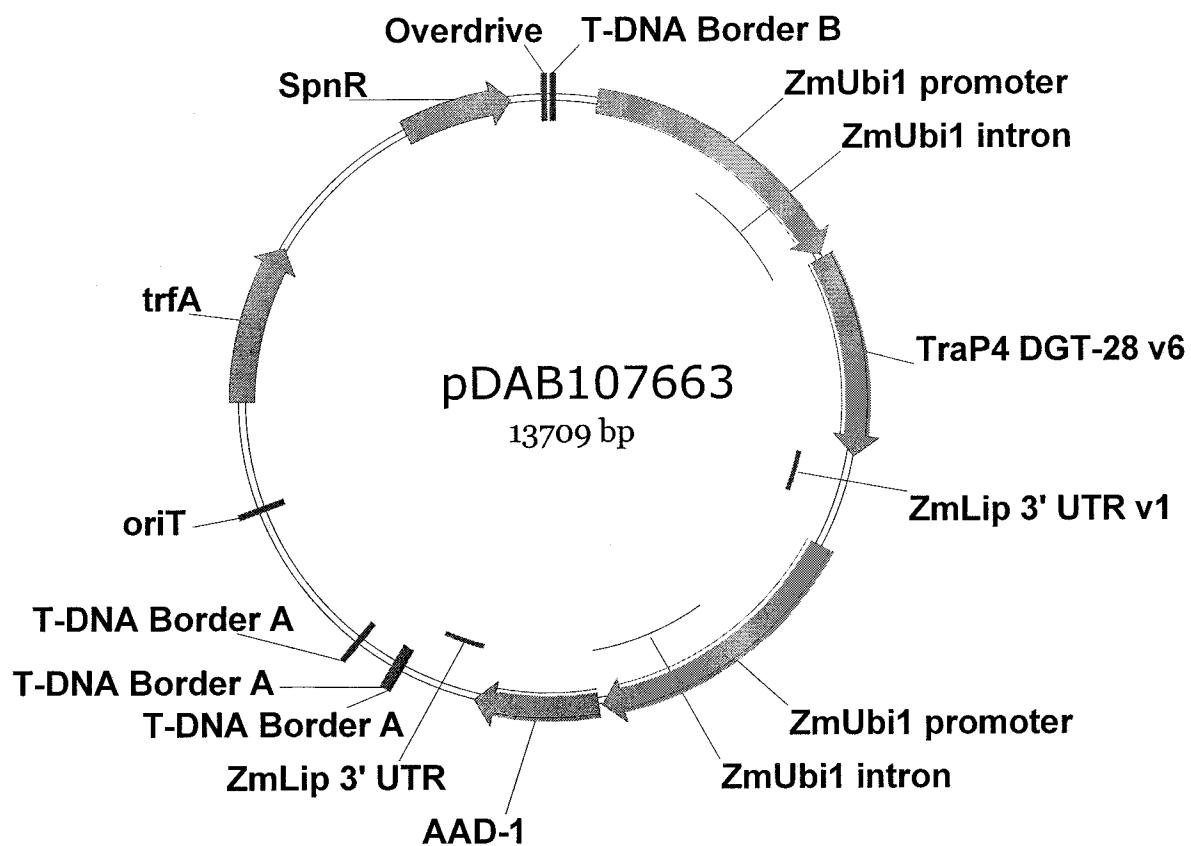


FIG. 34

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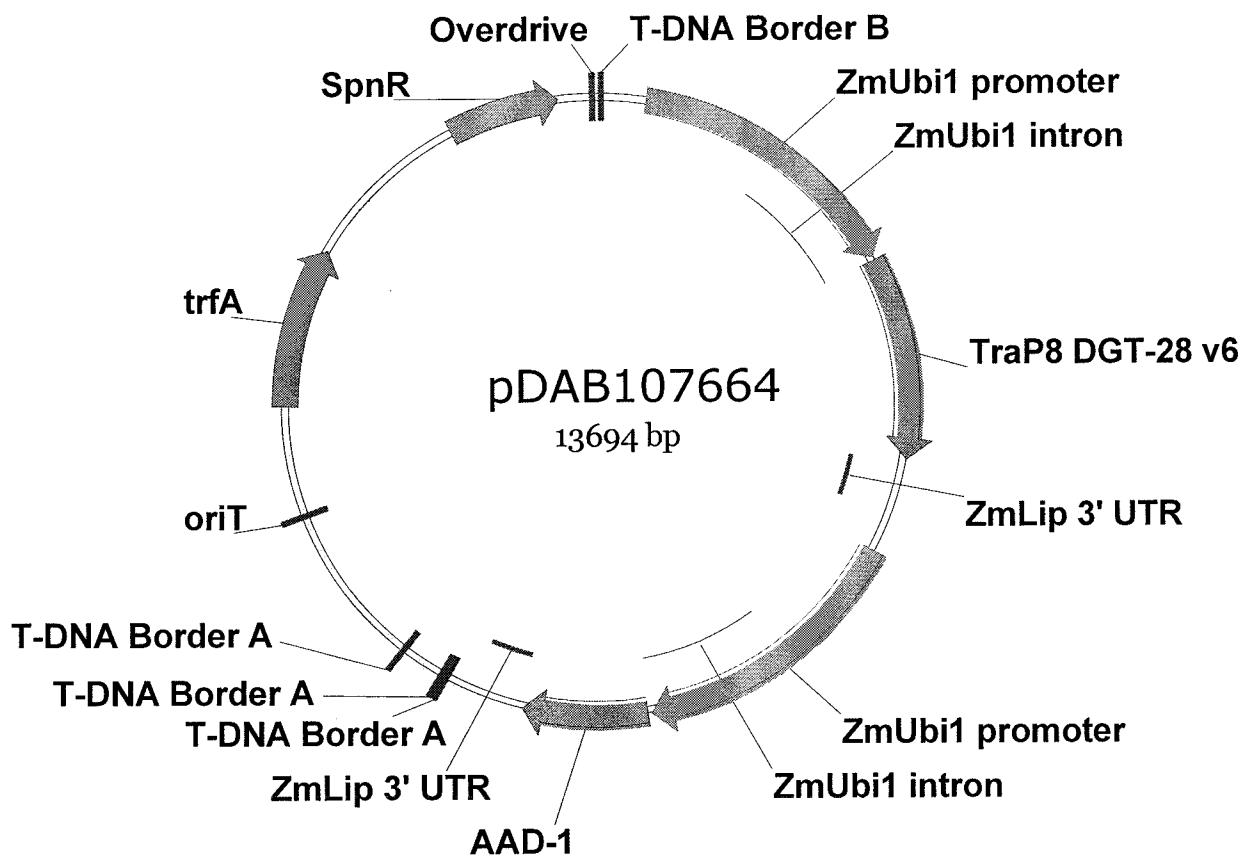


