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(54) Title: ALS INHIBITOR HERBICIDE TOLERANT BETA VULGARIS MUTANTS

(57) Abstract: The present invention relates to an ALS inhibitor herbicide tolerant Beta vulgaris plant and parts thereof comprising a mutation of an endogenous acetolactate synthase (ALS) gene, wherein the ALS gene encodes an ALS polypeptide containing an amino acid different from tryptophan at a position 569 of the ALS polypeptide.



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ALS inhibitor herbicide tolerant Beta vulgaris mutants

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## Description

The present invention relates to ALS inhibitor herbicide tolerant Beta vulgaris plants and parts thereof as well as a method for their manufacture.

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Cultivated forms of Beta vulgaris (as defined in Ford-Lloyd (2005) Sources of genetic variation, Genus Beta. In: Biancardi E, Campbell LG, Skaracis GN, De Biaggi M (eds) Genetics and Breeding of Sugar Beet. Science Publishers, Enfield (NH), USA, pp25-33) are important agricultural crops in temperate and subtropical regions. For example, about 20 % of the world sugar production is based on sugar beet. Because beet seedlings and juvenile plants during their first 6-8 weeks of their life are susceptible for strong competition caused by fast growing weeds, which outcompete the young crop plants, reliable weed control measures are imperative in these crop areas.

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Since more than 40 years, herbicides are the preferred tools to control weeds in cultured beets. The products used for this purpose, like phenmedipham, desmedipham and metamilon allow to suppress the growth of weeds in beet fields without damaging the crop. Nevertheless under adverse environmental conditions the efficacy of these products leaves room for improvements, especially if noxious weeds like Chenopodium album, Amaranthus retroflexus and/or Tripleurospermum inodorata germinate over an extended period of time.

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Innovative herbicidal active ingredients are highly desirable in order to improve the weed control options in beet. Such compounds should act against a broad weed spectrum, preferably from weed germination until full development of the weed plants, without affecting the beet crop irrespective of its developmental stage. Via the classical herbicide screening approach no selective herbicidal active ingredient was

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discovered for beet during the past decades which fulfils all these stringent properties in an agronomically superior way.

Some chemicals inhibit the enzyme "acetohydroxyacid synthase" (AHAS), also  
5 known as "acetolactate synthase" (ALS [EC 4.1.3.18]). ALS is the site of action of five structurally diverse herbicide families belonging to the class of ALS inhibitor herbicides, like (a) sulfonylurea herbicides (Beyer E.M et al. (1988), Sulfonylureas in Herbicides: Chemistry, Degradation, and Mode of Action; Marcel Dekker, New York, 1988, 117-189), (b) sulfonylaminocarbonyltriazolinone herbicides (Pontzen, R.,  
10 Pflanz.-Nachrichten Bayer, 2002, 55, 37-52), (c) imidazolinone herbicides (Shaner, D.L., et al., Plant Physiol., 1984, 76, 545-546; Shaner, D.L., and O'Connor, S.L. (Eds.) The Imidazolinone Herbicides, CRC Press, Boca Raton, FL, 1991), (d) triazolopyrimidine herbicides (Kleschick, W.A. et al., Agric. Food Chem., 1992, 40, 1083-1085), and (e) pyrimidinyl(thio)benzoate herbicides (Shimizu, T.J., Pestic.  
15 Sci., 1997, 22, 245-256; Shimizu, T. et al., Acetolactate Synthase Inhibitors in Herbicide Classes in Development, Böger, P., Wakabayashi, K., Hirai, K., (Eds.), Springer Verlag, Berlin, 2002, 1-41).

ALS is involved in the conversion of two pyruvate molecules to an acetolactate  
20 molecule and carbon dioxide. The reaction uses thiamine pyrophosphate in order to link the two pyruvate molecules. The resulting product of this reaction, acetolactate, eventually becomes valine, leucine and isoleucine (Singh (1999) "Biosynthesis of valine, leucine and isoleucine", in Plant Amino Acids, Singh, B.K., ed., Marcel Dekker Inc. New York, New York, pp. 227-247).

