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(54) Title: HERBICIDE TOLERANT PLANTS

(57) Abstract: The present invention relates to methods for the identification and/or selection of glyphosate tolerant plants. The present invention also relates to glyphosate tolerant plants and uses thereof, such as for breeding and/or for the production of crops.

HERBICIDE TOLERANT PLANTS

PRIORITY CLAIM

This application claims priority to Australian provisional patent application 2014902626, filed 8 July 2014, the content of which is hereby incorporated by reference.

FIELD

The present invention relates to methods for the identification and/or selection of glyphosate tolerant plants. The present invention also relates to glyphosate tolerant plants and uses thereof, such as for breeding and/or for the production of crops.

BACKGROUND

Weed species have long been a problem in crops. Although once a labor intensive operation, weed control has been made easier by the availability of efficient weed killing chemical herbicides. 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 plants that are tolerant to certain broad spectrum herbicides.

Glyphosate is one particular broad-spectrum herbicide that has been used extensively for controlling weeds prior to crop planting or 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 of chemical herbicides available for use in agriculture.

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 inhibits this pathway by binding to and inhibiting activity of the enzyme 3-enolpyruvylshikimate-3-phosphate synthase, commonly referred to as EPSP synthase, or EPSPS.

One method that has been used 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 genetic engineering techniques. However, although glyphosate tolerant crop plants have been produced using genetic engineering techniques, commercial acceptance of such crops has been hindered in some countries by resistance to genetically modified organisms (GMO) as food sources.

Accordingly, methods to identify naturally occurring glyphosate tolerant plants and/or methods for producing glyphosate tolerant plants via means other than genetic engineering would be desirable.

DESCRIPTION

Nucleotide and amino acid sequences are referred to herein by a sequence identifier number (SEQ ID NO:). A summary of the sequence identifiers is provided below:

Sequence Identifier	Description
SEQ ID NO: 1	1763 bp promoter fragment marker nucleotide sequence
SEQ ID NO: 2	EPS-1.F primer nucleotide sequence
SEQ ID NO: 3	EPS-1.R primer nucleotide sequence
SEQ ID NO: 4	EPS-2.F primer nucleotide sequence
SEQ ID NO: 5	EPS-2.R primer nucleotide sequence
SEQ ID NO: 6	actF primer nucleotide sequence

SEQ ID NO: 7	actR primer nucleotide sequence
SEQ ID NO: 8	GAPDH_F primer nucleotide sequence
SEQ ID NO: 9	GAPDH_R primer nucleotide sequence
SEQ ID NO: 10	SNP1 tolerant nucleotide sequence
SEQ ID NO: 11	SNP1 intolerant nucleotide sequence
SEQ ID NO: 12	SNP2 tolerant nucleotide sequence
SEQ ID NO: 13	SNP2 intolerant nucleotide sequence
SEQ ID NO: 14	LuGly-wt.F primer nucleotide sequence
SEQ ID NO: 15	LuGly-mu.F primer nucleotide sequence
SEQ ID NO: 16	LuGly.R primer nucleotide sequence

A sequence listing is also provided at the end of the specification.

The present inventors have identified a marker nucleotide sequence that is associated with glyphosate tolerance in plants. The identified marker nucleotide sequence is a fragment of the EPSPS gene promoter in the genome of the plant. Without limiting the present invention to any one particular mode of action, the marker nucleotide sequence is associated with elevated EPSPS gene expression in the plant and this elevated expression is associated with glyphosate tolerance in the plant.

In a first aspect, the present invention provides a method for identifying a plant having a glyphosate tolerant phenotype, the method comprising determining the presence or absence of a marker nucleotide sequence in the genome of said plant, wherein the marker nucleotide sequence comprises one or more of:

(i) SEQ ID NO: 1 or a homolog thereof;

(ii) a fragment of the nucleotide sequence set out at (i); and/or

(iii) a nucleotide sequence genetically linked to the nucleotide sequence set out at (i) in the genome of said plant;

wherein the presence of the marker nucleotide sequence indicates that the plant has a glyphosate tolerant phenotype.

In some embodiments, the method further comprises a step of subsequently selecting plants having a glyphosate tolerant phenotype on the basis of the presence of a marker nucleotide sequence in the genome of the plant.

As set out above the present invention provides a method for identifying a "plant" having a glyphosate tolerant phenotype. In some embodiments, reference herein to a "plant" should be understood to include any member of the kingdom *Plantae*. In some embodiments, "plant" refers to a vascular plant, including a monocotyledonous ('monocot') or dicotyledonous ('dicot') angiosperm plant, or a gymnosperm plant.

In some embodiments the plant is a dicot plant. Exemplary dicots include, for example, *Arabidopsis* spp., *Nicotiana* spp., *Medicago* spp., soybean, canola, oil seed rape, sugar beet, mustard, sunflower, potato, safflower, cassava, yams, sweet potato, other Brassicaceae such as *Thellungiella halophila*, among others.

In some embodiments, the plant is a leguminous plant. A "leguminous plant" as referred to herein should be understood as any member of the Fabaceae (or Leguminosae). Examples of leguminous plants include:

Medicago spp., such as *Medicago sativa* (also referred to as lucerne or alfalfa);

Pisum spp., such as *Pisum abyssinicum* (syn. *P. sativum* subsp. abyssinicum), *Pisum fulvum*, *Pisum sativum*, *Pisum sativum* subsp. *elatius* (syn. *P. elatius*, *P. syriacum*) and *Pisum sativum* subsp. *sativum*;

Glycine spp., such as *Glycine max*, *Glycine albicans, Glycine aphyonota, Glycine arenaria, Glycine argyrea, Glycine canescens, Glycine clandestine, Glycine curvata, Glycine cyrtoloba, Glycine falcate, Glycine gracei, Glycine hirticaulis, Glycine hirticaulis* subsp. *leptosa, Glycine lactovirens, Glycine latifolia, Glycine latrobeana, Glycine microphylla, Glycine montis-douglas, Glycine peratosa, Glycine pescadrensis, Glycine pindanica, Glycine pullenii, Glycine rubiginosa, Glycine*

stenophita, Glycine syndetika, Glycine tabacina, Glycine tomentella and *Glycine soja;*

Cicer spp., such as *Cicer arietinum*;

Vicia spp., such as *V. faba*;

Vigna spp., such as *V. aconitifolia, V. angularis, V. mungo, V. radiate, V. subterranean, V. umbellatta* or *V. unguiculata*

Lathyrus spp., such as *Lathyrus sativus* or *Lathyrus tuberosus*;

Lens spp., such as *L. culinaris*

Lablab spp., such as *L. purpureus*;

Phaseolus spp., such as *P. acutifolius, P. coccineus, P. lunatus, P. vulgaris, P. polyanthus* or *P. dumosus*;

Psophocarpus spp., such as *P. tetragonolobus*;

Cajanus spp., such as *C. cajan;*

Stizolobium spp.;

Cyamopsis spp., such as *C. tetragonoloba*;

Canavalia spp., such as *C. ensiformis* or *C. gladiata*;

Macrotyloma spp., such as *M. uniflorum*;

Lupinus spp., such as *L. mutabilis* or *L. albus*; or

Erythrina spp., such as *E. herbacea.*

In some embodiments, the plant is a *Medicago* spp. plant. In some embodiments, the plant is a *Medicago sativa*, lucerne or alfalfa plant.

As set out above, the present invention provides a method for selecting a plant having a "glyphosate tolerant phenotype".

Reference herein to "glyphosate" should be understood to refer to any of glyphosate, *N*-(phosphonomethyl)glycine or 2-[(phosphonomethyl)amino]acetic acid, and all such names should be considered synonymous and interchangeable.

A "glyphosate tolerant phenotype" as referred to herein should be understood to

include wherein a plant is able to survive the application of glyphosate at a higher rate or concentration than a sensitive cultivar of the same species. In some embodiments, a glyphosate tolerant phenotype refers to wherein a plant is able to survive an application of glyphosate of at least 200, 300, 400, 500, 540, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500 or 1600 g/ha. In some embodiments, a glyphosate tolerant phenotype refers to wherein a plant is able to survive an application of glyphosate of at least 200, 300, 400, 500, 540, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500 or 1600 g/ha during spring, optionally when the plant is at the 3-5 trifoliate leaf growth stage.

As set out above, the method of the first aspect of the invention comprises determining the presence or absence of a marker nucleotide sequence in the genome of a plant.

Determining the presence or absence of a marker nucleotide sequence in the genome of a plant should be understood to include determining the presence or absence of the marker nucleotide sequence in the genome of at least one cell of the plant.

As set out above, the marker nucleotide sequence comprises:

(ii) SEQ ID NO: 1 or a homolog thereof;

(ii) a fragment of the nucleotide sequence set out at (i); and/or

(iii) a nucleotide sequence genetically linked to the nucleotide sequence set out at (i) or (ii) in the genome of said plant;

As set out above, in some embodiments the marker nucleotide sequence may comprise SEQ ID NO: 1 or a homolog thereof. In some embodiments, a "homolog" of SEQ ID NO: 1 refers to any nucleotide sequence which exhibits sequence identity to SEQ ID NO: 1 and which is associated with a glyphosate tolerant phenotype in a plant.

In some embodiments, reference to a "homolog" of SEQ ID NO: 1 refers to any nucleotide sequence in the genome of a plant which comprises at least 90% nucleotide sequence identity to SEQ ID NO: 1. In some embodiments the homolog of SEQ ID NO: 1 comprises at least 90%, at least 90.5%, at least 91%, at least 91.5%, at least 92%, at least 92.5%, at least 93%, at least 93.5%, at least 94%, at least 94.5%, at least 95%, at least 95.5%, at least 96%, at least 96.5%, at least 97%, at least 97.5%, at least 98%, at least 98.5%, at least 99% at least 99.5% or 100% nucleotide sequence identity to SEQ ID NO: 1.

When comparing nucleic acid sequences to SEQ ID NO: 1 to calculate a percentage identity, the compared nucleotide sequences should be compared over a comparison window of at least 100 nucleotide residues, at least 200 nucleotide residues, at least 500 nucleotide residues, at least 1000 nucleotide residues or over the full length of SEQ ID NO: 1. The comparison window may comprise additions or deletions (ie. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms such the BLAST family of programs as, for example, disclosed by Altschul *et al.* (*Nucl. Acids Res.* 25: 3389-3402, 1997). A detailed discussion of sequence analysis can be found in Unit 19. 3 of Ausubel *et al.* ("*Current Protocols in Molecular Biology*" John Wiley & Sons Inc, 1994-1998, Chapter 15,1998).

The term "homolog" may also comprise a nucleic acid which hybridises to a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO: 1 under stringent conditions. As used herein, "stringent" hybridisation conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least 30°C. Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Stringent hybridisation

conditions may be low stringency conditions, medium stringency conditions or high stringency conditions. Exemplary low stringency conditions include hybridisation with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C., and a wash in 1× to 2×SSC (20×SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridisation in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C., and a wash in 0.5× to 1×SSC at 55 to 60°C. Exemplary high stringency conditions include hybridisation in 50% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.1×SSC at 60 to 65°C. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity of hybridisation is also affected by post-hybridization wash conditions, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the Tm can be approximated from the equation of Meinkoth and Wahl (*Anal. Biochem.* 138: 267-284, 1984), ie. Tm=81.5°C.+16.6 (log M)+0.41 (% GC)−0.61 (% form)−500/L; 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. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Tm is reduced by about 1°C. for each 1% of mismatching; thus, Tm, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of different degrees of complementarity. For example, sequences with ≧90% identity can be hybridised by decreasing the Tm by about 10°C. Generally, stringent conditions are selected to be about 5°C. lower than the thermal melting point (Tm) for the specific sequence and its complement at a defined ionic strength and pH. However, high stringency conditions can utilize a hybridisation and/or wash at, for example, 1, 2, 3, or 4°C. lower than the thermal melting point (Tm); medium stringency conditions can utilize a hybridization and/or wash at, for example, 6, 7,

8, 9, or 10°C. lower than the thermal melting point (Tm); low stringency conditions can utilize a hybridization and/or wash at, for example, 11, 12, 13, 14, 15, or 20°C. lower than the thermal melting point (Tm). Using the equation, hybridization and wash compositions, and desired Tm, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a Tm of less than 45°C. (aqueous solution) or 32°C. (formamide solution), the SSC concentration may be increased so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, Pt I, Chapter 2, Elsevier, New York, 1993), Ausubel *et al.*, eds. (*Current Protocols in Molecular Biology,* Chapter 2, Greene Publishing and Wiley-Interscience, N.Y., 1995) and Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1989).

In some embodiments, a "homolog" of SEQ ID NO: 1 comprises all or part of a promoter nucleotide sequence in the genome of a plant. In some embodiments, a "homolog" of SEQ ID NO: 1 comprises all or part of an EPSPS gene promoter nucleotide sequence in the genome of a plant.

As set out above, in some embodiments, the marker nucleotide sequence may comprise a "fragment" of SEQ ID NO: 1 or homolog thereof. Reference herein to a "fragment" should be understood as a nucleotide sequence of at least 10, 20, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600 or 1700 nucleotides (nt) in length.

As set out in some embodiments the marker nucleotide sequence may also comprise a nucleotide sequence genetically linked to SEQ ID NO: 1 or a homolog thereof.