FIG. 35

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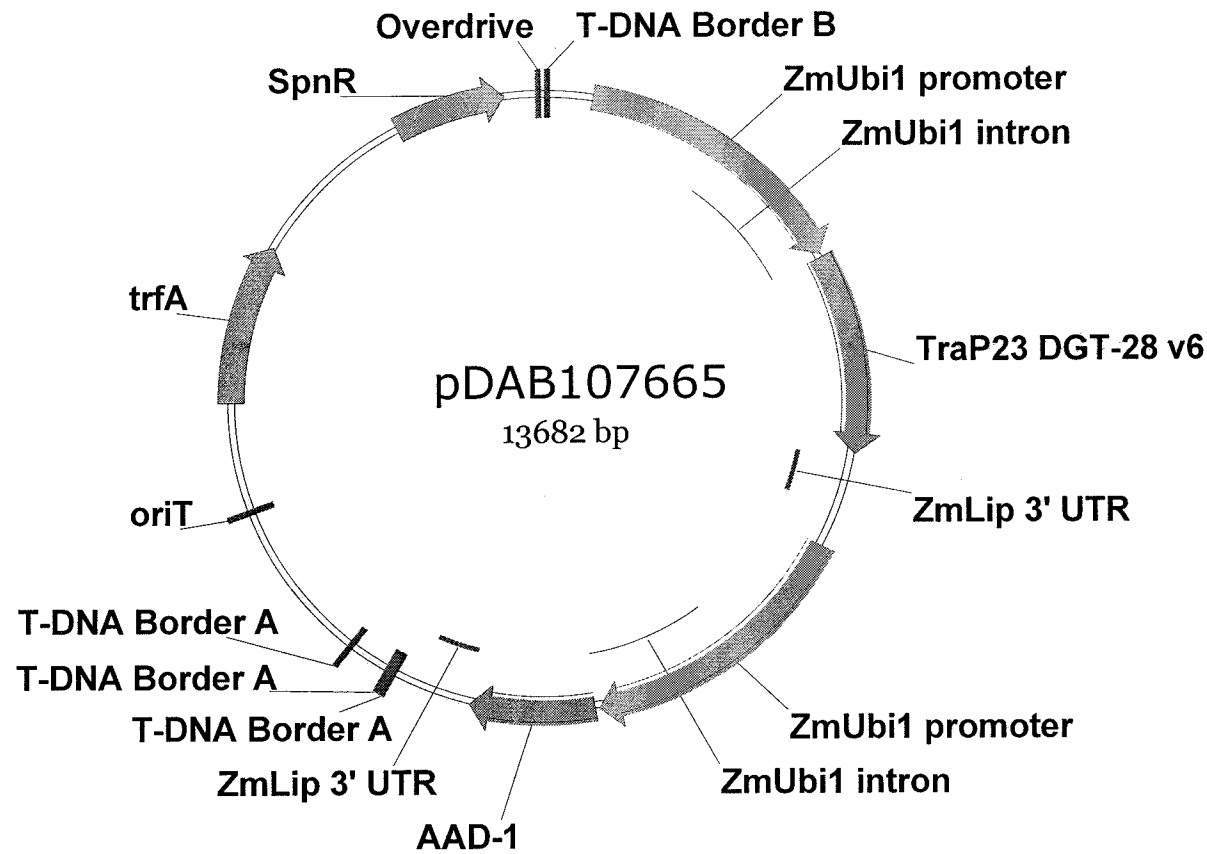
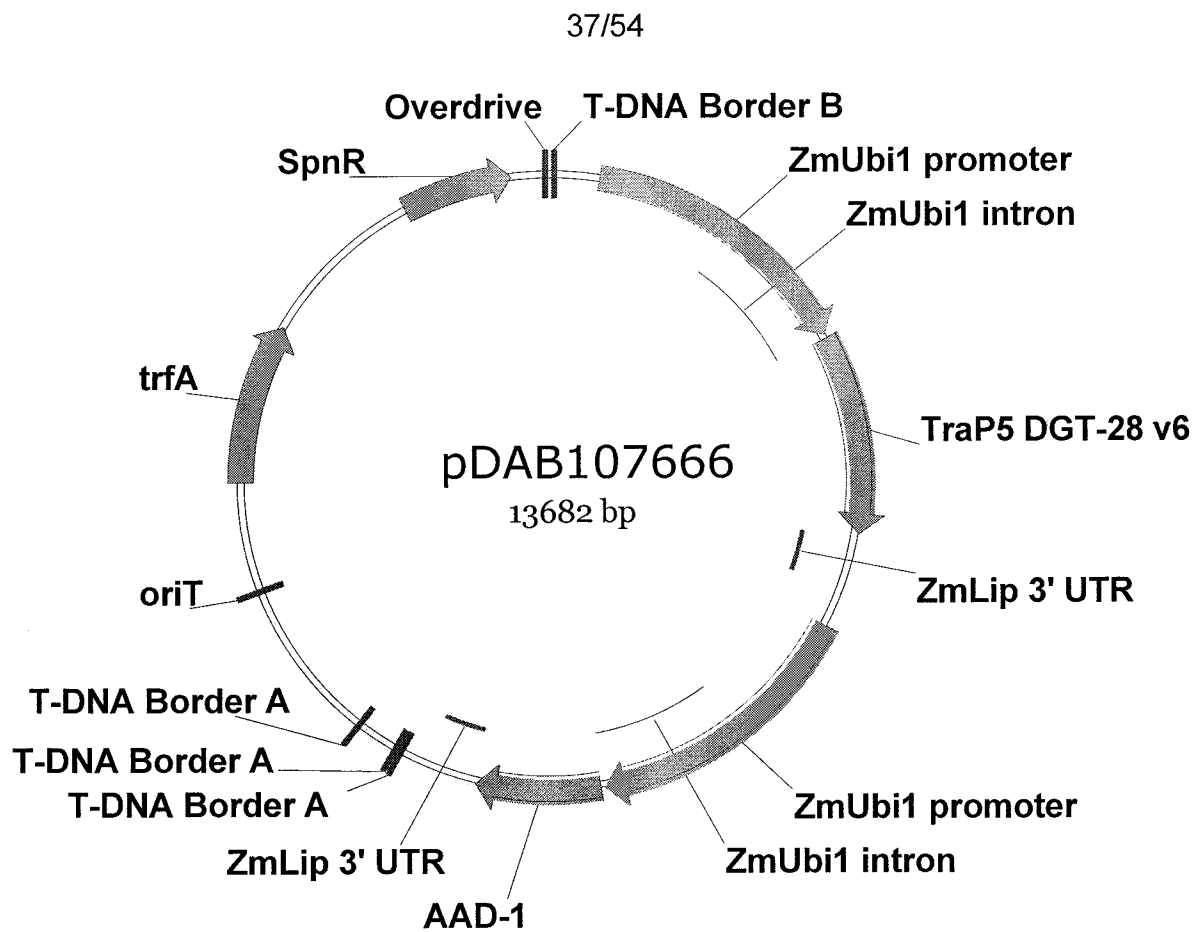


FIG. 36

**FIG. 37**

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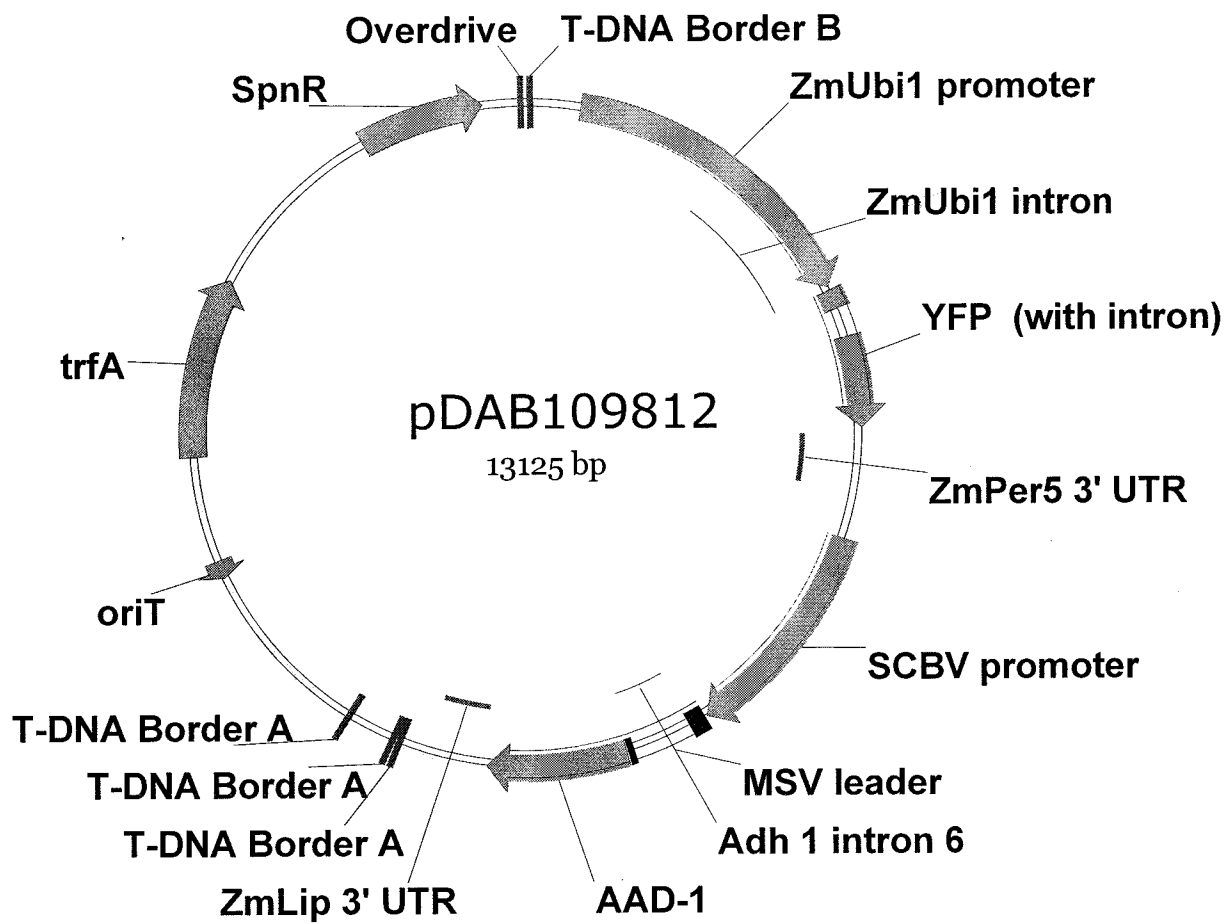


FIG. 38

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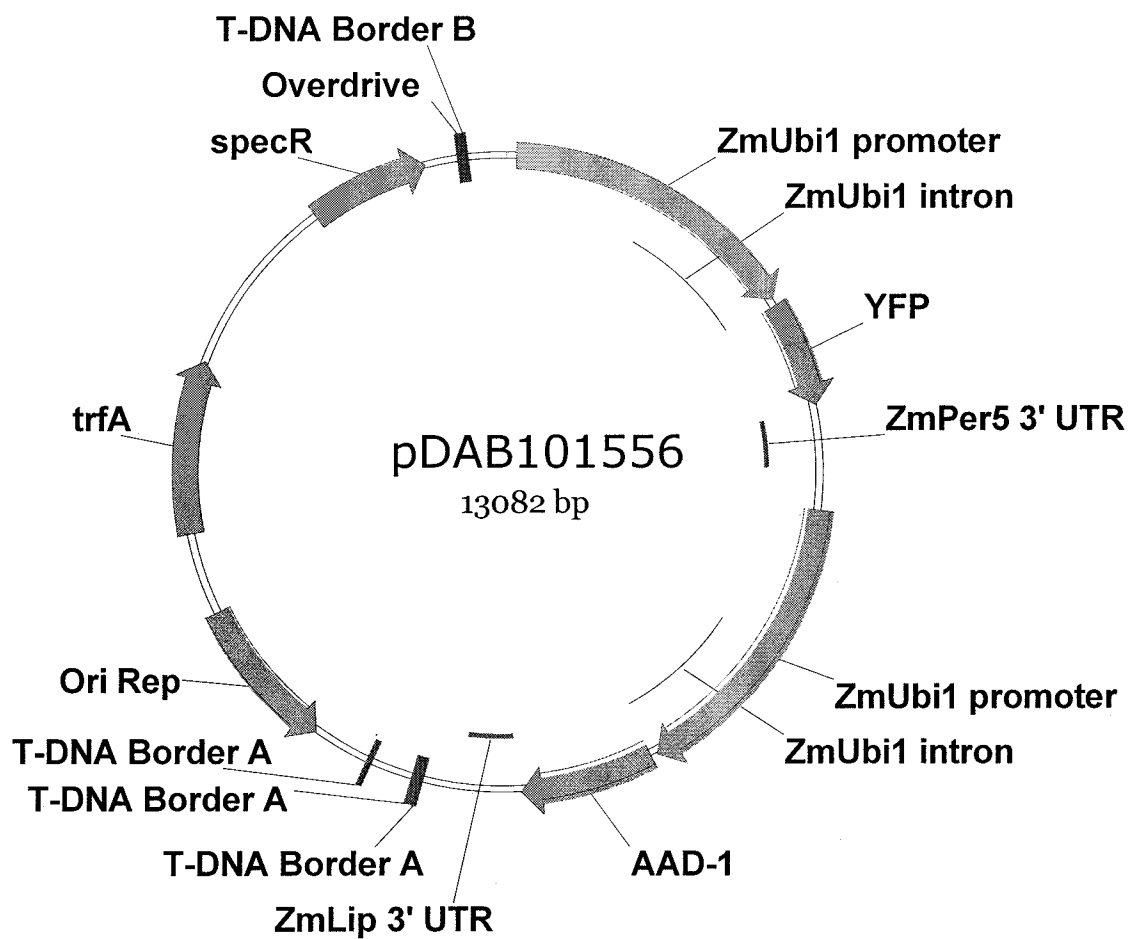