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Inhibitors of the ALS interrupt the biosynthesis of valine, leucine and isoleucine in plants. The consequence is an immediate depletion of the respective amino acid pools causing a stop of protein biosynthesis leading to a cessation of plant growth and eventually the plant dies, or – at least – is damaged.

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ALS inhibitor herbicides are widely used in modern agriculture due to their effectiveness at moderate application rates and relative non-toxicity in animals. By

inhibiting ALS activity, these families of herbicides prevent further growth and development of susceptible plants including many weed species. In order to provide plants with an increased tolerance to even high concentrations of ALS inhibitor herbicides that may be required for sufficient weed control, additional ALS-inhibiting herbicide-resistant breeding lines and varieties of crop plants, as well as methods and compositions for the production and use of ALS inhibiting herbicide-resistant breeding lines and varieties, are needed.

A broad variety of ALS inhibitor herbicides enable a farmer to control a wide range of weed species independently of their growth stages, but these highly efficient herbicides cannot be used in beet because conventional beet plants/commercial beet varieties are highly susceptible against these ALS inhibitor herbicides. Nevertheless, these ALS inhibitor herbicides show an excellent herbicidal activity against broadleaf and grass weed species. The first herbicides having the mode of action of inhibiting the ALS were developed for their use in agriculture already 30 years ago. Nowadays, active ingredients of this class of herbicides exhibit a strong weed control and are widely used in maize and cereals as well as in dicotyledonous crops, except beet.

The only ALS inhibitor herbicide that is known today to be applied in post-emergent application schemes in beet is Debut®. This herbicide (containing triflurosulfuron-methyl as the active ingredient plus specific formulation compounds) is degraded by beets before it can inhibit the beet endogenous ALS enzyme but it has severe gaps in weed control in beet growing areas.

Since ALS inhibitor herbicides were introduced into agriculture it was observed that susceptible plant species, including naturally occurring weeds, occasionally develop spontaneous tolerance to this class of herbicides. Single base pair substitutions at specific sites of the ALS gene usually lead to more or less resistant ALS enzyme variants which show different levels of inhibition by the ALS inhibitor herbicides.

Plants conferring mutant ALS alleles therefore show different levels of tolerance to ALS inhibitor herbicides, depending on the chemical structure of the ALS inhibitor herbicide and the site of the point mutation in the ALS gene.

For example, Hattori et al. (1995), Mol. Gen. Genet. 246: 419-425, describes a single mutation in the Trp 557 codon in a Brassica napus cell line (according to the numbering of the Arabidopsis thaliana sequence that is used in the literature in order to compare all ALS/AHAS mutants this refers to position "574") – which equals position 569 of the beet ALS sequence. These authors observed resistance to several members of sub-classes of ALS inhibitor herbicides, like sulfonylureas, imidazolinones and triazolopyrimidines.

Beet mutants were described conferring a point mutation in the Ala 122 codon which led to a certain tolerance to the ALS inhibitor herbicide subclass of imidazolinones (WO 98/02526) but which is not sufficient for weed control in agricultural application schemes. No cross-tolerance to other ALS inhibitor herbicide classes were described by employing this mutant. Furthermore, beet plants conferring a second point mutation in the Pro 197 codon showed a moderate tolerance to ALS inhibitor herbicides belonging to members of the subclass of sulfonylurea herbicides. Also double mutants of these two were described (WO 98/02527). However, none of these mutants were used for the market introduction of beet varieties because the level of herbicide tolerance to ALS inhibitor herbicides was not sufficiently high in these mutants to be exploited agronomically.

Stougaard et al. (1990), J. Cell Biochem., Suppl. 14E, 310 describe the isolation of ALS mutants in a tetraploid sugar beet cell culture. Two different ALS genes (ALS I and ALS II) were isolated which differed at amino acid position 37 only. Mutant 1 contained in its ALS I gene 2 mutations, while mutant 2 contained 3 mutations in its ALS II gene. After the mutations were separated to resolve which mutation would confer resistance against an ALS inhibitor, it was revealed that ALS synthesized from a recombinant E. coli was herbicide resistant if it contained a point mutation in the Trp 574 codon (according to the numbering of the Arabidopsis thaliana sequence that is used in the literature in order to compare all ALS mutants) – which equals

position 569 of the beet ALS sequence, leading to a replacement of the amino acid “Trp” by the amino acid “Leu”. Stougaard et al did not show in sugar beet that the mutation at position 569 of any of the sugar beet ALS genes is sufficient in order to obtain an acceptable level of tolerance to ALS inhibitor herbicides. Moreover,  
5 Stougaard et al did not regenerate or handle sugar beet plants comprising a mutation, including Trp -> Leu mutation at position 569 of sugar beet ALS.