Reference herein to a nucleotide sequence "genetically linked" to SEQ ID NO: 1

or a homolog thereof should be understood to include any genomic nucleotide sequence in a plant that is inherited together with SEQ ID NO: 1 or a homolog thereof during meiosis. In some embodiments, a nucleotide sequence which is genetically linked to SEQ ID NO: 1 or a homolog thereof is any nucleotide sequence within a genetic distance of less than 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.01 or 0.001 centimorgans (cM) of SEQ ID NO: 1 or a homolog thereof in the genome of the plant. In some embodiments a nucleotide sequence "genetically linked" to SEQ ID NO: 1 or a homolog thereof is a nucleotide sequence which is 100% linked to SEQ ID NO: 1, that is a nucleotide sequence which always is inherited with SEQ ID NO: 1 or a homolog thereof during meiosis.

In some embodiments, a nucleotide sequence genetically linked to SEQ ID NO: 1 or a homolog thereof refers to a nucleotide sequence within a promoter of which SEQ ID NO: 1 or a homolog thereof is a part; and/or a nucleotide sequence in a gene which is transcriptionally linked to SEQ ID NO: 1 or a homolog thereof.

In some embodiments, a nucleotide sequence genetically linked to SEQ ID NO: 1 or a homolog thereof may include one or more sequence polymorphisms in the EPSPS gene of the plant which are genetically linked to SEQ ID NO: 1 in the genome of the plant. In some embodiments, the sequence polymorphism comprises a Single Nucleotide Polymorphism (SNP) in an EPSPS gene of the plant.

An "EPSPS gene" should be understood as any gene which encodes a 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme. A 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme should be understood as any enzyme that catalyzes the reaction that converts shikimate-3-phosphate plus phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate (EPSP). Specific sequences for a range of EPSPS genes would be readily ascertained by those skilled in the art. For example, the NCBI GENE database may be searched for EPSPS or EPSP synthase to identify a range of EPSPS genes. Non-limiting

examples of plant EPSPS genes in the NCBI database include Gene ID numbers: 542727 (*Z. mays*), 819138 (*A. thaliana*) and 100232912 (*V. vinifera*).

In some embodiments, the present inventors have identified a number of SNPs in the EPSPS gene of lucerne that are genetically linked to the presence of SEQ ID NO: 1 in the genome of the plant.

In some embodiments, the SNP is positioned within an intron of an EPSPS gene. In some embodiments, the SNP is positioned within an intron between exon 3 and exon 4 of an EPSPS gene.

In some embodiments, a tolerant plant comprises the following nucleotide sequence, or a SNP-bearing fragment or homolog thereof, within an intron between exon 3 and exon 4 of the EPSPS gene:
ATCTACTGGCATAAGCACTTATGAGACTGTTTGGTATAG**T**TTATGAAAACAATTTATGACAT GTCCCTGAGAGCT (SEQ ID NO: 10).

In some embodiments, an intolerant plant comprises the following nucleotide sequence within an intron between exon 3 and exon 4 of the EPSPS gene:
ATCTACTGGCATAAGCACTTATGAGACTGTTTGGAATAG**C**TTATGAAAACAATTTATGACAT GTCCCTGAGAGCT (SEQ ID NO: 11).

In some embodiments, a tolerant plant comprises the following nucleotide sequence, or a SNP-bearing fragment or homolog thereof, within an intron between exon 3 and exon 4 of the EPSPS gene:
TAAAGCACAACAAATTGTGATGTTGCACTTGA**T**TGTGCATTTGAATTACTGGATAACTCTCA CATCAGTTACAGT (SEQ ID NO: 12).

In some embodiments, an intolerant plant comprises the following nucleotide sequence within an intron between exon 3 and exon 4 of the EPSPS gene:
TAAAGCACAACAAATTGTGATGTTGCACTTGA**C**TGTGCATTTGAATTACTGGATAACTCTCA CATCAGTTACAGT (SEQ ID NO: 13)

A "SNP-bearing fragment or homolog" of SEQ ID NO: 10 or SEQ ID NO: 12 should be considered as any fragment or homolog of SEQ ID NO: 10 or SEQ ID NO: 12 which includes the polymorphic locus, as indicated with bold and underline in each of SEQ ID NO: 10, 11, 12 and 13 above. Specifically, in tolerant plants, a T is present at each of the polymorphic loci, as shown in each of SEQ ID NO: 10 and SEQ ID NO: 12.

In some embodiments, in intolerant plants, a C is present at each of the polymorphic loci, as shown in each of SEQ ID NO: 11 and SEQ ID NO: 13. However, in other embodiments, a G or A may also be present at the polymorphic loci in an intolerant plant.

In some embodiments, a "fragment" of SEQ ID NO: 10 or SEQ ID NO: 12 may be at least 10, 20, 30, 40, 50, 60 or 70 nucleotides in length. In some embodiments a "homolog" of SEQ ID NO: 10 or SEQ ID NO: 12 comprises at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% nucleotide sequence identity to SEQ ID NO: 10 or SEQ ID NO: 12.

The present invention contemplates any suitable method for determining the presence or absence of a nucleotide sequence in the genome of a plant, as would be readily ascertained by those skilled in the art. For example, suitable methods may involve extraction of genomic DNA from one or more cells of the plant to be tested followed by the use of a polymerase chain reaction based method and/or Southern blotting based method on the extracted DNA in order to determine the presence or absence of the marker nucleotide sequence in the extracted genomic DNA.

In a second aspect, the present invention provides a plant having a glyphosate tolerant phenotype, wherein said plant has been identified and/or selected according to the method of the first aspect of the invention.

In some embodiments, the plant of the second aspect of the invention may be any plant as described above with reference to the first aspect of the invention.

In a third aspect, the present invention provides a method for breeding a progeny plant comprising a glyphosate tolerant phenotype, the method comprising:

identifying and/or selecting one or more parent plants which exhibit a glyphosate tolerant phenotype according to the method of the first aspect of the invention; and

producing one or more progeny plants from said parent, wherein one or more of said progeny plant exhibits a glyphosate tolerant phenotype.

In some embodiments, the plant of the third aspect of the invention may be any plant as described above with reference to the first aspect of the invention.

Methods for breeding various plants would be readily ascertained by those skilled in the art. By way of example, methods for breeding a range of agricultural crop plants are described in *Breeding field crops* (Sleper & Poehlman Eds., 5th Edition, Blackwell Publishing, 2006).

In some embodiments, a "progeny plant" as referred to herein should be understood to include any plant that is the product of breeding between two parent plants. In some embodiments, the term "progeny plant" may also include progeny plants produced by selfing one or more plants. As such, in some embodiments, a "progeny plant" may include the product of a breeding program and/or such plants which have been multiplied for cultivar increase, maintenance or seed production.

In a fourth aspect, the present invention provides a method for producing a crop of plants, the method comprising:

sowing seed obtained from one or more plants which exhibit a glyphosate

tolerant phenotype, wherein said plant or plants are: (i) identified and/or selected according to the method of the first aspect of the invention and/or (ii) produced by breeding according to the method of the third aspect of the invention;

cultivating plants from said seed to produce a crop of plants; and

controlling weeds in said crop by one or more applications of glyphosate to said crop.

In some embodiments, the plant of the fourth aspect of the invention may be any plant as described above with reference to the first aspect of the invention.

Methods for the sowing and cultivation of a wide range of plants would be readily ascertained by those skilled in the art. By way of example, cultivation of lucerne is described in detail in *Alfalfa, Botany cultivation and utilization* (Leonard Hill [Books] Ltd., London : and Interscience Publishers, Inc, New York, 1962).

Methods for controlling weeds in crops using glyphosate would also be readily ascertained by those skilled in the art. For example, methods for the application of glyphosate to a wide range of crops are described by glyphosate manufacturers including for example in the *Monsanto herbicide application handbook* (available at: http://www.monsanto.com/sitecollectiondocuments/ito/2009%20herbicide%20handbook%20(2).pdf).

In a fifth aspect, the present invention provides a glyphosate tolerant plant wherein said plant comprises:

glyphosate tolerance conferred by overexpression of an EPSPS gene; and

one or more agronomic traits selected from: a winter activity score (WAc) of greater than 1; resistance to an insect pest; resistance to a fungal disease; or a high seed yield.

In some embodiments, the plant of the fifth aspect of the invention may be a

plant as previously described with reference to the first aspect of the invention.

In some embodiments, the plant of the fifth aspect of the invention may be produced according to the breeding method of the third aspect of the invention.

As set out above, the plants of the fifth aspect of the present invention comprise glyphosate tolerance conferred by overexpression of an EPSPS gene. "Overexpression" of an EPSPS gene should be understood to comprise transcription of an EPSPS gene in a plant at a level of at least 1.5x, 2x, 2.5x, 3x, 3.5x, 4x, 4.5x, 5x, 6x, 7x, 8x, 9x or 10x of the level of EPSPS in a glyphosate sensitive cultivar of the same species of plant. In some embodiments, overexpression of the EPSPS gene in the glyphosate tolerant plant comprises basal and/or constitutive overexpression of the EPSPS gene, that is overexpression of the EPSPS gene even when the plant is not exposed to glyphosate.

Expression of an EPSPS gene in a plant may be determined using any suitable method. Exemplary methods of the detection of expression include methods such as quantitative or semi-quantitative reverse-transcriptase PCR (eg. see Burton *et al.*, *Plant Physiology* 134: 224-236, 2004), *in-situ* hybridization (eg. see Linnestad *et al.*, *Plant Physiology* 118: 1169-1180, 1998); northern blotting (eg. see Mizuno *et al.*, Plant Physiology 132: 1989-1997, 2003); indirect methods such as the method of the first aspect of the invention; and the like.

As set out above, the plants of the present invention comprise one or more agronomic traits selected from: a winter activity score (WAc) of greater than 1; resistance to an insect pest; resistance to a fungal disease; or a high seed yield.

In at least lucerne, winter activity is a highly critical trait. For example high WAc is desirable for intensive grazing areas but with the consequence that the crop is less persistent in such a growing area. Winter activity (WAc) may be scored on

the basis of growth of a plant during a winter season. For example, in lucerne, the WAc score of the plant refers to the length of plant regrowth in inches per 6 weeks during the winter season. For example, a WAc level of 1, means regrowth over 6 weeks in winter of 1 inch. In some embodiments, the plants of the present invention have a WAc score of at least 2, 3, 4, 5, 6, 7, 8, 9 or 10.

"Resistance to an insect pest" and/or "resistance to a fungal disease" would be readily identified in a plant of the present invention by a person skilled in the art using any assay suitable to assess the level of a particular disease in the plant.

In some embodiments, resistance to an insect pest comprises resistance to Spotted Alfalfa Aphid (SAA) and/or resistance to Blue Green Aphid (BGA). By way of example, resistance to either of these insect pests may be assessed using the methods of Humphries *et al.* (*Crop and Pasture Science* 63: 893-901, 2012) and/or Nair *et al.* (*Australian Journal of Experimental Agriculture* 43: 1345-1349, 2003).

In some embodiments, resistance to a fungal disease comprises resistance to Anthracnose (*Colletotrichum trifolii*) and/or Phytophthora root rot (*Phytophtora medicaginis)*. By way of example, resistance to either of these fungal diseases may be assessed using the methods of Mackie and Irwin (*Australian Journal of Experimental Agriculture* 38: 41-44, 1998).

"High seed yield" as defined herein should be understood as a plant having at least 50%, 60%, 70%, 80%, 90% or 100% of the seed yield observed in an existing commercially-released variety of the plant of the same species. In some embodiments, "high seed yield" may comprise a plant which has 50%, 60%, 70%, 80%, 90% or 100% of the average seed yield observed in at least 3, 5 or 10 commercially released varieties of a plant of the same species.

In respect of lucerne, "high seed yield" comprises a plant which has at least 50%,

60%, 70%, 80%, 90% or 100% of the seed yield of a commercially-released variety with comparable winter activity score in the absence of disease. In some embodiments, the commercially-released variety for comparison of seed yield comprises SARDI 5, SARDI 7 or SARDI 10, which may be obtained from the South Australian Research & Development Institute (Waite campus, Plant Research Centre, Hartley Grove, Urrbrae SA 5064).

The present invention is further described with reference to the following non-limiting examples:

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows phenotypic variation in *Medicago sativa* plants that differentially express EPSPS 3 weeks after application of glyphosate at a rate of 1.2 L/ha (540g glyphosate/L). From left to right: P1: Intolerant parent plant (breeders line) with superior agronomic performance; K108: intolerant plant used as crossing parent with superior resistance to BGA and SAA; K150.65: tolerant selection; K150.57: tolerant selection.

FIGURE 2 shows the expression of EPSPS in a range of glyphosate intolerant *M. sativa* plant lines and a range of glyphosate tolerant *M. sativa* plants prior to application of glyphosate to the plants.

FIGURE 3 shows the expression of EPSPS in a range of glyphosate intolerant *M. sativa* plant lines and a range of glyphosate tolerant *M. sativa* plants 24 h after application of glyphosate to the plants.

FIGURE 4 shows schematic diagrams of the 6kb promoter region (A) and the 4kb promoter region (B) identified in glyphosate tolerant lucerne.

FIGURE 5 shows a schematic diagram of a range of EPSPS promoter fragments to be tested in order to identify EPSPS gene promoter regions associated with increased EPSPS expression and glyphosate tolerance.

FIGURE 6 shows a schematic representation of the pGUS UBQ dsRED transformation vector used for hairy root transformation.

FIGURE 7 is a graphical representation showing relative GUS reporter gene expression in hairy root cultures transformed with the GUS reporter gene under the transcriptional control of a range of promoter fragments from glyphosate tolerant and intolerant plants. Int6B = 6B promoter fragment from intolerant lucerne; Int6C = 6C promoter fragment from intolerant lucerne; Int4B = 4B promoter fragment from intolerant lucerne; Int4C = 4C promoter fragment from intolerant lucerne; EV = empty vector control; Tol6B = 6B promoter fragment from tolerant lucerne; Tol6C = 6C promoter fragment from tolerant lucerne; Tol4A = 4A promoter fragment from tolerant lucerne; Tol4B = 4B promoter fragment from tolerant lucerne; Tol4C = 4C promoter fragment from tolerant lucerne.