FIG. 39

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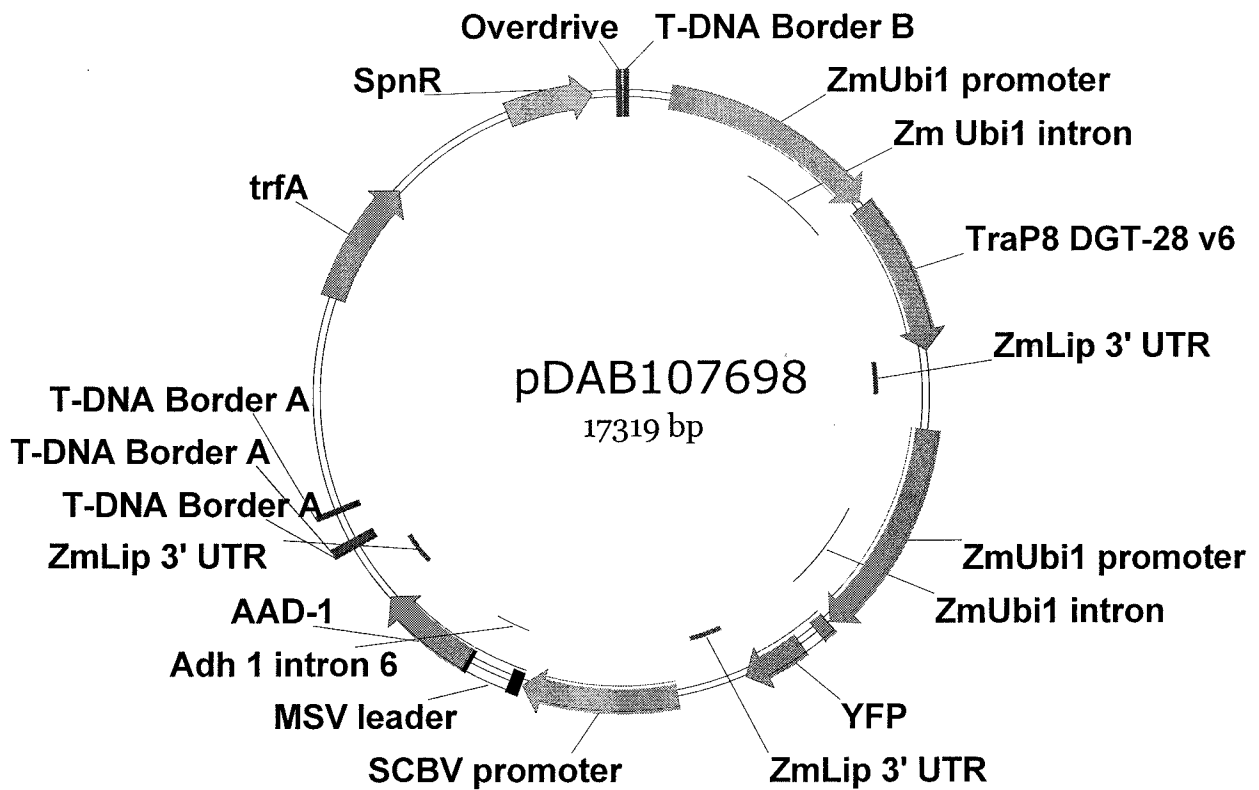


FIG.40

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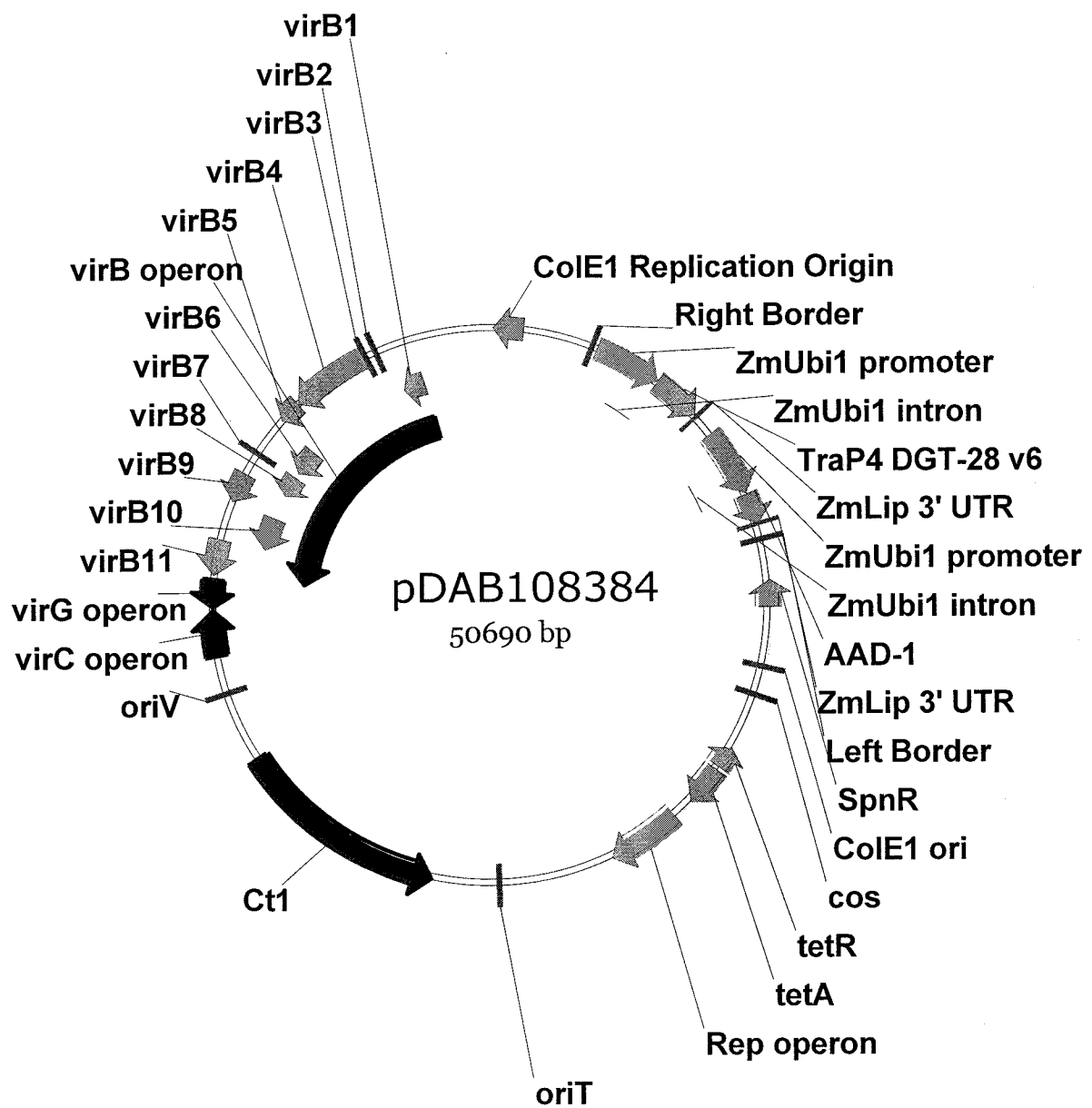