Knowing this, Stougaard et al. constructed plant transformation vectors containing different ALS genes for use in plant transformation. However, up to now, no further  
10 data - especially not concerning the effects of the application of ALS inhibitor herbicides to plants and/or agricultural areas comprising this mutation in Beta vulgaris plants have been disclosed by these or other authors either in genetically engineered or mutant plants over more than 20 years, thereafter.

15 WO 99/57965 generally describes sulfonylurea resistant sugar beet plants and methods for obtaining them by EMS (Ethylmethanesulfonate) mutagenesis. However, apart from the research that is required to obtain such mutants, this publication does neither provide such plants, nor describes any specific location in the ALS gene that may be relevant for obtaining ALS inhibitor herbicide tolerant  
20 mutants, nor discloses any correlated agronomical use of such. Furthermore, there is a strong likelihood that - by employing such strong mutagenic compound like EMS – various further mutations may occur elsewhere in the genome and which might lead to disadvantages up to non-fertility and/or growth retardation of such obtained plants. Moreover, due to its chemical interaction with the DNA, the EMS application may  
25 have gaps of inducing specific mutations, like converting the triplet TGG into TTG, because EMS always converts a guanosine into an adenosine.

In some weed species as Amaranthus, the Trp 574 Leu mutation could be detected in plant populations which were repeatedly exposed to ALS inhibitor herbicides.

30 These Trp 574 Leu mutants show a high level of tolerance to several chemical classes of ALS inhibitor herbicides, like those selected from the group consisting of sulfonylureas and sulfonylaminocarbonyltriazolinones.

WO 2008/124495 discloses ALS double and triple mutants. According to WO 2009/046334, specific mutations in the ALS gene were provided. However, agronomically exploitable herbicide tolerant Beta vulgaris mutants containing such mutations according to WO 2009/046334 have not been obtained so far.

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Moreover, in view of the fact that, for example, sugar beet accounts for about 20% of the world sugar production, it would also be highly desirable to have available sugar beet plants which have a growth advantage versus highly potent weeds. It would thus be highly desirable to have available, with respect to the ALS gene, non-transgenic Beta vulgaris plants including sugar beet plants which are tolerant to ALS inhibitor herbicides. Hence, there is a need for such non-transgenic Beta vulgaris plants, in particular sugar beet plants which are tolerant to ALS inhibitor herbicides at an agronomically exploitable level of ALS inhibitor herbicides.

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15 Thus, the technical problem is to comply with this need.

The present invention addresses this need and thus provides as a solution to the technical problem an ALS inhibitor herbicide tolerant Beta vulgaris plant and parts thereof comprising a mutation of an endogenous acetolactate synthase (ALS) gene, wherein the ALS gene encodes an ALS polypeptide containing an amino acid different from tryptophan at a position 569 of the ALS polypeptide.

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Seeds according to present invention have been deposited with the NCIMB, Aberdeen, UK, under Number NCIMB 41705 on March 12, 2010.

25

By applying various breeding methods, high yielding commercial varieties highly competitive in all specific markets with the add-on of a robust ALS inhibitor herbicide tolerance can be developed subsequently by using the originally obtained mutant plants.

30

It must be noted that as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example,

reference to "a reagent" includes one or more of such different reagents and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

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All publications and patents cited in this disclosure are incorporated by reference in their entirety. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material.

10

Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such

15 equivalents are intended to be encompassed by the present invention.

20

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. The word "comprise" and its variations on the one side and "contain" and its analogous variations on the other side can be used interchangeably without a preference to any of them.