FIGURE 8 is a schematic representation of showing the position of the 1763bp region relative to the 6B and 6C promoter fragments.

FIGURE 9 shows the survival of glyphosate sensitive and glyphosate tolerant *M. sativa* plants at two different rates of glyphosate application (0.8 l/ha and 1.2 l/ha). Falcata = naturally occurring glyphosate tolerant *Medicago* spp. identified in accordance with the present invention; K116, K117, K118 = glyphosate tolerant *M. sativa* selections produced by breeding according to the present invention; S5, S7, S10 = glyphosate intolerant cultivars SARDI 5, SARDI 7 and SARDI 10 that have increased winter activity.

FIGURE 10 shows two selected glyphosate tolerant cultivars of *M. sativa* and a glyphosate sensitive *M. sativa* cultivar growing in the field after application of glyphosate (540g in 1L/ha sprayed at the seedling stage of 3-5 trifoliates). A: A glyphosate tolerant selection produced from crossing between Falcata and breeders lines with improved agronomic performance; B: Intolerant SARDI 7 variety as negative control; C: selection of glyphosate tolerance from within negative control SARDI 7 in the absence of using the Falcata as a parent.

FIGURE 11 shows a schematic of the breeding plan for generating agronomically useful lucerne varieties, which carry the marker nucleotide sequence and glyphosate tolerance trait of the present invention.

FIGURE 12 shows a schematic representation of the intron-exon structure of *M. truncatula* EPSPS gene *Medtr4g024620*.

FIGURE 13 shows the ability of SNP 2, which is linked to the presence or absence SEQ ID NO: 1 in the genome of lucerne plants, to distinguish between glyphosate tolerant and intolerant plants. Synthetic heterozygous: equimolar amounts of DNA extracted from tolerant and intolerant plants are mixed to test the ability of the marker to distinguishing homozygous and heterozygous plants.

FIGURE 14 shows a schematic of the breeding plan for generating glyphosate tolerant lucerne line GHT5.

FIGURE 15 shows a schematic of the breeding plan for generating glyphosate tolerant lucerne line GHT9.

EXAMPLE 1 – EPSPS EXPRESSION IN INTOLERANT AND TOLERANT *M. SATIVA*

Figure 1 shows phenotypic variation in *Medicago sativa* plants that differentially express EPSPS 3 weeks after application of glyphosate at a rate of 1.2 L/ha (540g

glyphosate/L). As can be seen in Figure 1, the selected glyphosate tolerant plants exhibit substantially improved growth and overall plant health compared to the intolerant plants.

Figures 2 and 3 show the expression of EPSPS in glyphosate tolerant and intolerant plants prior to glyphosate application (Figure 2) and 24 h after glyphosate application (Figure 3).

Expression of the EPSPS gene in lucerne was assessed using two types of quantitative real-time PCR (qPCR) analyses on total RNA extracted from seedlings (8 trifoliate stage) prior (0 hours; Experiment 1 and 2) and after (24 hours Exp. 2) spraying with glyphosate at 1.2 L/ha (540 g/L conc. of active ingredient as in Roundup Powermax). Total RNA was extracted using Trizol (Invitrogen, Victoria, Australia), RNA quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Wilmington, DE, USA) and quality of RNA samples checked by gel electrophoresis. cDNA was synthesised from 3 µg of RNA using 200 U of SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions with quality being confirmed via PCR detection of transcripts from the *M. truncatula* gene actin using primers previously described (Kuppusamy *et al.*, *Plant Physiology* 136: 3682–3691, 2004).

Two primer pairs specific to the EPSPS gene sequence in lucerne were designed for quantitative real-time RT PCR analysis as set out below:
EPS-1.F: 5′-TGTGGAAGGCAGTGGTGGGT-3′ (SEQ ID NO: 2)
EPS-1.R: 5′-GGACGCATTGCAGTACCGGC-3′ (SEQ ID NO: 3)
(60°C annealing temperature, 84 bp amplicon size)

EPS-2.F: 5′-GAAGGGAGGGCTTCCAGGGG-3′ (SEQ ID NO: 4)
EPS-2.R: 5′-GGGCCAACGGAGCTGCCATA-3′ (SEQ ID NO: 5)
(60°C annealing temperature, 92 bp amplicon size)

Primers of *actin* gene used for normalization:
actF: 5'-ATGTTGCTATTCAGGCCG-3' (SEQ ID NO: 6)
actR: 5'-GCTCATAGTCAAGGGCAAT-3' (SEQ ID NO: 7)

Experiment 1

Total RNA was treated with DNase to remove potential genomic DNA contamination as per manufacturer's recommendations (DNA-free kit, Ambion). cDNA was synthesised from 3 µg of RNA using 200 U of SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions with quality being confirmed via PCR detection of transcripts from the *M. truncatula* gene actin using primers previously described (Kuppusamy *et al.,* 2004, *supra*). Quantification in experiment 1 was performed in a RG 3000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, Sydney, Australia) according to the procedure previously described by Bogacki *et al.* (*BMC Plant Biology* 13:54, DOI 10.1186/1471-2229-13-54, 2013).

Before a comparison between tolerant and intolerant plants, initial qPCR runs compared the amplification efficiency of primer pairs EPS-1F/-1R and EPS-2F/-2R and showed very similar results for both pairs so that one primer pair, EPS-1F/-1R, was used in this first experiment.

The first qPCR experiment comparing *EPSPS* expression was carried out with cDNA samples from 10 phenotypically intolerant (no glyphosate tolerant line in parentage) in comparison to Falcata and 9 tolerant progeny plants. In general, expression data are relative data and normalized to the housekeeping *actin* gene amplified by actF/actR. In this first experiment, the tolerant lines showed on average a 8.2-fold higher expression level (range 6.1 to 10.9-fold) of the *EPSPS* gene compared to the intolerant lines.

Experiment 2

The second experiment was carried out with RNA from tolerant and intolerant lucerne plants immediately before (0 h) and after (24 h) glyphosate treatment. Total RNA was extracted as described above using three trifoliates per plant of eight month old plants, 19 with an intolerant phenotype and 20 tolerant plants plus Falcata. cDNAs were synthesized according to the protocol described by Nattrass *et al.* (*Journal of Animal Science* 92(2): 443-455, 2014) in sections 'Reverse Transcription' using the oligodT-VN primers but no gene-specific primers. The qPCR runs were performed using both *EPSPS* qPCR primer pairs, generating comparable results, on 384-well real-time PCR machines (7900, Applied Biosystems) using cDNA concentrations and cycling parameters as described in Nattrass *et al.* (2014, *supra*).

For normalization, qPCR primers for the lucerne sequence of gene *GAPDH* were used:
GAPDH_F: 5'-TCCCAACCGTCGATGTTTCAGT-3' (SEQ ID NO: 8)
GAPDH_R: 5'-TTGCCCTCTGATTCCTCCTTG-3' (SEQ ID NO: 9)

As shown in Figures 2 and 3, at time point 0 hours, average EPSPS gene expression in tolerant plants was 9-fold higher than that in intolerant plants. Expression in Falcata was 4-fold compared to average intolerant plants. After 24 hours of treatment, average EPSPS expression in tolerant plants was still 5-fold compared to intolerant plants and 10-fold in Falcata.

EXAMPLE 2 – IDENTIFICATION OF EPSPS GENE PROMOTER FRAGMENTS ASSOCIATED WITH INCREASED EPSPS EXPRESSION IN *M. SATIVA*

In lucerne, which is tetraploid, the EPSPS gene is duplicated and driven under the control of different promoter regions. As shown in Figure 4, these promoter regions are referred to herein as the 4kb promoter region (4kbPro) and the 6kb promoter region (6kbPro) on the basis of their respective sizes.

A range of promoter fragments was selected for testing in order to identify promoter regions associated with increased EPSPS expression and glyphosate tolerance. These promoter fragments are shown in Figure 5 and identified as:

6B – 2586 bp fragment of the 6kb promoter

6C – 823 bp fragment of the 6kb promoter

4A – 3319 bp fragment of the 4kb promoter

4B – 2161 bp fragment of the 4kb promoter

4C – 842 bp fragment of the 4 kb promoter

EXAMPLE 3 – SCREENING OF PROMOTER FRAGMENTS IN A HAIRY ROOT EXPRESSION SYSTEM

Each of fragments 6B, 6C, 4A, 4B and 4C from glyphosate intolerant lucerne and glyphosate tolerant lucerne, respectively, were tested for their ability to drive expression of a GUS reporter gene in a hairy root expression system.

The protocols for the hairy root system transformation and the MUG assay were carried out as previously been described in Kim and Nam (*Journal of Plant Physiology* 170: 291-302, 2013) using the same A17 *Medicago truncatula* genotype with minor modifications. Modifications were that between 30 – 80 mg of root tissue were used with the initial extraction volume of 500 µL. 10 µL aliquots of extracts were used with an additional 10 µL extraction buffer and 180 µL assay buffer. Aliquots of 10 µL were removed at time points 10, 40 and 70 minutes and 90 µL of Na_2CO_3 stop solution immediately added. Protein concentrations were quantified using Bradford solution according to the manufacturer's directions (Bio-Rad). For each promoter construct three different tissue samples (repeats) were measured at all three time points (10, 40, 70 min). The measurements of all promoter constructs (three repeats each) were repeated five times. Fluorescence was measured on plate reader POLARstar OPTIMA, (BMG Labtech, Offenburg, Germany) with 355 excitation/460 emission filters.

In short, each of the test fragments was cloned into the pGUS UBG dsRED vector (see Figure 6) and this vector was then transformed into a *M. truncatula* hairy root culture. Within this vector, each test fragment was operably connected to a GUS reporter gene and thus the ability of the promoter fragment to drive gene expression was represented by expression of the GUS reporter gene in the hairy root system.

Results are shown in Figures 7 & 8. As shown in Figure 7, promoter fragment 6B from glyphosate tolerant *M. sativa* drove expression of the GUS reporter gene at a level substantially higher than any other promoter fragment. Particularly, promoter fragment 6B drove expression of the reporter gene at a level substantially higher than promoter fragment 6C from the glyphosate tolerant plants. This indicates that all or part of the additional 1763 bp region of fragment 6B from the glyphosate tolerant plants (see Figure 8) is responsible for the substantially higher expression of the EPSPS gene in glyphosate tolerant *M. sativa* and thus involved in the glyphosate tolerance phenotype.

The sequence of the 1763bp region (5′-3′) is set out below:

gacagtgtctccctcatcaacagtcgctgccgattctcaccaacccactcattcccctccta
ccaacccattctttagataaataaaacccactcattcccctcttaccaacccattctttaga
taaataaatgaagaaatattttatcacatatacgcagttcctctatattaattatttaaagt
taaataaatttttttatctcataaatatttttcccaaaaaaaaaaaaaaatcctttccatatt
ttaaatttttctagactttttttttcatctaaatattttaaatttcaaatattttctgtcacg
atttttggtctatattttcaagtgaactcatacatatttttcaaaaacttgagttaatttttt
ttagacaagtttaatagatcatagaaattattctttccctaaaaaaagttattgttcttgca
aaagttaatttttttttttttaataaaagattaattgaatgtgaactctgatgcctaacctgtc
tcttgacttataaaaaaaatatgaatttttaagtttttaacaccaacaaatttctttttaaat
cataatttgttaaaaaaattccaatttttttatagtagataatgtgttataatattctaaaag
tctttgtaaatatttattaaataatatgaatgatacacatgagttgatataaggggaattag
tcactttagtctctgaatagaaaataagtagtcactttagtccctgaatgcagagaaattgc
aaaacaatccttgaatgtagactctgttggtcaatttcagggactaaagtgactaacggagt
ctacattcaggaactaaattgactaacggagtctgcattcaggggctaaattgactaacata
gtctacattcagggactattttgcaatttatctgcattcatagactaaagtgacaactcctt
tcctattcagggactaaagtgactaattccccgttgatttaatgacaaaacatttgaataga
agaaggatatgcatttgcatttggaagatatctttgtgctagcaaaaacaatttctatttct
ccaatgtgctcaatactggtgggggaagctttgagtttatatcatgctaaggaatggttgag
cgatactcttatttgataatgtggattttgcttcagatttcaaggttactatggatgcttga
ttgctttccatcatgatcgtgtgatttcacagaatttgatcagatattttgcgtctgcagaa

gattgtttatcactcacttcactaactttaaggtcgagttcaatcgatgacaaataaatgac
gtaactcaccacctaacagaaattgtcacattatcagctagtctcaatatccatttttatgc
accttattgtatcgaataattattaataaaatgttataaggatctttcttttcaaaaaatgt
tgctgctaagaataaaacagtaatagtggaggctgtgaatattactctctttcctctatatc
tgagttgttttgttatgagtttttttaaggggaattgtttttttatgagttgttaatttgt
tattatgaattggaatgtaaacatttatacaatttgtctatttatttgataaaatttaaatt
agcatatatagtgtaatttattcaatgctatttaatttttttttatttttcttaatataaaat
aattaacttttcctattttattcatttcttcaaaattcatgacatttctttaataatttttt
ttaaggaacatttctttaataataaca (SEQ ID NO: 1)

EXAMPLE 4 – IDENTIFICATION OF SNPs IN EPSPS GENE LINKED TO INCREASED EPSPS EXPRESSION AND GLYPHOSATE TOLERANCE

As at 19 June 2014, no lucerne (*Medicago sativa*) sequence of the EPSPS gene is available in the public database; to illustrate the position of two diagnostic SNPs, the sequence of the *EPSPS* gene *Medtr4g024620* in *M. truncatula* (gene as in clone mth2-6b12, BAC AC119419), a close relative of *M. sativa*, serves as a reference (see Figure 12). Vertical arrows indicate the position of two diagnostic SNPs between exon no. 3 and 4.