FIG. 41

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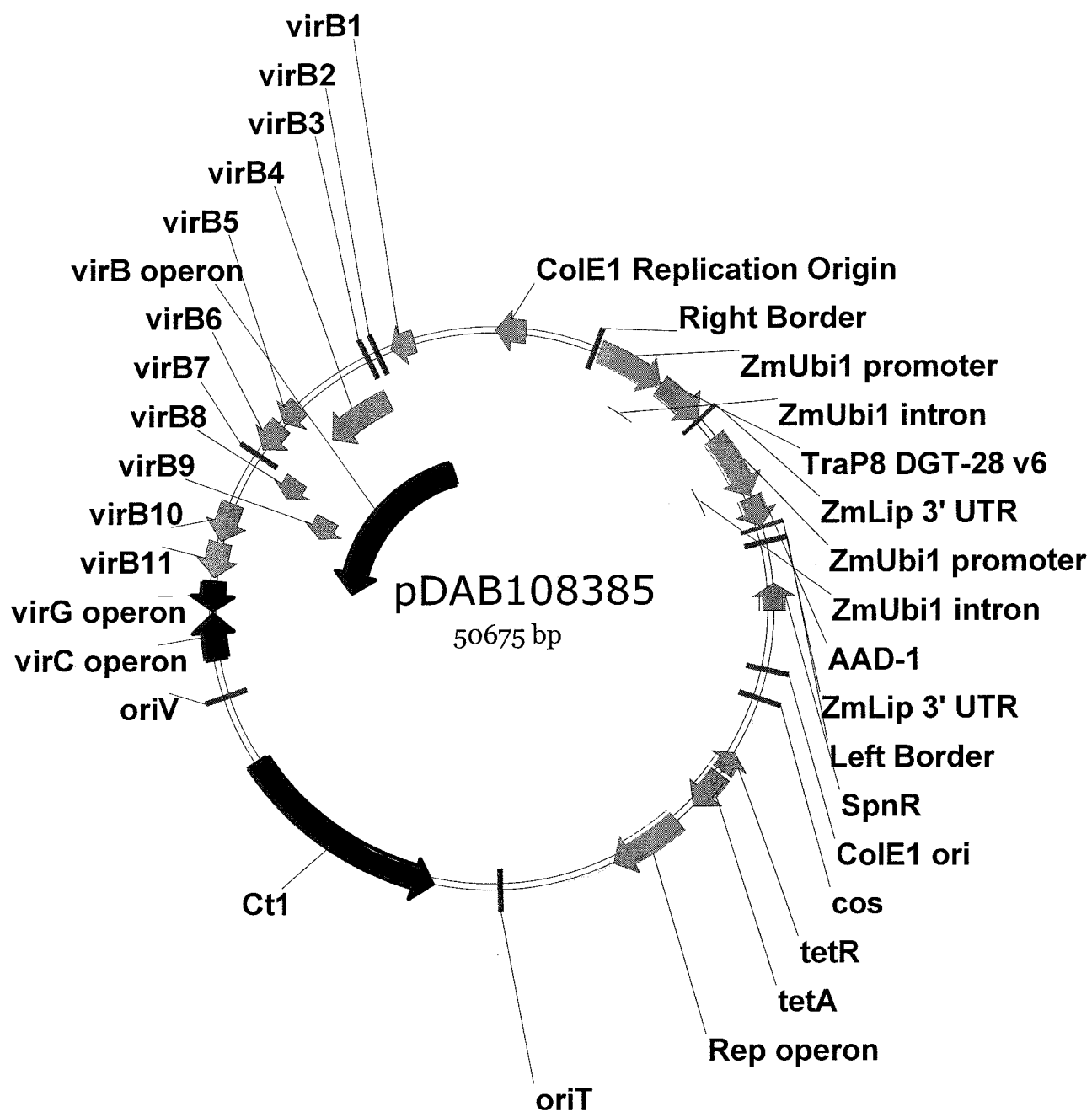


FIG. 42

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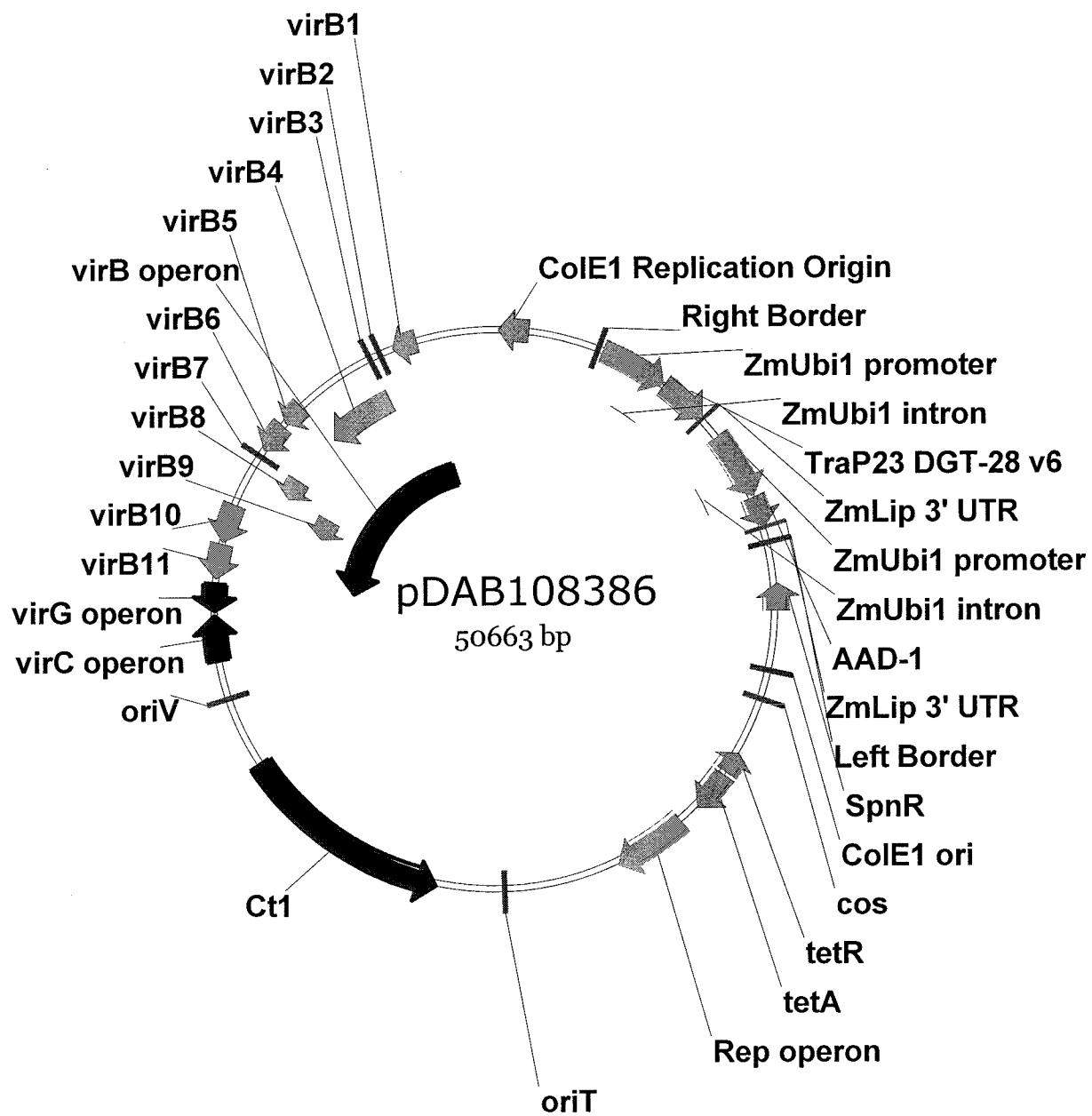


FIG. 43

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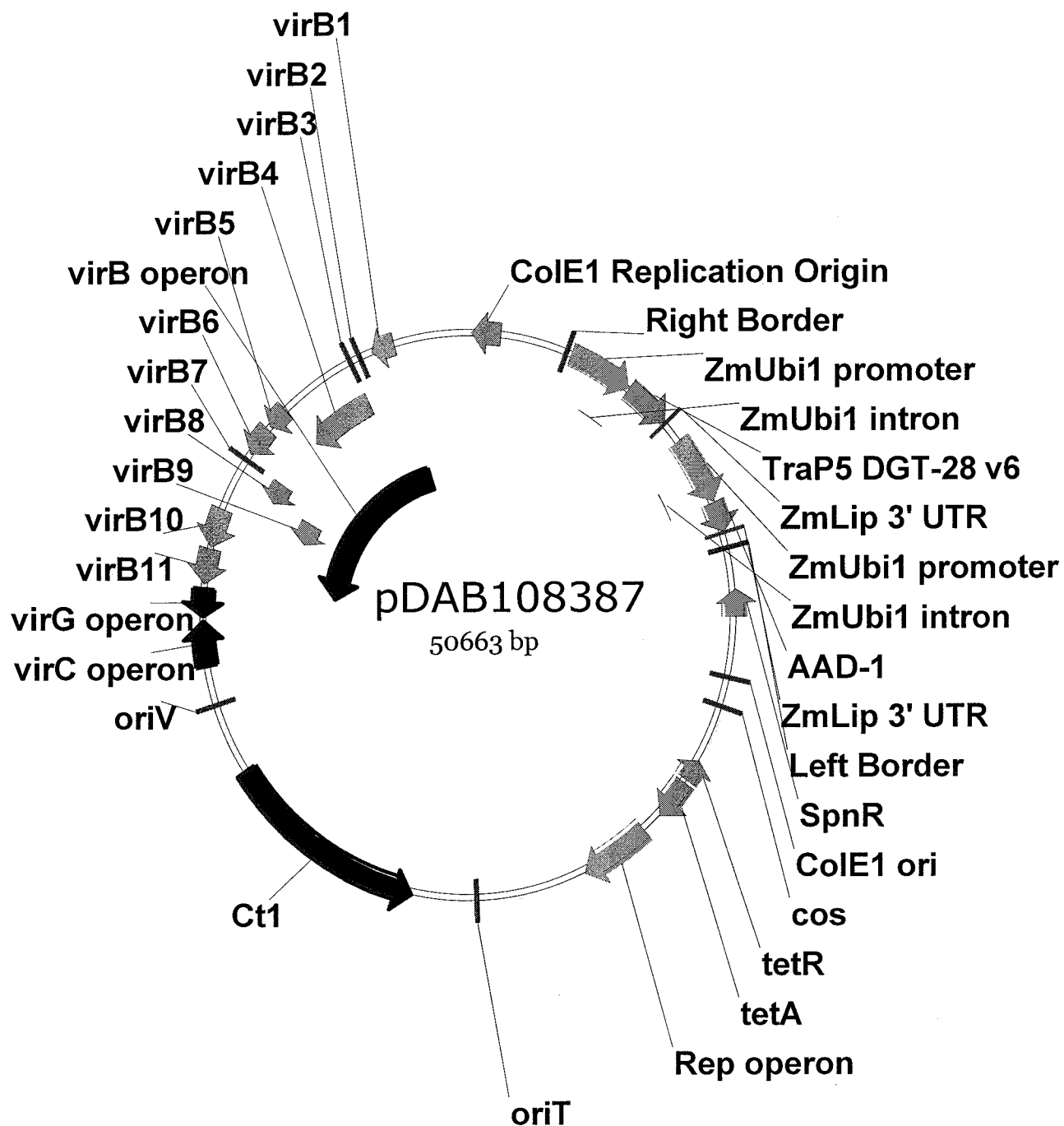