25

In the present invention, beet plants were obtained which comprise an altered endogenous ALS gene (also referred to as "AHAS" gene), carrying a point mutation in the Trp 569 codon (in relation to the Beta vulgaris ALS amino acid reference sequence shown in SEQ ID NO: 2; this equals position 574 of the referenced Arabidopsis thaliana sequence as shown in SEQ ID NO: 6) and which point mutation

30 was obtained by several circles of selection on specifically elected ALS inhibitor herbicides.



Due to the fact that the *B. vulgaris* plants of the present invention were obtained by isolating spontaneous mutant plant cells, which were directly regenerated to fully fertile beet plants having a point mutation as described herein in more detail. These plants are non-transgenic as far as the ALS gene is concerned.

- 5 Moreover, the plants of the present invention themselves as well as their offspring are fertile and thus useful for breeding purposes without any further manipulation that may cause stress induced further alterations of the genetic background. Such plants obtained according to the selection procedure applied herein can directly be employed in order to generate beet varieties and/or hybrids conferring
- 10 agronomically useful levels of tolerance to ALS inhibitor herbicides, thus allowing innovative weed control measures in beet growing areas.

When used herein, the term "transgenic" means that a gene – which can be of the same or a different species – has been introduced via an appropriate biological

15 carrier, like *Agrobacterium tumefaciens* or by any other physical means, like protoplast transformation or particle bombardment, into a plant and which gene is able to be expressed in the new host environment, namely the genetically modified organism (GMO).

In accordance to the before definition, the term "non-transgenic" means exactly the

20 contrary, i.e. that no introduction of the respective gene has occurred via an appropriate biological carrier or by any other physical means. However, a mutated gene can be transferred through pollination, either naturally or via a breeding process to produce another non-transgenic plant concerning this specific gene.

- 25 An "endogenous" gene means a gene of a plant which has not been introduced into the plant by genetic engineering techniques.

The term "sequence" when used herein relates to nucleotide sequence(s), polynucleotide(s), nucleic acid sequence(s), nucleic acid(s), nucleic acid molecule,

30 peptides, polypeptides and proteins, depending on the context in which the term "sequence" is used.

The terms "nucleotide sequence(s)", "polynucleotide(s)", "nucleic acid sequence(s)", "nucleic acid(s)", "nucleic acid molecule" are used interchangeably herein and refer to nucleotides, either ribonucleotides or deoxyribonucleotides or a combination of both, in a polymeric unbranched form of any length. Nucleic acid sequences include  
5 DNA, cDNA, genomic DNA, RNA, synthetic forms and mixed polymers, both sense and antisense strands, or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art.

When used herein, the term "polypeptide" or "protein" (both terms are used  
10 interchangeably herein) means a peptide, a protein, or a polypeptide which encompasses amino acid chains of a given length, wherein the amino acid residues are linked by covalent peptide bonds. However, peptidomimetics of such proteins/polypeptides wherein amino acid(s) and/or peptide bond(s) have been replaced by functional analogs are also encompassed by the invention as well as  
15 other than the 20 gene-encoded amino acids, such as selenocysteine. Peptides, oligopeptides and proteins may be termed polypeptides. The term polypeptide also refers to, and does not exclude, modifications of the polypeptide, e.g., glycosylation, acetylation, phosphorylation and the like. Such modifications are well described in basic texts and in more detailed monographs, as well as in the research literature.  
20 The polypeptide (or protein) that is preferably meant herein is the B. vulgaris ALS polypeptide (or ALS protein) [SEQ ID NO: 2].

Amino acid substitutions encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring amino acid residue. Such substitutions  
25 may be classified as "conservative", in which an amino acid residue contained in the wild-type ALS protein is replaced with another naturally-occurring amino acid of similar character, for example Gly↔Ala, Val↔Ile↔Leu, Asp↔Glu, Lys↔Arg, Asn↔Gln or Phe↔Trp↔Tyr. Substitutions encompassed by the present invention may also be "non-conservative", in which an amino acid residue which is present in  
30 the wild-type ALS protein is substituted with an amino acid with different properties, such as a naturally-occurring amino acid from a different group (e.g. substituting a charged or hydrophobic amino acid with alanine. "Similar amino acids", as used