The SNPs were identified by sequencing regions of the lucerne EPSPS gene in 347 plants with known tolerance/intolerance to the herbicide glyphosate. It is most likely that there are further diagnostic SNPs in other regions of the gene but in our analyses we have used the following two (bold and underlined) which have 100% linkage between the SNP, SEQ ID NO: 1 and a glyphosate tolerant phenotype in lucerne.

Diagnostic SNP no. 1

Tolerant donor line and all derived tested tolerant lucerne progeny plants:
ATCTACTGGCATAAGCACTTATGAGACTGTTTGGTATAG**T**TTATGAAAACAATTTATGACAT
GTCCCTGAGAGCT (SEQ ID NO: 10)

Intolerant plants incl. current intolerant varieties:
ATCTACTGGCATAAGCACTTATGAGACTGTTTGGAATAG**C**TTATGAAAACAATTTATGACAT
GTCCCTGAGAGCT (SEQ ID NO: 11)

Diagnostic SNP no. 2

Tolerant donor line and all derived tested tolerant lucerne progeny plants:
TAAAGCACAACAAATTGTGATGTTGCACTTGA**T**TGTGCATTTGAATTACTGGATAACTCTCA
CATCAGTTACAGT (SEQ ID NO: 12)

Intolerant plants incl. current intolerant varieties:
TAAAGCACAACAAATTGTGATGTTGCACTTGA**C**TGTGCATTTGAATTACTGGATAACTCTCA
CATCAGTTACAGT (SEQ ID NO: 13)

SNP 2 was utilized to derive a high-throughput PCR marker that is able to distinguish the tolerant from the intolerant EPSPS genomic sequence (allele). This KASPar marker is diagnostic and a versatile tool to identify plants that carry the homozygous tolerant gene version. Primers below LuGly-wt.F, LuGly-mu.F and LuGly.R are used together to differentiate the intolerant (wt) allele and tolerant (mu) allele. The amplified lucerne gene sequence is 61 bp with an additional 21 bp added by the fluorescent tag sequence for VIC (=HEX) and FAM labels. The marker assays have been performed on Bio-Rad CFX thermal cyclers according to the protocol described in the Bio-Rad booklet 'KASP_Bio-Rad manual_v2'.

LuGly-wt.F (5'-3'):
GAAGGTCGGAGTCAACGGATTCAACAAATTGTGATGTTGCACTTGAC (SEQ ID NO: 14)
LuGly-mu.F (5'-3'):
GAAGGTGACCAAGTTCATGCTCAACAAATTGTGATGTTGCACTTGAT (SEQ ID NO: 15)
LuGly.R (5'-3'):
CTGATGTGAGAGTTATCCAGTAATTCA (SEQ ID NO: 16)

As shown in Figure 13, the LuGly marker is able to identify homozygous tolerant plants among intolerant and heterozygous plants. It does not distinguish between heterozygous and homozygous intolerant plants. It is useful to select pure breeding tolerant plants and detect the tolerant varieties.

EXAMPLE 5 – GLYPHOSATE TOLERANCE OF PLANTS SELECTED USING A MARKER NUCLEOTIDE SEQUENCE

Herbicide selections were carried out in the field and the glasshouse with different rates of glyphosate. Intolerant varieties such as SARDI 5, 7 or 10 were used as negative controls that were grown simultaneously with the tolerant selections so that developmental stages (physiological stage) between tolerant and intolerant plants were matching. Selections were made by spraying at rates of 180 g active ingredient (ai) per ha, 360 g ai/ha, 540 g ai/ha, 900 g ai/ha and 1620 g ai/ha. The commercial product Roundup Powermax (Monsanto) with a concentration of 540 g ai/L was used in spraying assays.

The rate screening occurred:

- for seedling testing, at plant stage of 3-5 trifoliates, in field and glasshouse;
- for mature plants in the field with plants >8 months old;
- to achieve the desired concentration of ai, desired volumes of Roundup Powermax were diluted at a rate of 100 L water /ha as carrier. Total volume was adjusted to the actual size of the area sprayed, e.g. spray volume was 20 L for a trial site size of 2000 m^2 sprayed as 2x 10L to achieve good coverage.
- to achieve good coverage, plants were sprayed twice with ½ rate.
- survival inspection occurred weekly with final assessment at 6-8 weeks after spraying for mature plants and scoring of sprayed seedlings after 3 weeks at the latest.
- controls were SARDI lines with matching winter activity (SARDI 5, 7, 10 with 10 being the most actively growing variety in winter).

Results from these experiments are shown in Figures 9 and 10. As can be seen from these figures, selected glyphosate tolerant plants exhibited substantially improved plant survival and growth after application of glyphosate at all rates

tested.

EXAMPLE 6 – MARKER ASSISTED BREEDING OF GLYPHOSATE TOLERANT LUCERNE PLANTS

Crosses to combine glyphosate tolerance and desirable agronomical traits occurred either in the glasshouse by manual pollination, in the field or in insect proof tents containing bee hives for pollination during the entire flowering period of lucerne (ca. 6 weeks).

Figure 11 shows an overview of the crosses used to produce advanced material carrying the glyphosate tolerance trait and agronomically relevant traits (namely Winter active class 7 cultivar (WAc 7) starting with a cross between the original herbicide tolerant selection, *Mypolonga falcata* and germplasm grown in the SARDI Nursery to reduce the unfavorable traits from *M. falcata* such as prostrate growth, poor biomass etc. by 50%.

2008-2010

Seed from the original plant was produced under open pollination in the SARDI nursery. We discovered that the plant will not self seed, so seed should be assumed to be from crosses with random pollen from our nursery.

The first seed, G6701, was then selected for tolerance to glyphosate (GHT) and survivors used as maternal plants to hand cross with random pollen from highly winter active breeders lines with the aim of improving its winter activity, forage and seed yield. This produced line K127 with 15 half sib families. K127 was sequentially selected in the greenhouse for tolerance to bluegreen aphid (BGA) and spotted alfalfa aphid (SAA). Surviving plants were then further refined for tolerance to glyphosate. 36 plants with the highest tolerance to glyphosate were used as maternal parents and crossed with bulk pollen from highly winter active breeders lines to produce K148 with 36 half sib families. K148 half sib families

were then sown in a row trial and refined for glyphosate tolerance, winter activity and seed yield. Plants with similar winter activity ratings were grouped into three winter activity classes "3-5, 6-7 and 8-9", intermated in these groups to produce 'K150 WAc 5, K150 WAc 7 and K150 WAc9' for use in cultivar development with these target winter activity ratings.

2010-2015

The winter activity class 6-7 K150 half sib families were selected for resistance to bluegreen and spotted alfalfa aphids, Phytophthora (PRR) and Collletotrichum (Anthracnose, Anth) and glyphosate. Surviving plants were cloned and then crossed using open pollination with winter active breeders lines to produce seed of half sib families, K168-K172 (harvested autumn 2011).

K168-172 were sown in punnets 50 seeds, selected for glyphosate tolerance at 1.5 true leaves, and then refined using sequential selection for resistance to BGA, SAA, PRR and Anth. Selected plants sown intermated with controlled pollination using an insect proof mesh cage and honeybees for pollination, and maternal parents harvested individually as K212-1,132 (harvested autumn 2012).

K212 (132 half sibs) families were sown in a replicated row experiment at the lucerne nursery (Bay 8) in autumn 2012 and evaluated over an 18 month period. In the first year the plants were selected for tolerance to glyphosate in late winter and spring (high selection pressure), and then seed production components of surviving plants were measured in March 2013 (pod coiling, seeds per pod, pods per plant, seed yield per plant) to eliminate plants with poor seed yield. The remaining plants were further refined in April-October 2013 for leafiness (visual estimate), winter activity, and forage yield following application of glyphosate. Cuttings were also taken of best plants in autumn 2013 to identify resistance to BGA, SAA, PRR and Anth. Results of greenhouse disease and insect testing combined with phenotyping in the field experiments were used to refine the number of parents to 64, which were then confirmed to contain the glyphosate

tolerant genotype (SEQ ID NO: 1) and intermated with honeybees in a controlled pollination environment using vegetative clones to replicate and randomise the mating design, to produce pre-breeders seed 'K266'. The pre-breeders seed was sown at Howlong, NSW in September 2014, and following removal of plants susceptible to glyphosate in 2014/15 will be used to produce breeders seed in March 2016.

Given the assumption that 50% of genes in each generation will be contributed from the maternal parent, the final cultivar is expected to have approximately 6.25% genes contributed from the original falcata parent.

Crosses in 2011, 2012, and 2013 were carried out in the glasshouse, tents and under nursery conditions to glyphosate tolerance and disease resistances with an emphasis on seed yield increase that was particularly weak due to the remaining Falcata genome fragments, which would be on average 6.25% or 3.12% in the different advanced breeders' lines. During all breeding cycles selections occurred also based on 'soft' criteria such as healthy, vigorous plant appearance and leafiness.

Line K266 is the most advanced lucerne line carrying the desirable agronomical traits (superior resistance to SAA, BGA, Anthracnose, Phytophthora, high seed yield, leafiness and WAc 7) combined with glyphosate tolerance.

EXAMPLE 6 – MARKER ASSISTED BREEDING OF GLYPHOSATE TOLERANT LUCERNE LINE GHT5

A scheme for the breeding of a glyphosate tolerant, semi-winter active class 5, lucerne cultivar (pre-breeders seed to be produced in April 2017) is shown in Figure 14.

2008-2010

The steps to produce seed of K150 are common to the development of the winter activity class 7 cultivar described in Example 5.

2010-2015

The semi-winter activity class 5 K150 half sib families were selected for resistance to bluegreen and spotted alfalfa aphids, Phytophthora (PRR) and Collletotrichum (Anthracnose, Anth) and glyphosate. Surviving plants were cloned and then crossed using open pollination with winter active breeders lines to produce seed of half sib families, K163-K168 (harvested autumn 2011).

K163-168 were sown in punnets 50 seeds, selected for glyphosate tolerance at 1.5 true leaves, and then refined using sequential selection for resistance to BGA, SAA, PRR and Anth. Selected plants sown intermated with controlled pollination using an insect proof mesh cage and honeybees for pollination, and 64 maternal parents harvested individually as K211 (harvested autumn 2012).

K211 (64 half sibs) families were sown in a replicated row experiment at the lucerne nursery (Bay 11) in autumn 2012 and evaluated over an 18 month period. In the first year the plants were selected for tolerance to glyphosate in late winter and spring (high selection pressure), and then seed production components of surviving plants were measured in March 2013 (pod coiling, seeds per pod, pods per plant, seed yield per plant) to eliminate plants with poor seed yield. A total of 32 single plants from K211 (and some K212 plants eliminated from the GHT7 cultivar development due to being too winter dormant) were selected from this trial, confirmed to contain the glyphosate tolerant genotype (SEQ ID NO: 1) and hand crossed in a greenhouse with high seed yield selections of SARDI Grazer to improved seed yield. Seed from the 32 field K211 maternal parents was harvested as K253 1-32.

The 32 K253 half sib families were sequentially selected in the greenhouse for resistance to BGA, SAA, Anth and PRR and tolerance to glyphosate. A total of 86

disease, insect and glyphosate tolerant plants were selected from the 32 half sib families, and intermated in a controlled pollination environment with honeybees to produce K273.

2015-2017

In 2015, 66 of the 86 K273 plants were sown on 8 May in a replicated and randomised plot experiment to identify parents over the next 18 months that are winter active, glyphosate tolerant and high seed yielding. Selected plants will be identified as final parents in a winter activity class 5 cultivar, with production of pre-breeders seed planned for autumn 2017.

Given the assumption that 50% of genes in each generation will be contributed from the maternal parent, the final cultivar is expected to have approximately 3.125% genes contributed from the original falcata parent.

EXAMPLE 7 – MARKER ASSISTED BREEDING OF GLYPHOSATE TOLERANT LUCERNE LINE GHT9

A scheme for the breeding of a glyphosate tolerant, Winter activity class 9, lucerne cultivar (pre-breeders seed to be produced in April 2017) is shown in Figure 15.

2008-2010

The steps to produce seed of K150 are common to the development of the winter activity class 7 cultivar described in Example 5.

2010-2015

The highly winter active class 9 K150 half sib families were selected for resistance to bluegreen and spotted alfalfa aphids, Phytophthora (PRR) and Collletotrichum (Anthracnose, Anth) and glyphosate. Surviving plants were cloned and then crossed using open pollination with winter active breeders lines to produce seed

of half sib families, K173-K175 (harvested autumn 2011).

K173-175 were sown in punnets with 50 seeds, selected for glyphosate tolerance at 1.5 true leaves, and then refined using sequential selection for resistance to BGA, SAA, PRR and Anth. Selected plants sown intermated with controlled pollination using an insect proof mesh cage and honeybees for pollination, and 192 maternal parents harvested individually as K213 (harvested autumn 2012).