FIG. 44

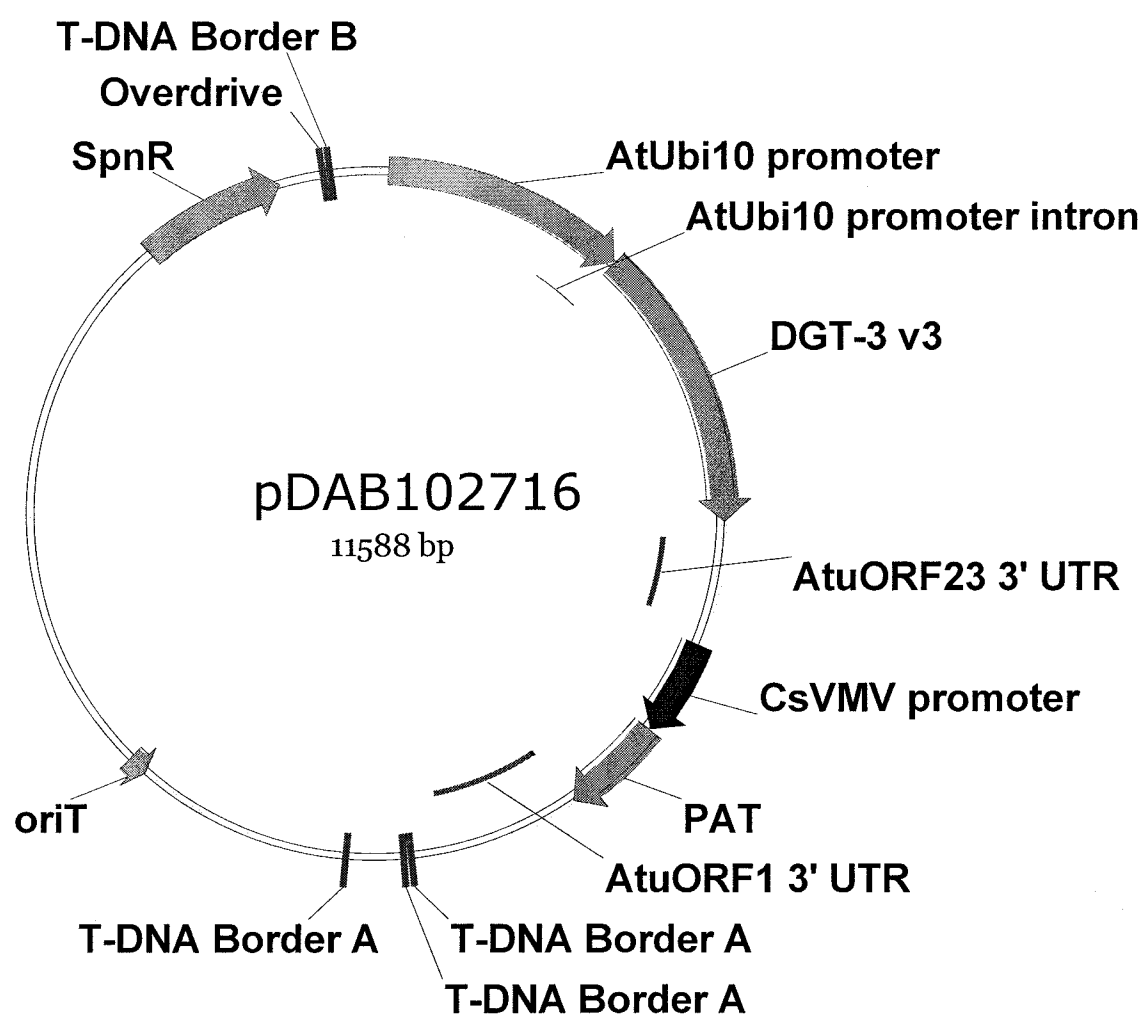


FIG. 45

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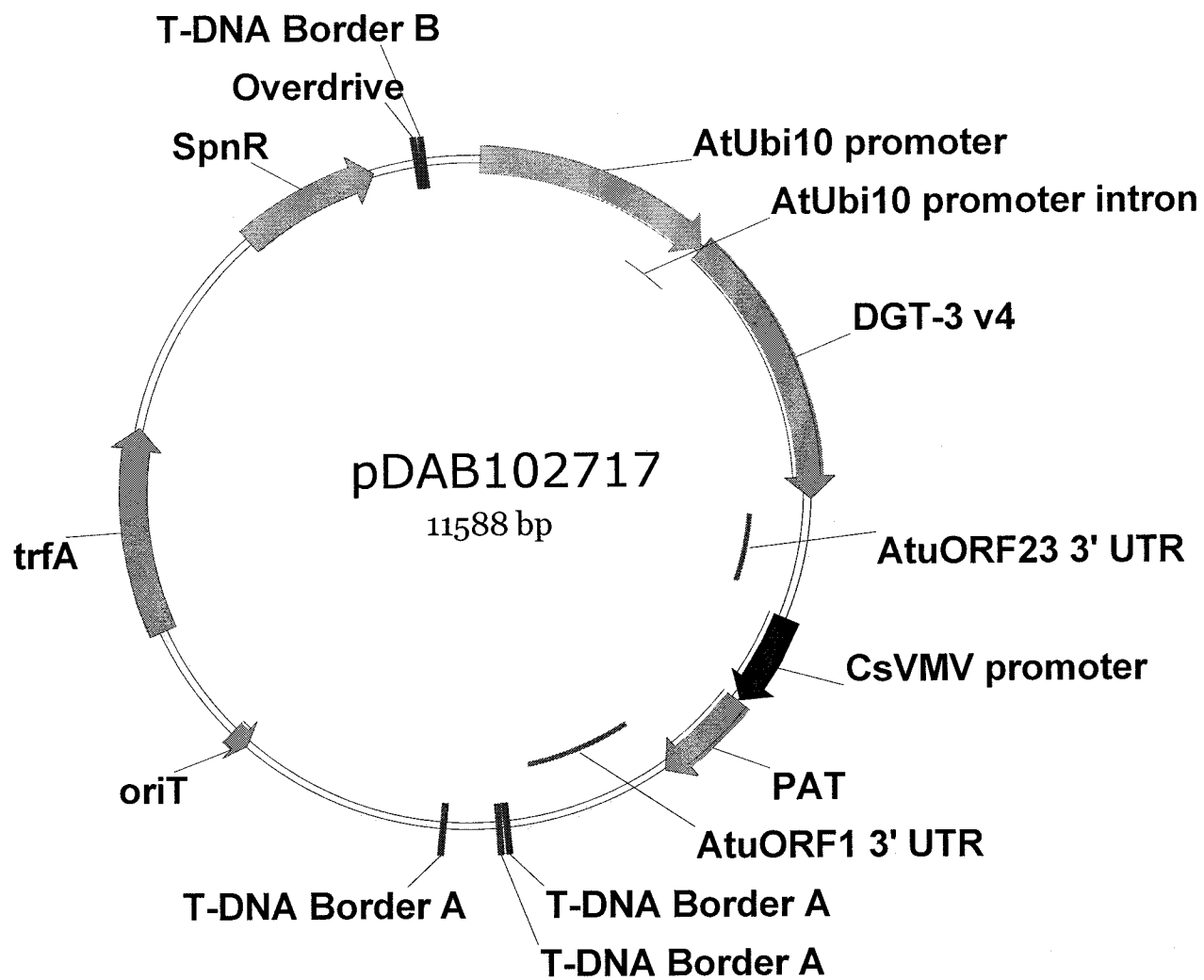