herein, refers to amino acids that have similar amino acid side chains, i.e. amino acids that have polar, non-polar or practically neutral side chains. "Non-similar amino acids", as used herein, refers to amino acids that have different amino acid side chains, for example an amino acid with a polar side chain is non-similar to an amino acid with a non-polar side chain. Polar side chains usually tend to be present on the surface of a protein where they can interact with the aqueous environment found in cells ("hydrophilic" amino acids). On the other hand, "non-polar" amino acids tend to reside within the center of the protein where they can interact with similar non-polar neighbours ("hydrophobic" amino acids"). Examples of amino acids that have polar side chains are arginine, asparagine, aspartate, cysteine, glutamine, glutamate, histidine, lysine, serine, and threonine (all hydrophilic, except for cysteine which is hydrophobic). Examples of amino acids that have non-polar side chains are alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, and tryptophan (all hydrophobic, except for glycine which is neutral).

Generally, the skilled person knows, because of his common general knowledge and the context when the terms ALS, ALSL, AHAS or AHASL are used, as to whether the nucleotide sequence or nucleic acid, or the amino acid sequence or polypeptide, respectively, is meant.

The term "gene" when used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. The term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, methylation, "caps", substitutions of one or more of the naturally occurring nucleotides with an analog. Preferably, a gene comprises a coding sequence encoding the herein defined polypeptide. A "coding sequence" is a nucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed or being under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleic acid sequences or genomic DNA, while introns may be present as well under certain circumstances.

When used herein the term "Beta vulgaris" is abbreviated as "B. vulgaris".

Furthermore, the term "beet" is used herein. Said three terms are interchangeably used and should be understood to fully comprise the cultivated forms of Beta

5 vulgaris as defined in Ford-Lloyd (2005) Sources of genetic variation, Genus Beta. In: Biancardi E, Campbell LG, Skaracis GN, De Biaggi M (eds) Genetics and Breeding of Sugar Beet. Science Publishers, Enfield (NH), USA, pp 25-33. Similarly, for example, the term "Arabidopsis thaliana" is abbreviated as "A. thaliana". Both terms are interchangeably used herein.

10

The term "position" when used in accordance with the present invention means the position of either an amino acid within an amino acid sequence depicted herein or the position of a nucleotide within a nucleotide sequence depicted herein. The term "corresponding" as used herein also includes that a position is not only determined

15 by the number of the preceding nucleotides/amino acids.

15

The position of a given nucleotide in accordance with the present invention which may be substituted may vary due to deletions or additional nucleotides elsewhere in the ALS 5'-untranslated region (UTR) including the promoter and/or any other regulatory sequences or gene (including exons and introns). Similarly, the position of

20 a given amino acid in accordance with the present invention which may be substituted may vary due to deletion or addition of amino acids elsewhere in the ALS polypeptide.

20

Thus, under a "corresponding position" in accordance with the present invention it is to be understood that nucleotides/amino acids may differ in the indicated number but may still have similar neighbouring nucleotides/amino acids. Said nucleotides/amino acids which may be exchanged, deleted or added are also comprised by the term "corresponding position".

25

In order to determine whether a nucleotide residue or amino acid residue in a given

30 ALS nucleotide/amino acid sequence corresponds to a certain position in the nucleotide sequence of SEQ ID NO: 1 or the amino acid sequence of SEQ ID NO: 2, the skilled person can use means and methods well-known in the art, e.g.,

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alignments, either manually or by using computer programs such as BLAST (Altschul et al. (1990), Journal of Molecular Biology, 215, 403-410), which stands for Basic Local Alignment Search Tool or ClustalW (Thompson et al. (1994), Nucleic Acid Res., 22, 4673-4680) or any other suitable program which is suitable to generate  
5 sequence alignments.

SEQ ID NO: 1 is the nucleotide sequence encoding Beta vulgaris wild type ALS.

SEQ ID NO: 2 is the Beta vulgaris amino acid sequence derived from SEQ ID NO: 1.

Accordingly, the codon at position 1705-1707 of the nucleotide sequence of SEQ ID  
10 NO: 1 encodes the amino acid at position 569 (i.e. the amino acid "Trp" according to the three letter code or "W" according to the one letter code) of SEQ ID NO: 2.