K211 (192 half sibs) families were sown in a replicated row experiment at the lucerne nursery (Bay 3) in autumn 2012 and evaluated over an 18 month period. In the first year the plants were selected for tolerance to glyphosate in late winter and spring (high selection pressure), and then seed production components of surviving plants were measured in March 2013 (pod coiling, seeds per pod, pods per plant, seed yield per plant) to eliminate plants with poor seed yield. A total of 30 single plants from K213 were selected from this trial, confirmed to contain the glyphosate tolerant genotype (SEQ ID NO: 1) and hand crossed in a greenhouse with high seed yield selections of highly winter active class 10 SARDI breeders lines to improved seed yield. Seed from the 30 field K213 maternal parents was harvested as K255 1-30.

The 30 K253 half sib families were sequentially selected in the greenhouse for resistance to BGA, SAA, Anth and PRR and tolerance to glyphosate. A total of 108 disease, insect and glyphosate tolerant plants were selected from the 30 half sib families, and intermated in a controlled pollination environment with honeybees to produce K274.

2015-2017

In 2015, 81 of the highest seed yielding 108 K273 plants were sown in May in a replicated and randomised plot experiment to identify parents over the next 18 months that are winter active, glyphosate tolerant and high seed yielding. Selected plants will be identified as final parents in a winter activity class 9

cultivar, with production of pre-breeders seed planned for autumn 2017.

Given the assumption that 50% of genes in each generation will be contributed from the maternal parent, the final cultivar is expected to have approximately 3.125% genes contributed from the original falcata parent.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the features referred to, or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the features.

Also, it must be noted that, as used herein, the singular forms "a", "an" and "the" include plural aspects unless the context already dictates otherwise.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Subheadings used in the description are for ease of reference only and should not affect interpretation or construction.

All documents referred to herein are hereby incorporated by reference.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for identifying a plant having a glyphosate tolerant phenotype, the method comprising determining the presence or absence of a marker nucleotide sequence in the genome of said plant, wherein the marker nucleotide sequence comprises one or more of:

(i) SEQ ID NO: 1 or a homolog thereof;

(ii) a fragment of the nucleotide sequence set out at (i); and/or

(iii) a nucleotide sequence genetically linked to the nucleotide sequence set out at (i) or (ii) in the genome of said plant;

wherein the presence of the marker nucleotide sequence indicates that the plant has a glyphosate tolerant phenotype.

2. The method of claim 1 wherein the method further comprises selecting a plant having a glyphosate tolerant phenotype on the basis of the presence of the marker nucleotide sequence in the genome of the plant.

3. The method of claim 1 or 2 wherein the plant is a dicot plant.

4. The method of claim 1 or 2 wherein the plant is a leguminous plant.

5. The method of claim 1 or 2 wherein the plant is of the genus *Medicago*.

6. The method of claim 1 or 2 wherein the plant is of the species *Medicago sativa*.

7. A plant having a glyphosate tolerant phenotype, wherein said plant has been identified and/or selected according to the method of any one of claims 1 to 6.

8. The plant of claim 7 wherein the plant is a dicot plant.

9. The plant of claim 7 wherein the plant is a leguminous plant.

10. The plant of claim 7 wherein the plant is of the genus *Medicago*.

11. The plant of claim 7 wherein the plant is of the species *Medicago sativa*.

12. A method for breeding a progeny plant comprising a glyphosate tolerant phenotype, the method comprising:

identifying and/or selecting one or more parent plants which exhibit a glyphosate tolerant phenotype according to the method of any one of claims 1 to 6; and

producing one or more progeny plants from said parent, wherein one or more of said progeny plant exhibits a glyphosate tolerant phenotype.

13. The method of claim 12 wherein the plant is a dicot plant.

14. The method of claim 12 wherein the plant is a leguminous plant.

15. The method of claim 12 wherein the plant is of the genus *Medicago*.

16. The method of claim 12 wherein the plant is of the species *Medicago sativa*.

17. A method for producing a crop of plants, the method comprising:

sowing seed obtained from one or more plants which exhibit a glyphosate tolerant phenotype, wherein said plant or plants are: (i) identified and/or selected according to the method of any one of claims 1 to 6 and/or (ii) produced by breeding according to the method of any one of claims 12 to 16;

cultivating plants from said seed to produce a crop of plants; and

controlling weeds in said crop by one or more applications of glyphosate

to said crop.

18. The method of claim 17 wherein the plant is a dicot plant.

19. The method of claim 17 wherein the plant is a leguminous plant.

20. The method of claim 17 wherein the plant is of the genus *Medicago*.

21. The method of claim 17 wherein the plant is of the species *Medicago sativa*.

22. A glyphosate tolerant plant wherein said plant comprises:
glyphosate tolerance conferred by overexpression of an EPSPS gene; and
one or more agronomic traits selected from: a winter activity score (WAc) of greater than 1; resistance to an insect pest; resistance to a fungal disease; or high seed yield.

23. The plant of claim 22 wherein the plant is a dicot plant.

24. The plant of claim 22 wherein the plant is a leguminous plant.

25. The plant of claim 22 wherein the plant is of the genus *Medicago*.

26. The plant of claim 22 wherein the plant is of the species *Medicago sativa*.

FIGURE 1

FIGURE 2

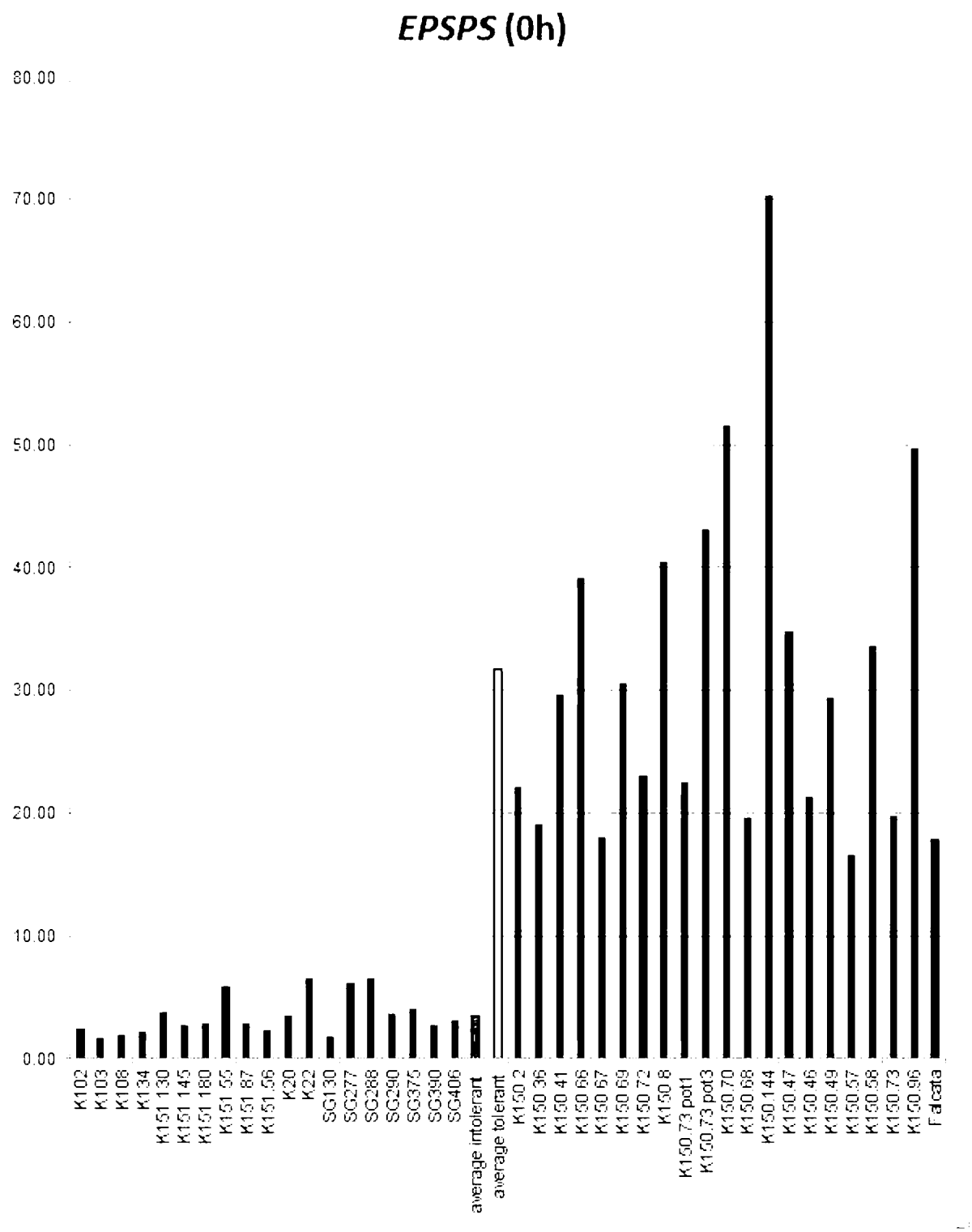

EPSPS (0h)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
K102
K103
K108
K134
K151 130
K151 145
K151 180
K151 55
K151 87
K151 56
K20
K22
SG130
SG277
SG288
SG290
SG375
SG390
SG406
average intolerant
average tolerant
K150 2
K150 36
K150 41
K150 66
K150 67
K150 69
K150 72
K150 8
K150 73 pot1
K150 73 pot3
K150 70
K150 68
K150 144
K150 47
K150 46
K150 49
K150 57
K150 58
K150 73
K150 96
Facata

FIGURE 3

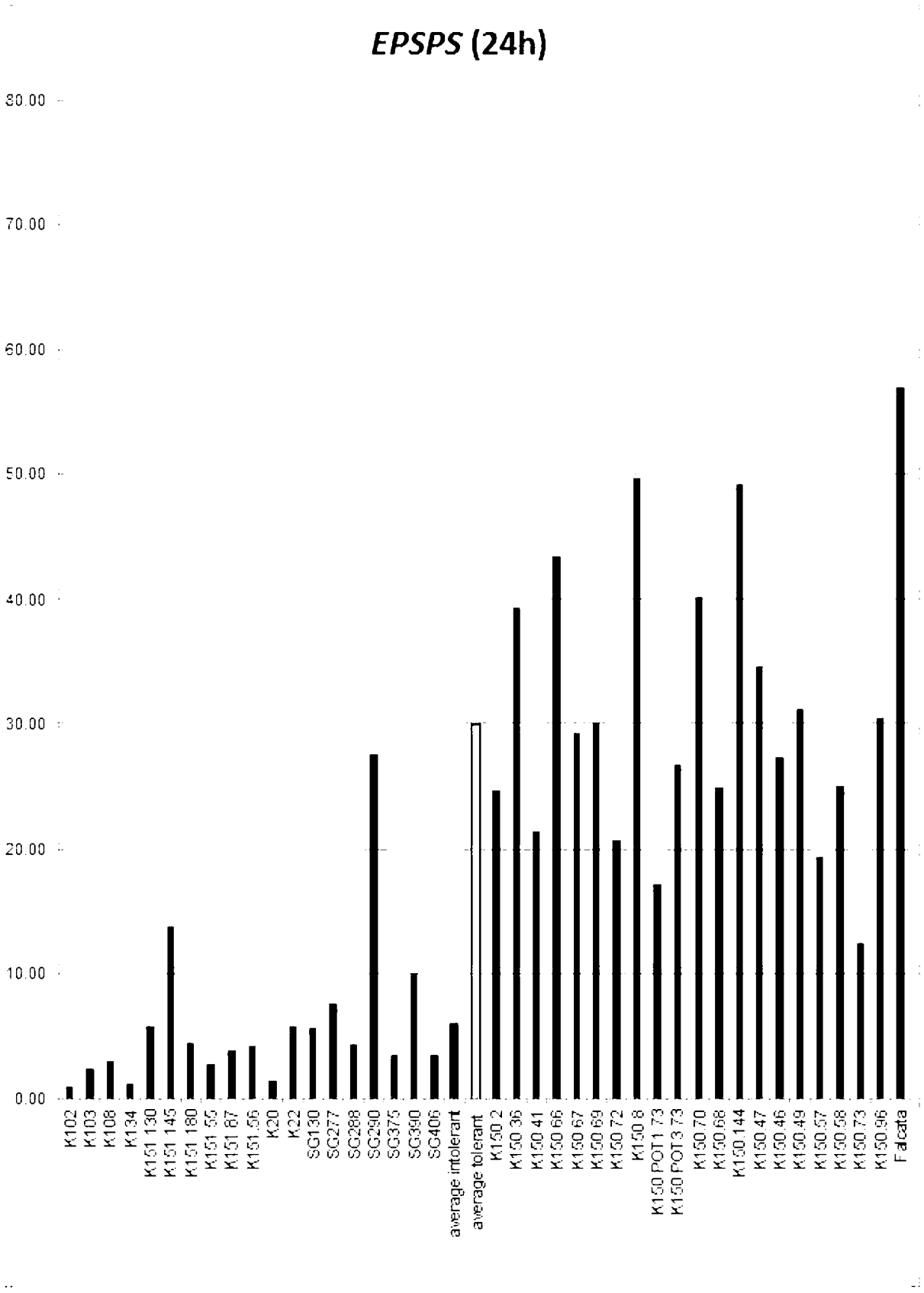
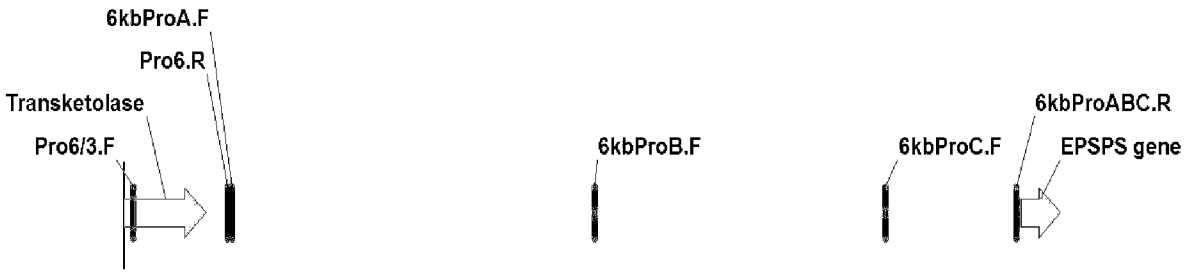