FIG. 46

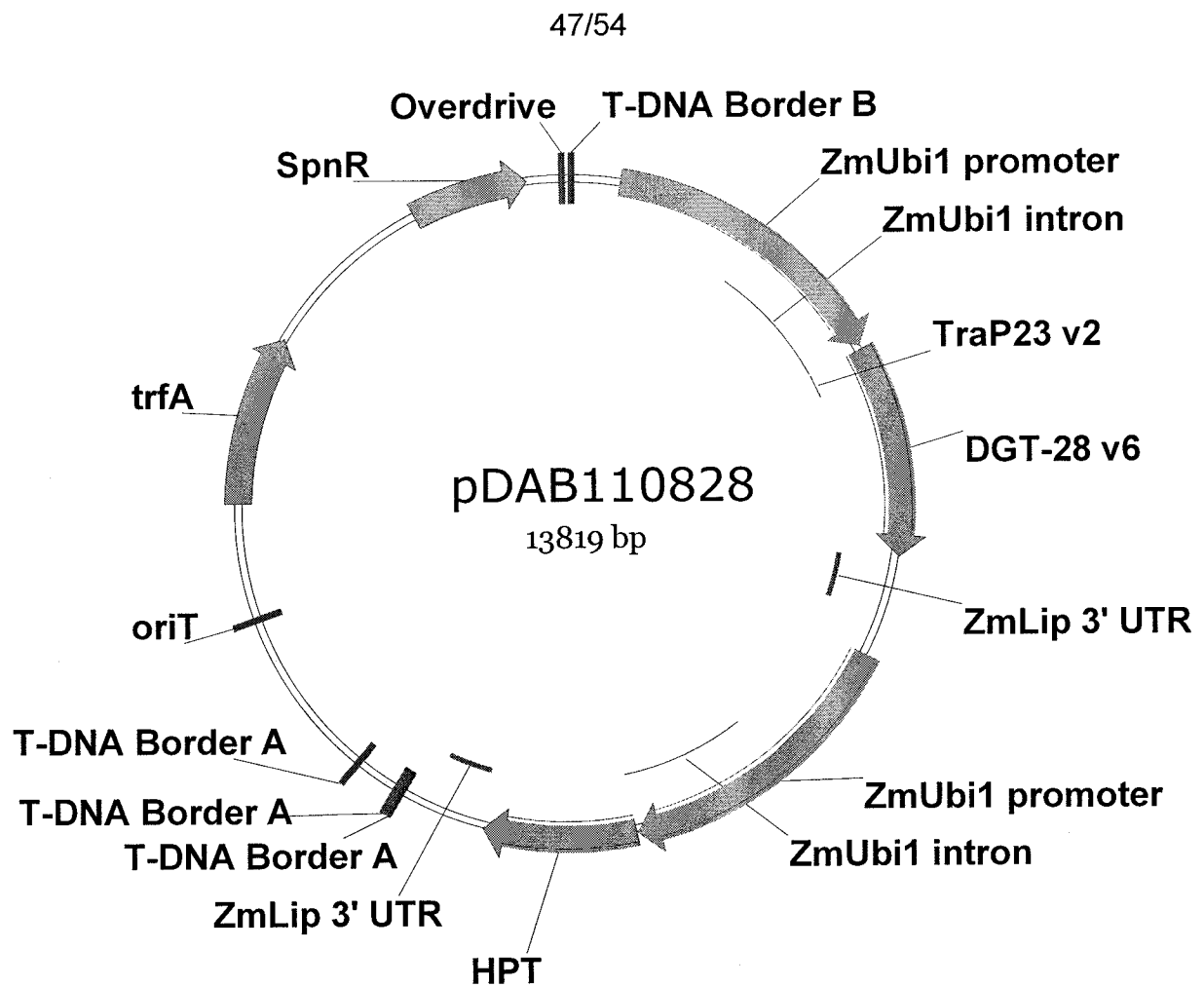


FIG. 47

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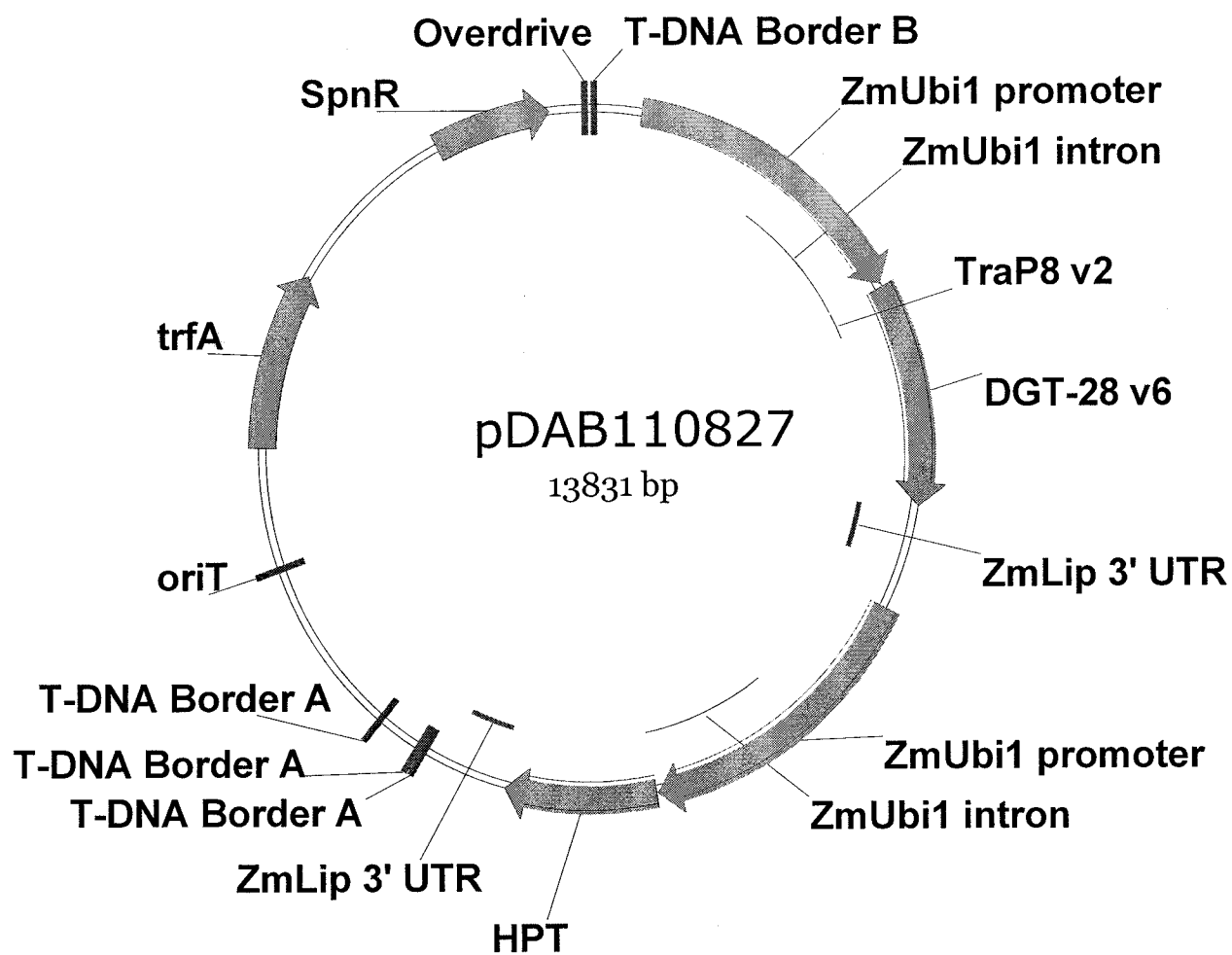
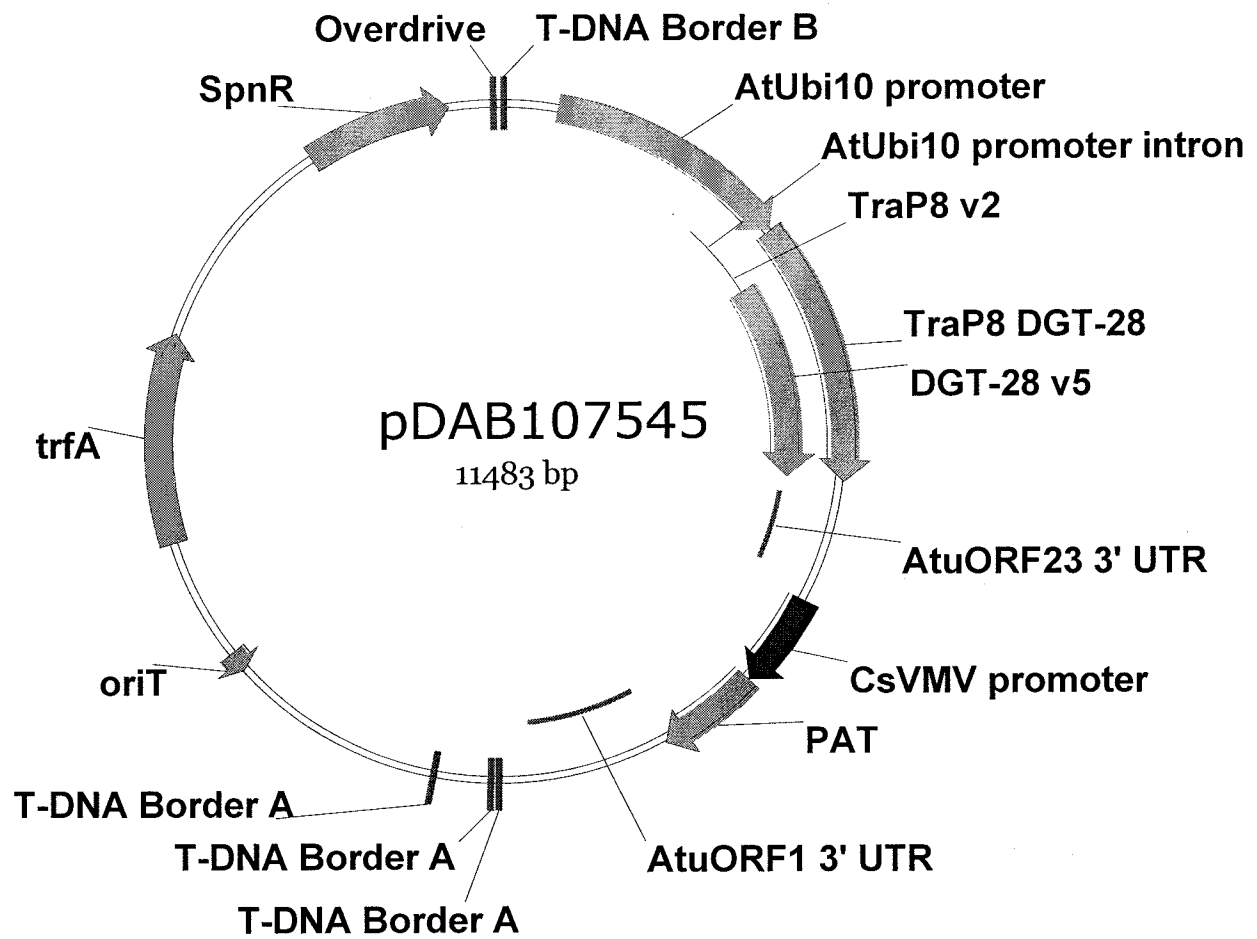