In the alternative to determine whether a nucleotide residue or amino acid residue in a given ALS nucleotide/amino acid sequence corresponds to a certain position in the  
15 nucleotide sequence of SEQ ID NO: 1, the nucleotide sequence encoding A. thaliana wild type ALS shown in SEQ ID NO: 5 can be used. SEQ ID NO: 6 is the A. thaliana amino acid sequence derived from SEQ ID NO: 5.

Accordingly, the codon at position 1720-1722 of the nucleotide sequence of SEQ ID  
20 NO: 5 encodes the amino acid at position 574 (i.e. the amino acid "Trp" according to the three letter code or "W" according to the one letter code) of SEQ ID NO: 6.

If the A. thaliana wild type ALS nucleotide sequence shown in SEQ ID NO: 5 is used as reference sequence (as it is done in most of the relevant literature and, therefore, is used to enable an easier comparison to such known sequences), the codon  
25 encoding an amino acid different from tryptophan is at a position corresponding to position 1720-1722 of the nucleotide sequence of the A. thaliana ALS gene shown in SEQ ID NO: 5.

However, SEQ ID NO: 1 is preferred as the reference nucleotide sequence and SEQ  
30 ID NO: 2 is preferred as the reference amino acid sequence in all of the subsequent disclosures.

The following table provides an overview on the reference sequences used herein when the position of the point mutation in a nucleotide sequence or the substitution in an amino acid sequence is determined:

SEQ ID NO:	Type of Sequence	Species
1	nucleotide sequence	<i>B. vulgaris</i>
2	amino acid sequence	<i>B. vulgaris</i>
3	nucleotide sequence (mutated)	<i>B. vulgaris</i>
4	amino acid sequence (mutated)	<i>B. vulgaris</i>
5	nucleotide sequence	<i>A. thaliana</i>
6	amino acid sequence	<i>A. thaliana</i>

5

Thus, in any event, the equivalent position could still be determined through alignment with a reference sequence, such as SEQ ID NO: 1 or 5 (nucleotide sequence) or SEQ ID NO: 2 or 6 (amino acid sequence).

- 10 In view of the difference between the *B. vulgaris* wild-type ALS gene and the ALS gene comprised by a *B. vulgaris* plant of the present invention, the ALS gene (or polynucleotide or nucleotide sequence) comprised by a *B. vulgaris* plant of the present invention may also be regarded as a "mutant ALS gene", "mutant ALS allele", "mutant ALS polynucleotide" or the like. Thus, throughout the specification,
- 15 the terms "mutant allele", "mutant ALS allele", "mutant ALS gene" or "mutant ALS polynucleotide" are used interchangeably.

Unless indicated otherwise herein, these terms refer to a nucleotide sequence that comprises a codon encoding an amino acid different from tryptophan at a position

20 corresponding to position 1705-1707 of the nucleotide sequence of the *B. vulgaris* ALS gene shown in SEQ ID NO: 1. When set in relation to the *A. thaliana* reference sequence shown in SEQ ID NO: 5, the position of the codon is 1720-1722.

Likewise, these terms refer to a nucleotide sequence that encodes an ALS protein having at a position corresponding to position 569 of the amino acid sequence of the Beta vulgaris ALS protein shown in SEQ ID NO: 2 an amino acid different from tryptophan. When set in relation to the A. thaliana reference sequence shown in  
5 SEQ ID NO: 6, the position is 574.

An "amino acid different from tryptophan" (indicated by "Trp" in the three letter code or "W" in the equivalently used one letter code) includes any naturally-occurring amino acid different from tryptophan. These naturally-occurring amino acids include alanine (A), arginine (R), asparagine (N), aspartate (D), cysteine (C), glutamine (Q),  
10 glutamate (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tyrosine (Y) or valine (V).

However, preferably, the amino acid different from tryptophan (belonging to the  
15 group of neutral-polar amino acids) is an amino acid with physico-chemical properties different from tryptophan, i.e. belonging to any of the amino acids showing neutral-nonpolar, acidic, or basic properties. More preferably, the amino acid different from tryptophan is selected from the group consisting of alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, valine, and arginine. Even  
20 more preferably, said amino acid is a neutral-nonpolar amino acid such as alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline or valine. Particularly preferred said amino acid is alanine, glycine, isoleucine, leucine, valine. Even more preferred is glycine and leucine. Most preferably, it is leucine.