EPSPS (24h)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
K102
K103
K108
K134
K151 130
K151 145
K151 180
K151 55
K151 87
K151 96
K20
K22
SG130
SG277
SG288
SG290
SG375
SG390
SG406
average intolerant
average tolerant
K150 2
K150 36
K150 41
K150 66
K150 67
K150 69
K150 72
K150 8
K150 POT1 73
K150 POT3 73
K150 70
K150 68
K150 144
K150 47
K150 46
K150 49
K150 57
K150 58
K150 73
K150 96
Falcata

FIGURE 4

A


6kbProA.F
Pro6.R
Transketolase
Pro6/3.F
6kbProB.F
6kbProABC.R
6kbProC.F
EPSPS gene

B

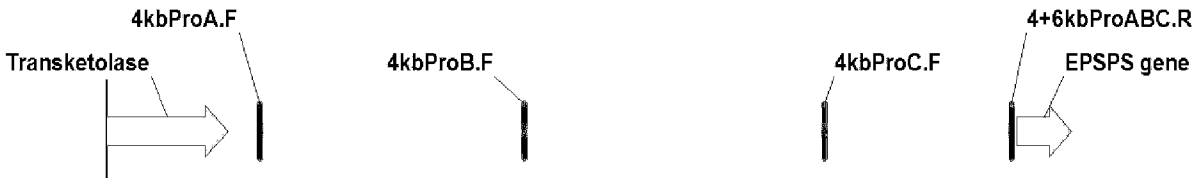

4kbProA.F
Transketolase
4kbProB.F
4+6kbProABC.R
4kbProC.F
EPSPS gene

FIGURE 5

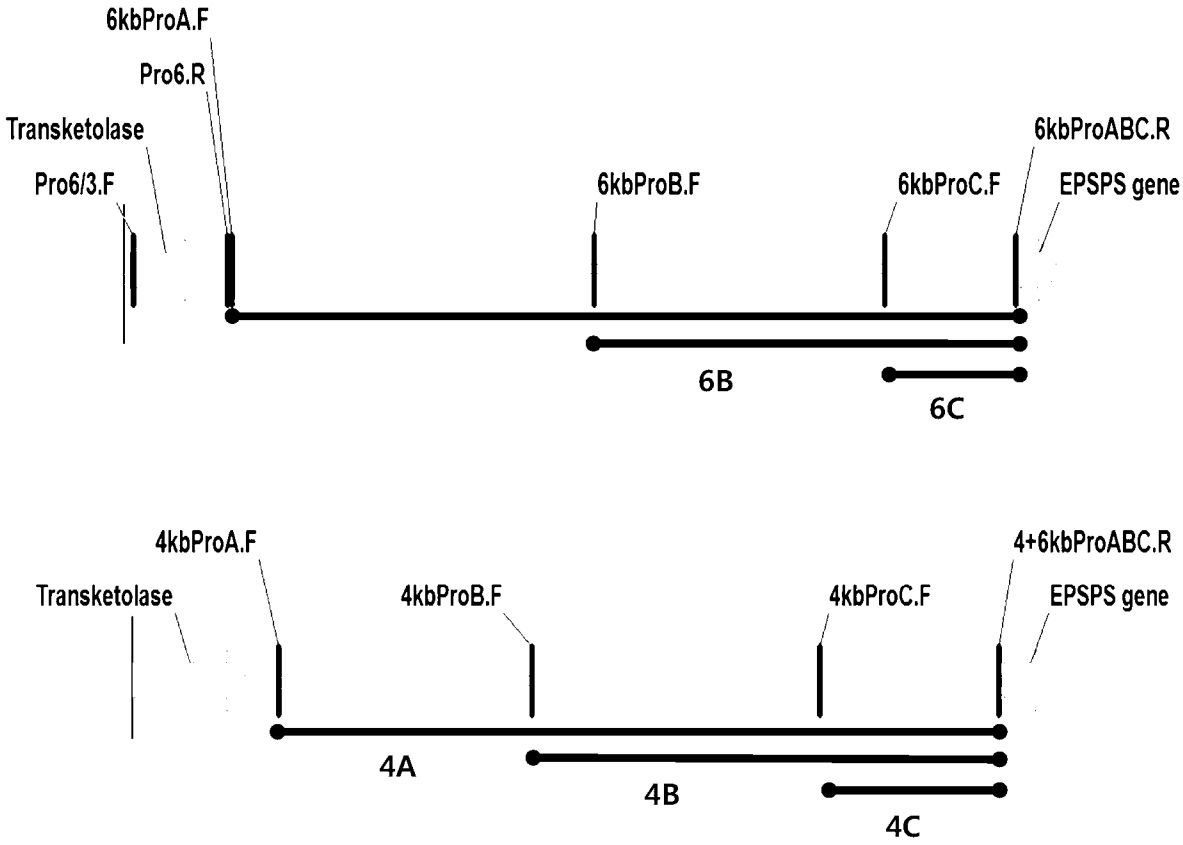

6kbProA.F
Pro6.R
Transketolase
Pro6/3.F
6kbProB.F
6kbProC.F
6kbProABC.R
EPSPS gene
6B
6C
4kbProA.F
Transketolase
4kbProB.F
4kbProC.F
4+6kbProABC.R
EPSPS gene
4A
4B
4C

FIGURE 6

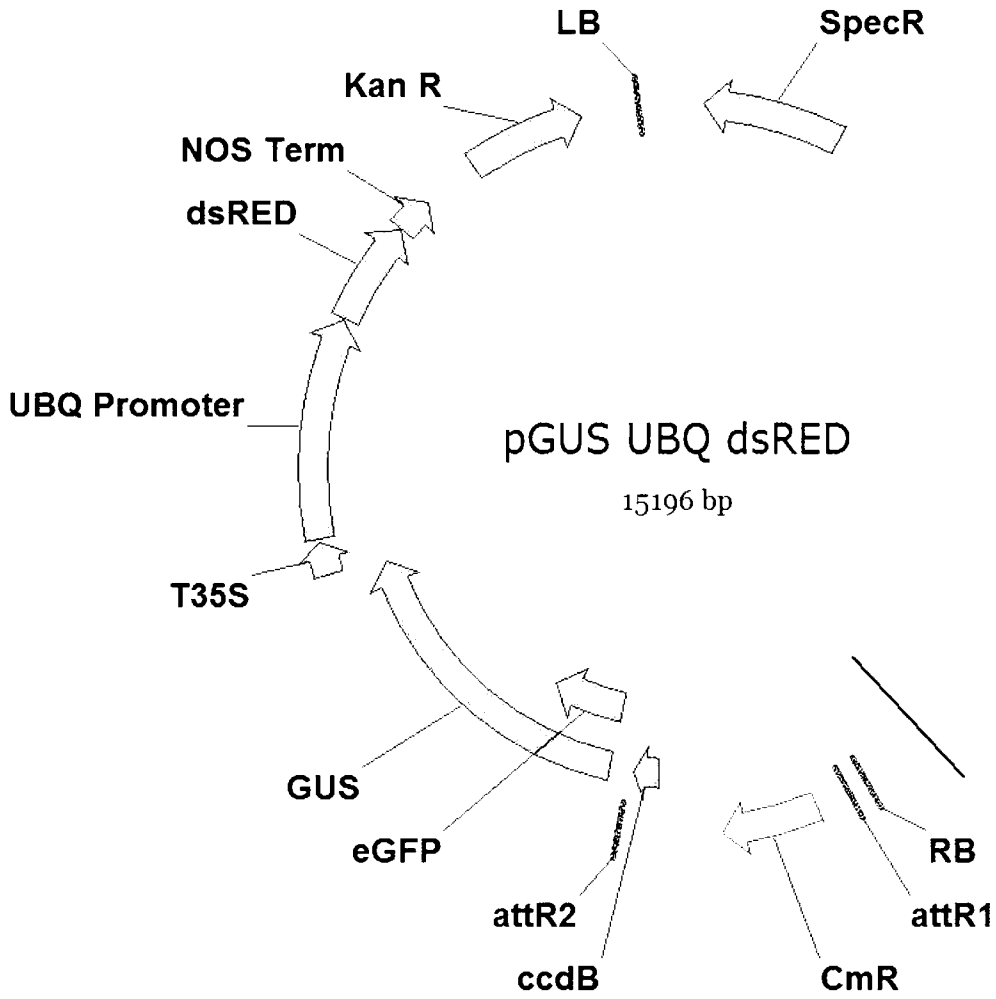

LB
SpecR
Kan R
NOS Term
dsRED
UBQ Promoter
pGUS UBQ dsRED
15196 bp
T35S
GUS
eGFP
attR2
ccdB
CmR
RB
attR1

FIGURE 7

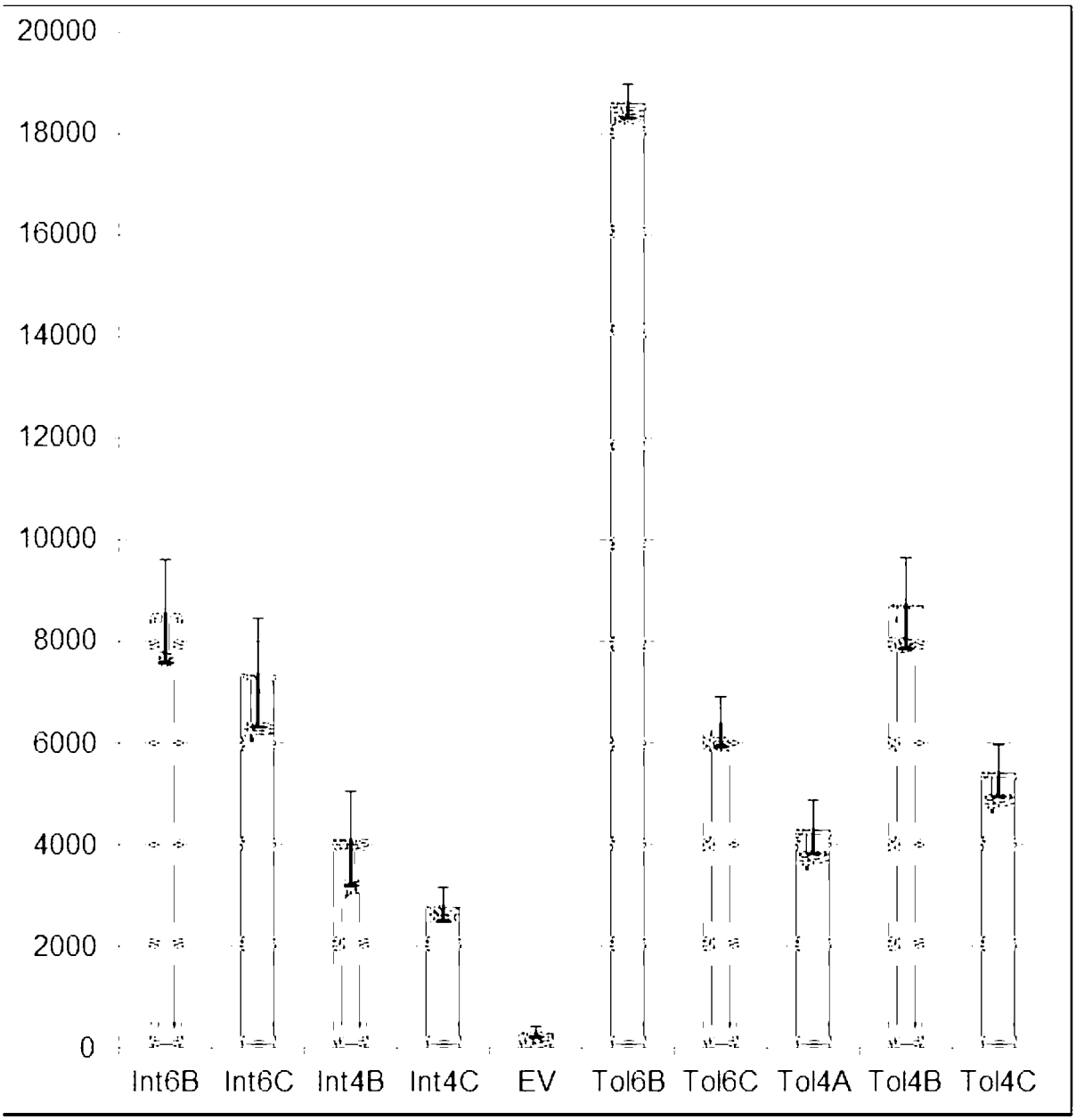

20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Int6B
Int6C
Int4B
Int4C
EV
Tol6B
Tol6C
Tol4A
Tol4B
Tol4C