FIG. 48

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**FIG. 49**

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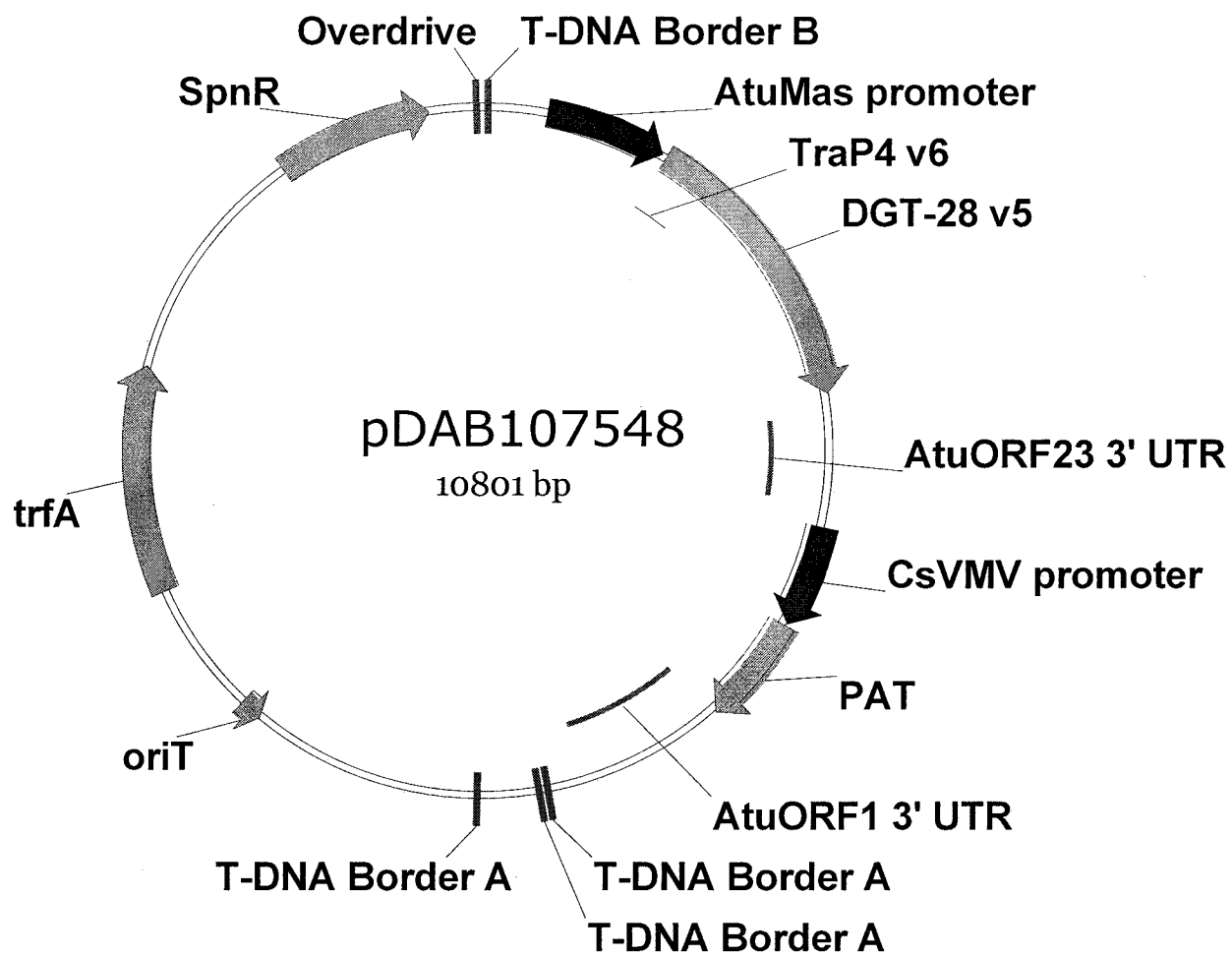


FIG. 50

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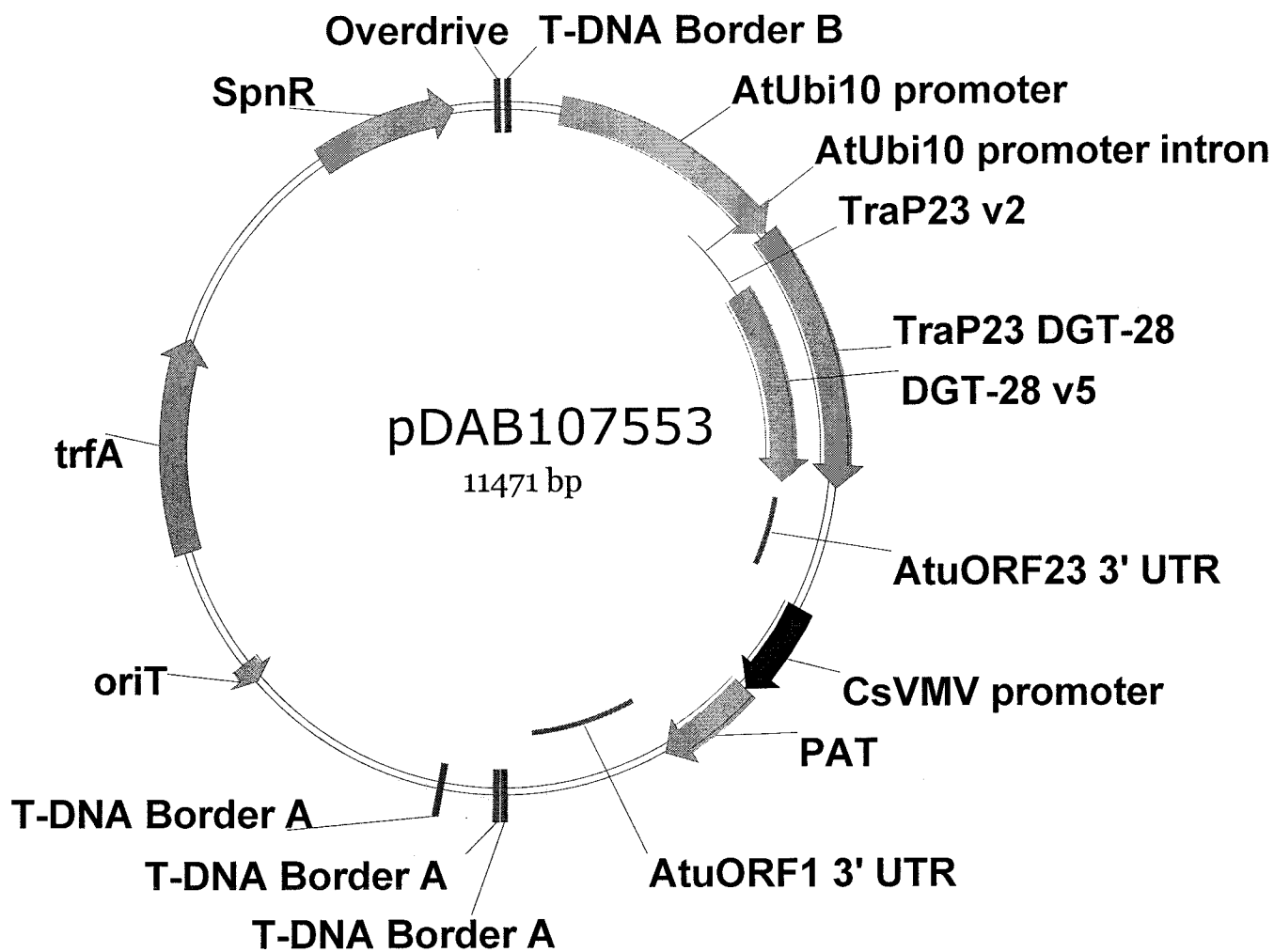


FIG. 51

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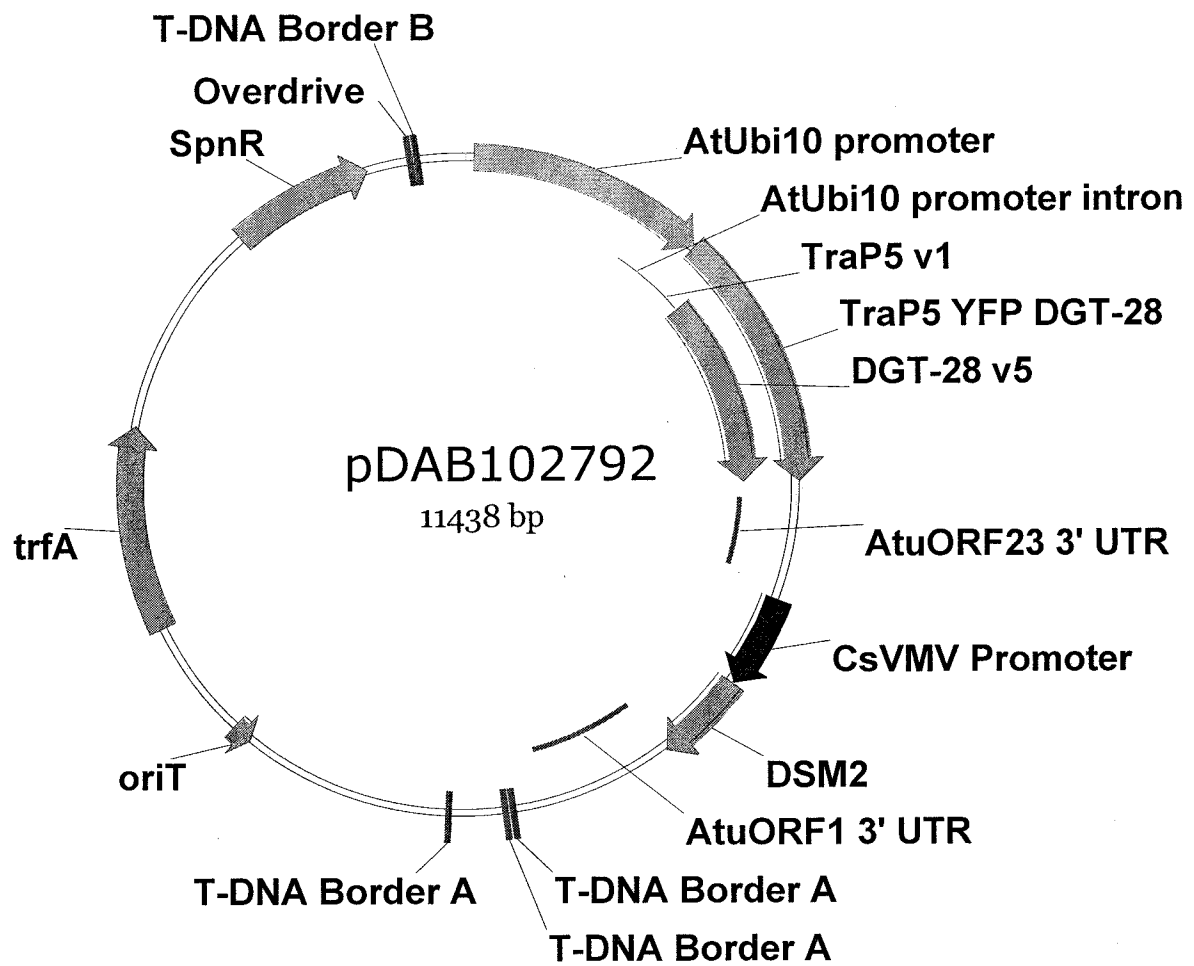


FIG. 52

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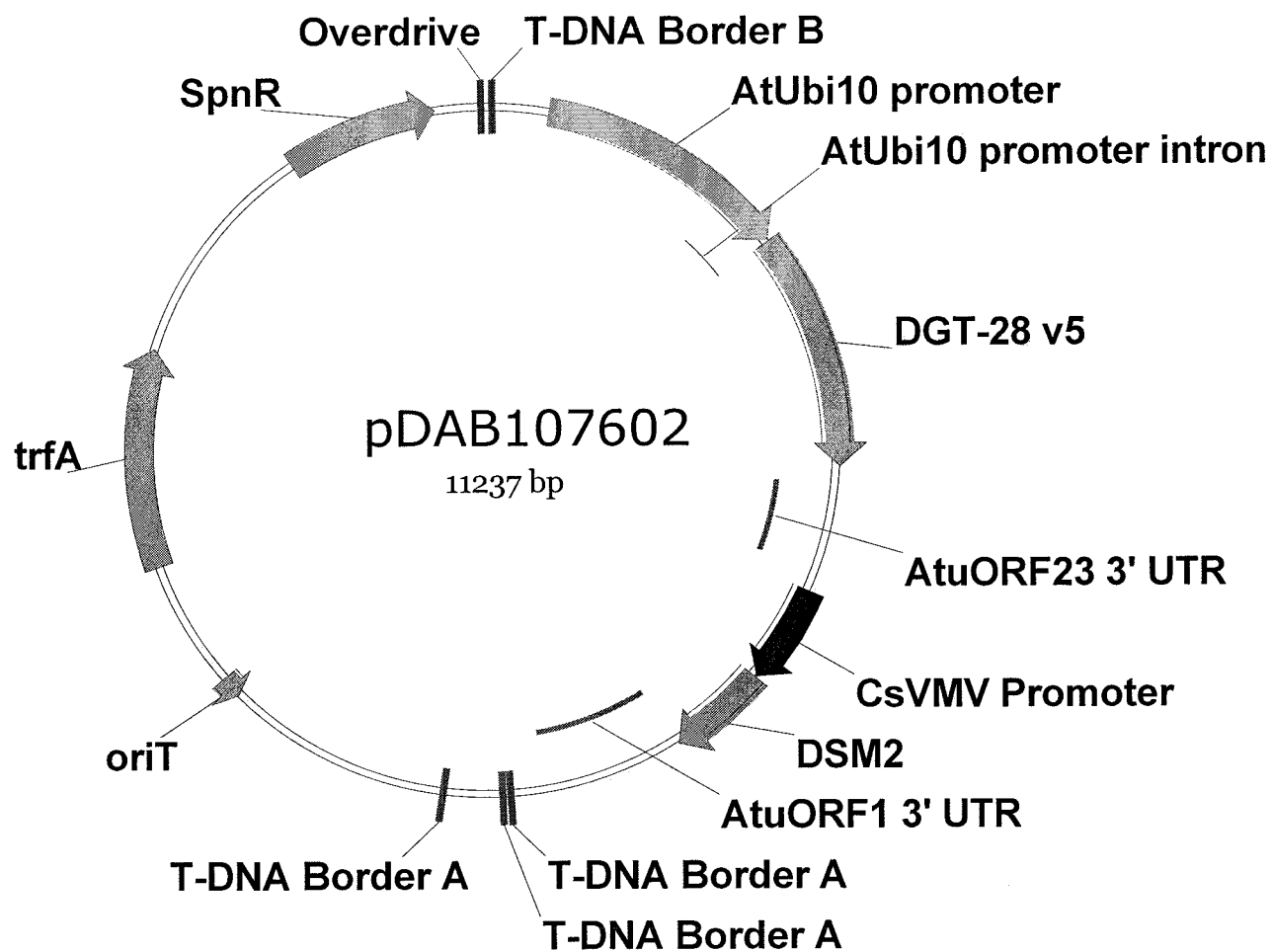


FIG. 53

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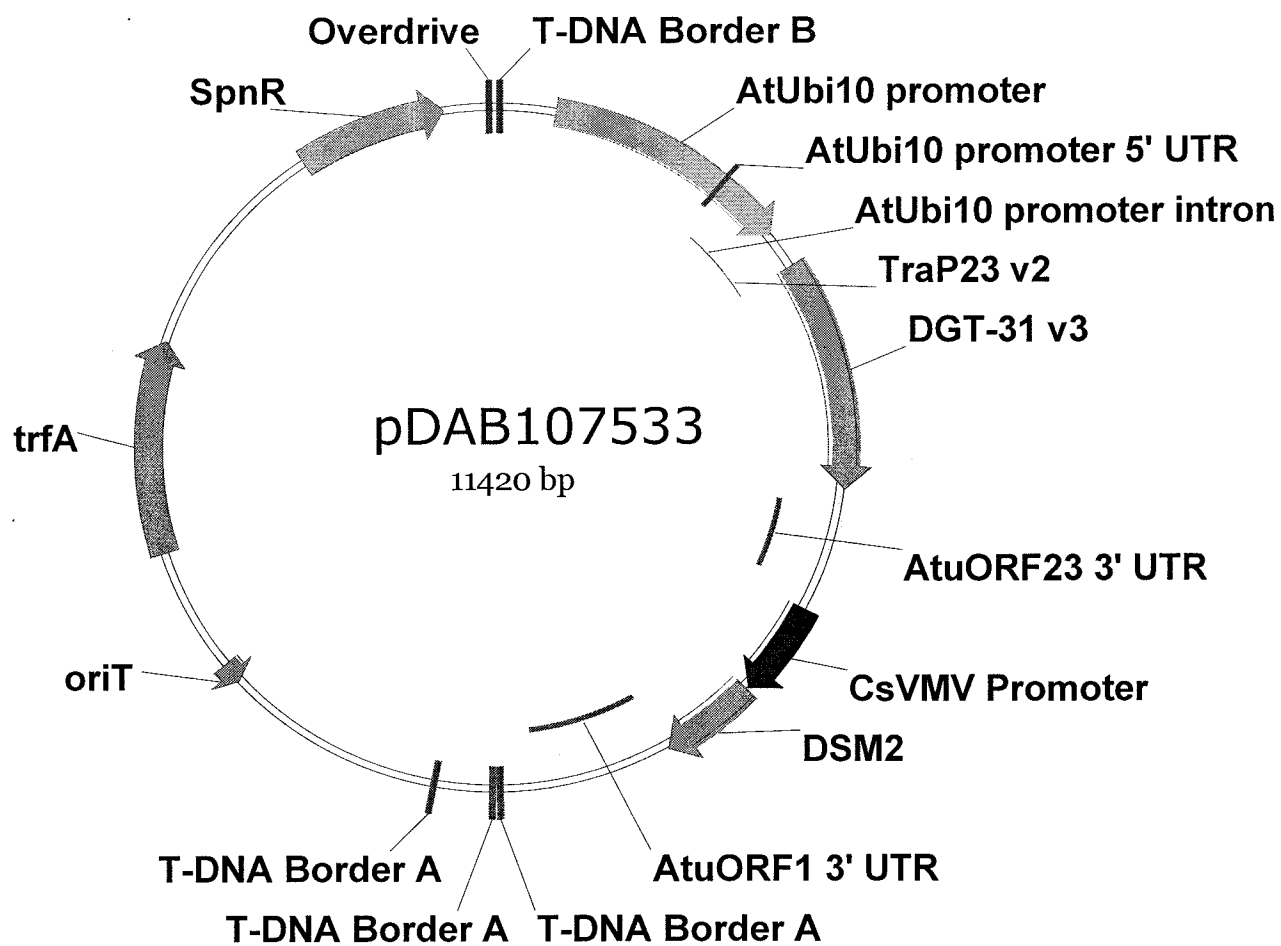


FIG. 54

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/024410**A. CLASSIFICATION OF SUBJECT MATTER****A01H 5/00(2006.01)i, C12N 15/29(2006.01)i, C12N 15/63(2006.01)i, C12N 15/82(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A01H 5/00; A01N 57/18; C12N 5/10; A01N 57/20; C12N 15/82; A01N 39/02

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: glyphosate resistant plant, herbicide-resistant plant, transgenic plant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NCBI GeneBank Accession No. ZP_06917240.1 (22 September 2011) See the whole document.	1-9, 12
Y		10, 13-16, 18-19, 21, 24-39
A		11, 17, 20, 22-23
Y	WRIGHT, TERRY R. et al., "Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes", PNAS, 23 September 2010, Vol. 107, No. 47, pp. 20240-20245, ISSN 0027-8424 See abstract; materials and methods.	10, 13-16, 18-19, 21, 24-39
A	US 2011-0124503 A1 (WRIGHT, TERRY R. et al.) 26 May 2011 See abstract.	1-39
A	US 2011-0289620 A1 (CUI, YUNXING. et al.) 24 November 2011 See abstract; claims 32 and 36.	1-39
A	US 2011-0195845 A1 (LIRA, JUSTIN M. et al.) 11 August 2011 See abstract.	1-39



Further documents are listed in the continuation of Box C.



See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

30 May 2013 (30.05.2013)

Date of mailing of the international search report

02 June 2013 (02.06.2013)

Name and mailing address of the ISA/KR

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Facsimile No. 82-42-472-7140

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YANG, In Su

Telephone No. 82-42-481-8131



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/024410

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/024410

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