FIGURE 8

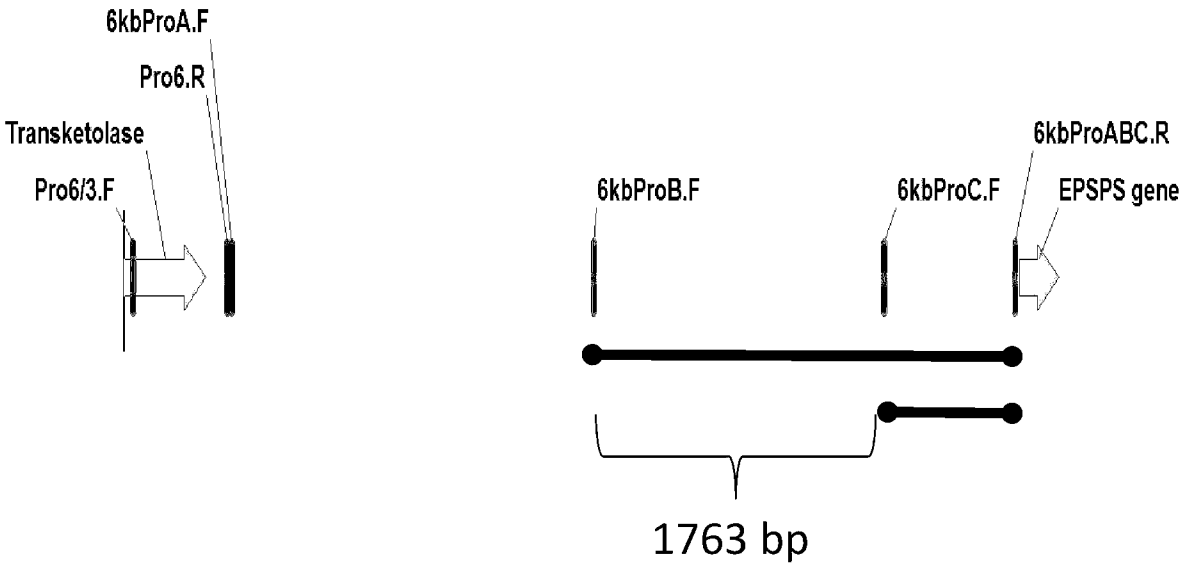

6kbProA.F
Pro6.R
Transketolase
Pro6/3.F
6kbProABC.R
6kbProB.F
6kbProC.F
EPSPS gene
1763 bp

FIGURE 9

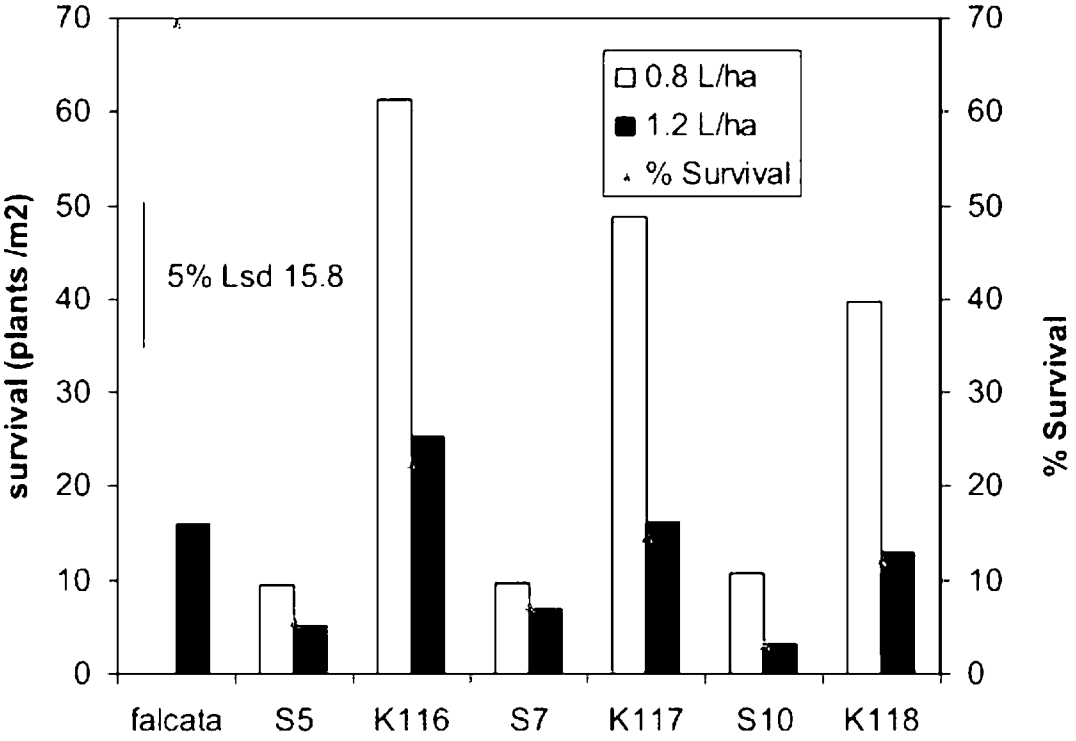

70
60
50
40
30
20
10
0
survival (plants /m2)
% Survival
0.8 L/ha
1.2 L/ha
% Survival
5% Lsd 15.8
falcata
S5
K116
S7
K117
S10
K118

FIGURE 10

A B C

FIGURE 11

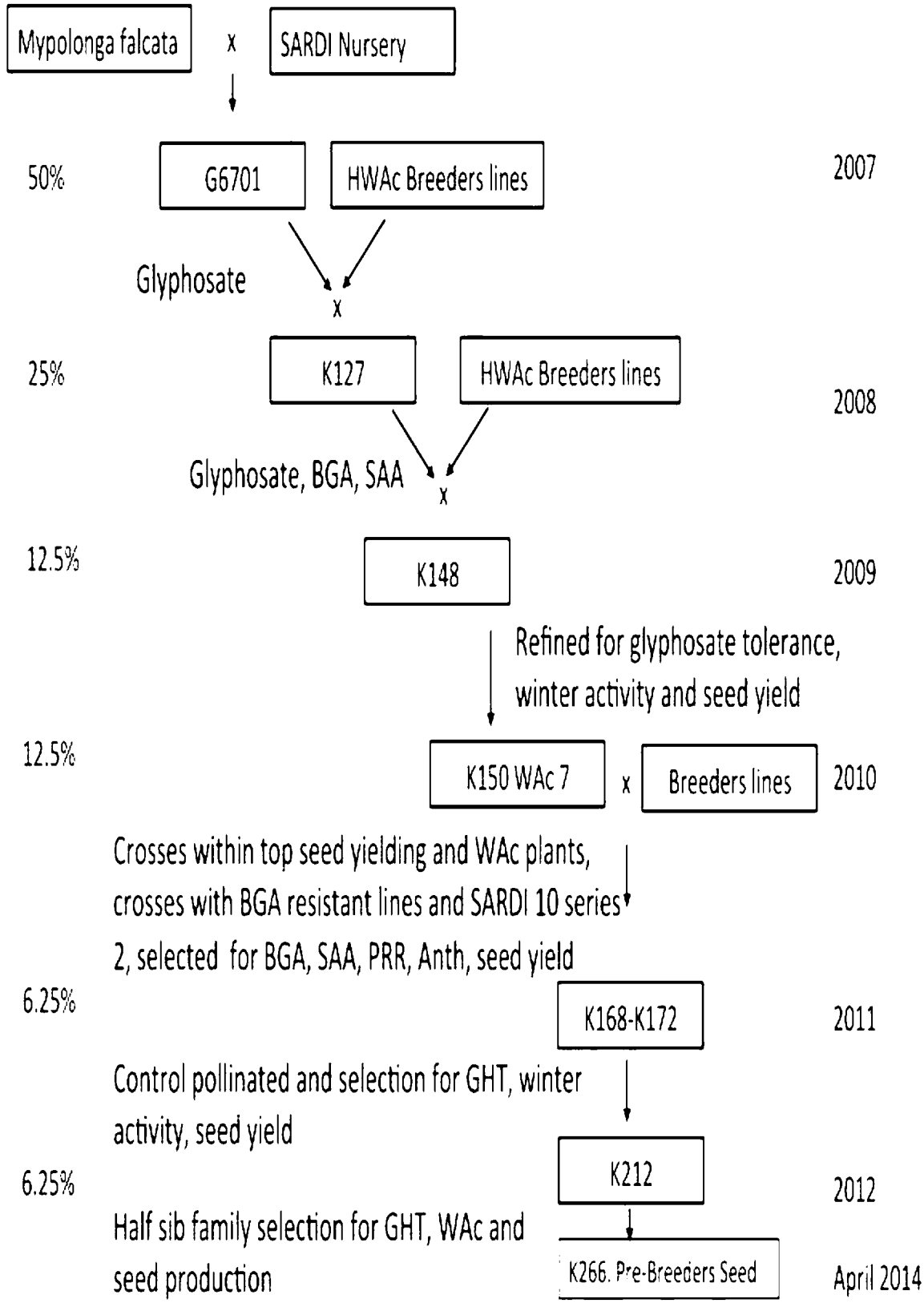

Mypolonga falcata
x
SARDI Nursery
50%
G6701
HWAc Breeders lines
2007
Glyphosate
x
25%
K127
HWAc Breeders lines
2008
Glyphosate, BGA, SAA
x
12.5%
K148
2009
Refined for glyphosate tolerance, winter activity and seed yield
12.5%
K150 WAc 7
x
Breeders lines
2010
Crosses within top seed yielding and WAc plants, crosses with BGA resistant lines and SARDI 10 series 2, selected for BGA, SAA, PRR, Anth, seed yield
6.25%
K168-K172
2011
Control pollinated and selection for GHT, winter activity, seed yield
6.25%
K212
2012
Half sib family selection for GHT, WAc and seed production
K266. Pre-Breeders Seed
April 2014

FIGURE 12

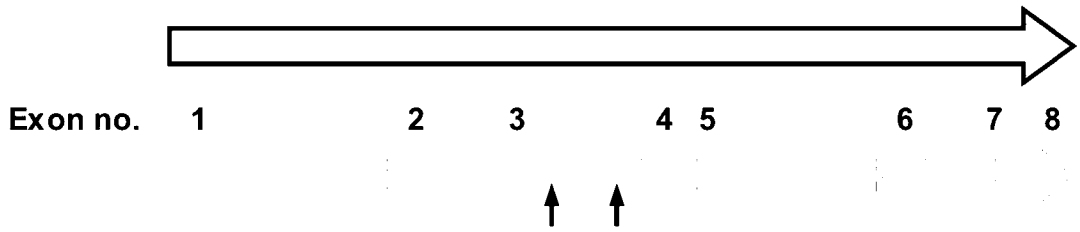


FIGURE 13

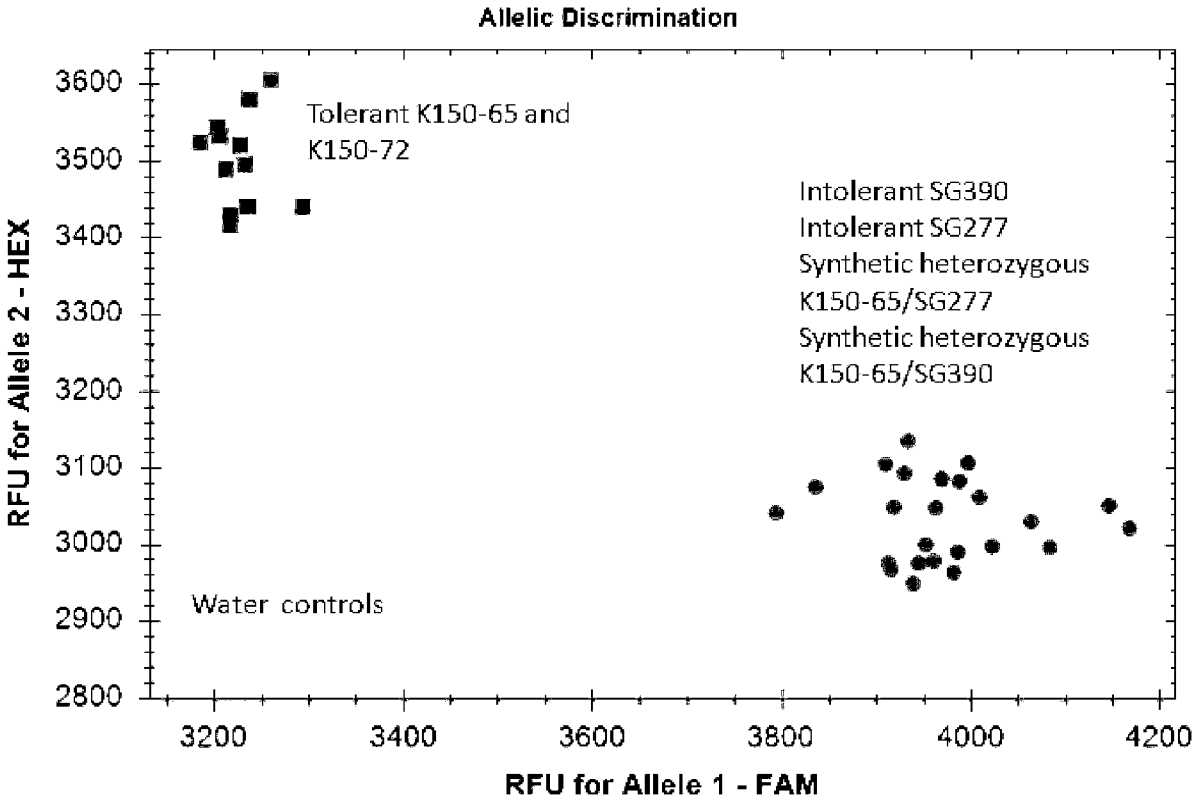

Allelic Discrimination
RFU for Allele 2 - HEX
RFU for Allele 1 - FAM
3600
3500
3400
3300
3200
3100
3000
2900
2800
3200
3400
3600
3800
4000
4200
Tolerant K150-65 and
K150-72
Intolerant SG390
Intolerant SG277
Synthetic heterozygous
K150-65/SG277
Synthetic heterozygous
K150-65/SG390
Water controls

FIGURE 14

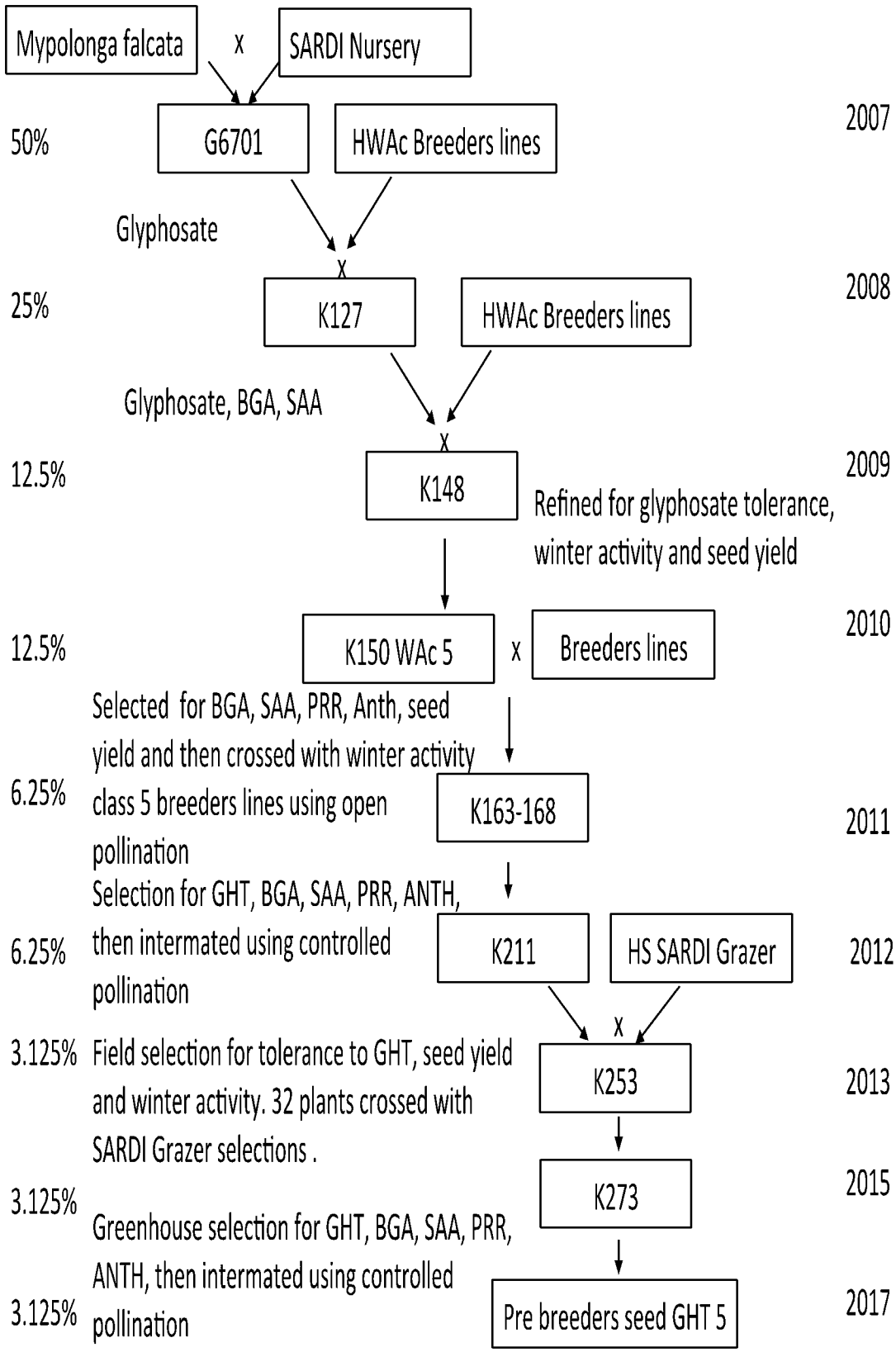
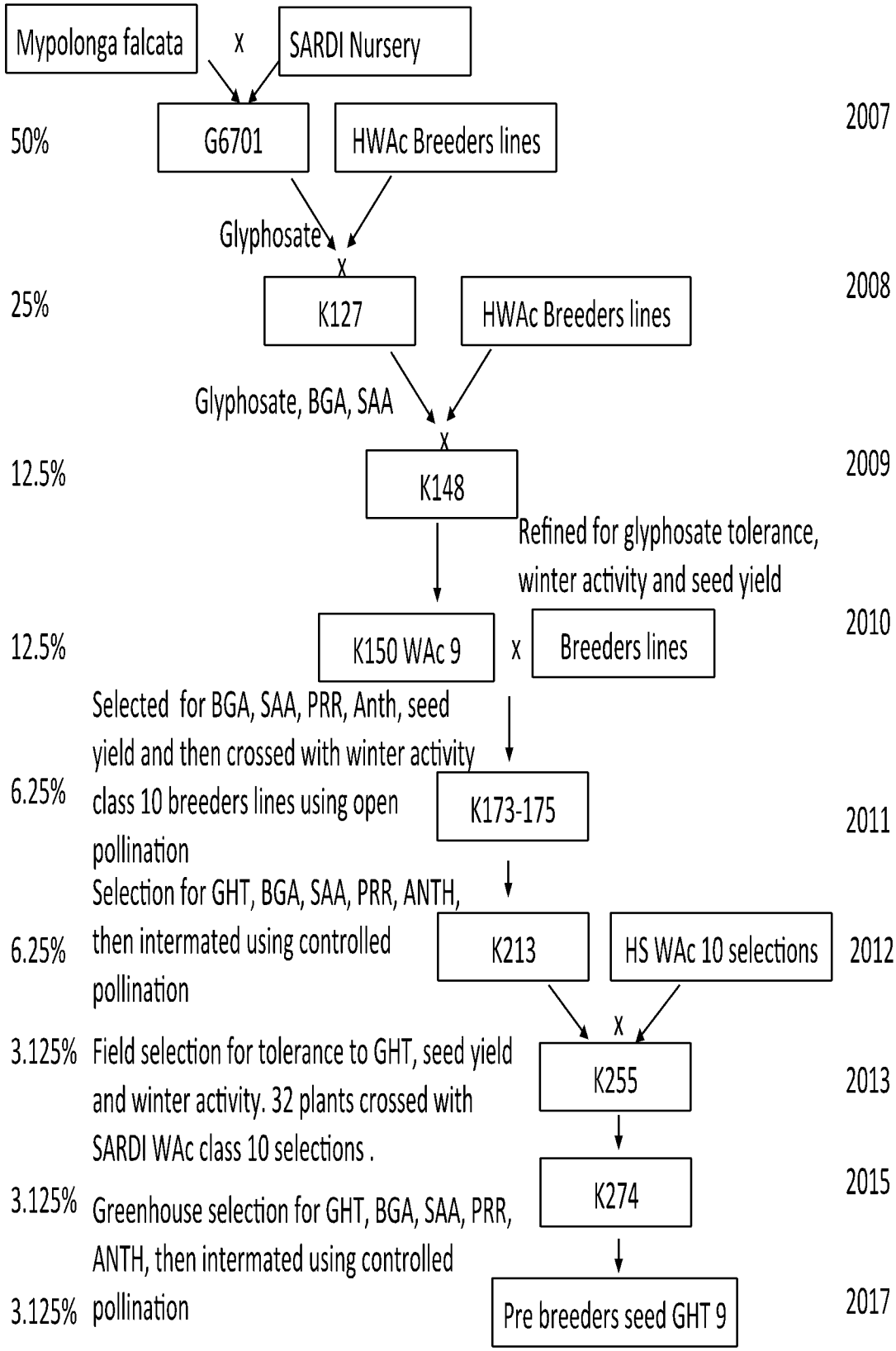

Mypolonga falcata
x
SARDI Nursery
50%
G6701
HWAc Breeders lines
2007
Glyphosate
x
25%
K127
HWAc Breeders lines
2008
Glyphosate, BGA, SAA
x
12.5%
K148
2009
Refined for glyphosate tolerance, winter activity and seed yield
12.5%
K150 WAc 5
x
Breeders lines
2010
Selected for BGA, SAA, PRR, Anth, seed yield and then crossed with winter activity
6.25%
class 5 breeders lines using open pollination
K163-168
2011
Selection for GHT, BGA, SAA, PRR, ANTH,
6.25%
then intermated using controlled pollination
K211
HS SARDI Grazer
2012
x
3.125%
Field selection for tolerance to GHT, seed yield and winter activity. 32 plants crossed with SARDI Grazer selections .
K253
2013
3.125%
K273
2015
Greenhouse selection for GHT, BGA, SAA, PRR, ANTH, then intermated using controlled
3.125%
pollination
Pre breeders seed GHT 5
2017

FIGURE 15


Mypolonga falcata
x
SARDI Nursery
50%
G6701
HWAc Breeders lines
2007
Glyphosate
x
25%
K127
HWAc Breeders lines
2008
Glyphosate, BGA, SAA
x
12.5%
K148
2009
Refined for glyphosate tolerance, winter activity and seed yield
12.5%
K150 WAc 9
x
Breeders lines
2010
Selected for BGA, SAA, PRR, Anth, seed yield and then crossed with winter activity
6.25%
class 10 breeders lines using open pollination
K173-175
2011
Selection for GHT, BGA, SAA, PRR, ANTH,
6.25%
then intermated using controlled pollination
K213
HS WAc 10 selections
2012
x
3.125%
Field selection for tolerance to GHT, seed yield and winter activity. 32 plants crossed with SARDI WAc class 10 selections .
K255
2013
3.125%
Greenhouse selection for GHT, BGA, SAA, PRR, ANTH, then intermated using controlled
K274
2015
3.125%
pollination
Pre breeders seed GHT 9
2017

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2015/000351

A. CLASSIFICATION OF SUBJECT MATTER

A01H 1/04 (2006.01) A01H 5/00 (2006.01)

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)

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

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

This search utilised the search strategy and terms of the corresponding earlier national application and was conducted back to 15th or 31 March 2015.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2015	26 June 2015

Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Alan Brownlee AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 62832943

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/AU2015/000351**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0096600 A1 (RUITER et al.) 19 April 2012 SEQ ID NO: 10; [0009], [0033], [0049], [0084], [0185]; claim8	1-21
X	CHANDRASEKHAR, K. et al., "Development of transgenic rice harboring mutated rice 5-enolpyruvylshikimate 3-phosphate synthase (Os-mEPSPS) and Allium sativum leaf agglutinin (ASAL) genes conferring tolerance to herbicides and sap-sucking insects", Plant Mol. Biol. Rep. 2014, vol. 32, pages 1146-1157 (published online 8 April 2014) Abstract; Figs 4, 8	22-26
X	WO 2000/066748 A1 (ZENECA LIMITED) 09 November 2000 Abstract; page 5, line 31 to page 6, line 30; page 11, line 13ff.; page 7, lines 7-9; Page 8, line 13; page 11, lines 29-32	7-11, 22-26
X	DILL, GM. et al., "Glyphosate-resistant crops: adoption, use and future considerations", Pest Manag. Sci. 2008, vol. 64, pages 326-331 Abstract; page 328, Section 2.5; page 330, Section 6; page 330, Section 4	7-11, 22-26
X	LEE, SI. et al., "Overproduction of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) confers resistance to the herbicide glyphosate in transgenic rice", J. Plant Biotechnol. 2011, vol. 38, pages 272-277 whole doc. in particular Abstract; Fig. 1	22-26
X	LONDO, JP. et al., "Changes in fitness-associated traits due to the stacking of transgenic glyphosate resistance and insect resistance in Brassica napus L.", Heredity, 2011, vol. 107, pages 328-337 Abstract; Fig 4; Table 2	22-26
X	WO 2013/116758 A1 (DOW AGROSCIENCES LLC) 08 August 2013 page 64, line 20-page 65, line 5; page 37, lines 21-32; page 38, lines 1-11; page 99, lines 6-7; claims	7-11, 22-26
A	SALAS, RA. et al., "EPSPS gene amplification in glyphosate-resistant Italian ryegrass (Lolium perenne ssp. multiflorum) from Arkansas", Pest Manag. Sci. 2012, vol. 68, pages 1223-1230 Abstract; Figs 1, 2; page 1226, sections 3.3 and 3.4; page 1228, col. 1, lines 16-33	1-21
A	GAINES, TA. et al., "Identification of genetic elements associated with EPSPS gene amplification", PLOS ONE, 2013, vol. 8 e65819 Whole doc. especially Fig. 4	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2015/000351

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box

Continuation of: **Box III**
This Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Claims 1-21 are directed to a method for identifying a plant having a glyphosate tolerant phenotype comprising determining the presence or absence of a marker comprising SEQ ID NO: 1 or a homologue or fragment thereof.. The feature of a method comprising determining the presence or absence of SEQ ID NO: 1 or a homologue or fragment thereof is specific to this group of claims.

- Claims 22-26 are directed to a glyphosate tolerant plant comprising tolerance conferred by overexpression of EPSPS, and one or more agronomic traits. The feature of a glyphosate tolerant plant comprising tolerance conferred by overexpression of EPSPS, and one or more agronomic traits is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a priori*.

As it was not considered to be unduly burdensome, both of the identified inventions (claims 1-21 nad claims 22-26) have been searched and reported on.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2015/000351**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 2012/0096600 A1	19 April 2012	AR 077305 A1	17 Aug 2011
		AU 2010268400 A1	02 Feb 2012
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