25 In contrast, unless indicated otherwise, the terms "wild-type allele," "wild-type ALS allele", "wild-type ALS gene" or "wild-type ALS polynucleotide" refer to a nucleotide sequence that encodes an ALS protein that lacks the W569 substitution in relation to SEQ ID NO: 2 (or W574 substitution in relation to SEQ ID NO: 6). These terms also refer to a nucleotide sequence comprising at a position corresponding to position  
30 1705-1707 of the nucleotide sequence of the B. vulgaris ALS gene shown in SEQ ID NO: 1, a codon encoding an amino acid different from tryptophan.

Such a "wild-type allele", "wild-type ALS allele", "wild-type ALS gene" or "wild-type ALS polynucleotide" may, or may not, comprise mutations, other than the mutation that causes the W569 substitution.

In essence, as regards the ALS gene, the only difference between a wild-type *B.*

5 *vulgaris* plant and the *B. vulgaris* plant of the present invention is preferably (and specifically) that at a position as specified herein (in particular at a position corresponding to position 1705-1707 of the nucleotide sequence of the *B. vulgaris* ALS gene shown in SEQ ID NO: 1), the *B. vulgaris* plant of the present invention comprises a codon encoding an amino acid different from tryptophan, preferably the  
10 codon encodes an amino acid as specified herein elsewhere. However, as mentioned above, further differences such as additional mutations may be present between wild-type and the mutant ALS allele as specified herein. Yet, these further differences are not relevant as long as the difference explained before is present.

15 Consequently, the W569 substitution (or W574 substitution when the *A. thaliana* ALS amino acid sequence of SEQ ID NO: 6 is used as reference) is a result of an alteration of the codon at a position corresponding to position 1705-1707 of the nucleotide sequence shown in SEQ ID NO: 1 (or at a position corresponding to position 1720-1722 of the nucleotide sequence shown in SEQ ID NO: 5,  
20 respectively).

Preferably, the substitution at position 569 is a W→L substitution, wherein "L" is encoded by any of the codons "CTT", "CTC", "CTA", "CTG", "TTA" or "TTG".

Most preferably, the substitution at position 569 is a W→L substitution, because of a transversion of the "G" nucleotide at a position corresponding to position 1706 of the  
25 nucleotide sequence shown in SEQ ID NO: 1 (or at a position corresponding to position 1721 of the nucleotide sequence shown in SEQ ID NO: 5, respectively), to a "T" nucleotide. Accordingly, the codon at a position corresponding to position 1705-1707 of the nucleotide sequence shown in SEQ ID NO: 1 (or at a position corresponding to position 1720-1722 of the nucleotide sequence shown in SEQ ID  
30 NO: 5, respectively) is changed from "TGG" to "TTG". While the codon "TGG" encodes tryptophan, the codon "TTG" encodes leucine.



Hence, in the most preferred embodiment, the present invention provides a Beta vulgaris plant comprising in the nucleotide sequence of the endogenous ALS gene, the codon TTG (encoding leucine) at a position corresponding to position 1705-1707 of the nucleotide sequence of the B. vulgaris ALS mutant gene shown in SEQ ID NO: 1, said nucleotide sequence comprising (or less preferably consisting of) SEQ ID NO: 3.

The B. vulgaris plants encoding an ALS polypeptide having at a position corresponding to position 569 of the amino acid sequence of the Beta vulgaris ALS protein shown in SEQ ID NO: 2 an amino acid different from tryptophan, preferably comprise in the nucleotide sequence of the endogenous ALS gene a codon encoding an amino acid different from tryptophan at a position corresponding to position 1705-1707 of the nucleotide sequence of the B. vulgaris ALS gene shown in SEQ ID NO: 1.

The term B. vulgaris "ALS" or "AHAS" gene also includes B. vulgaris nucleotide sequences which are at least 90, 95, 97, 98, or 99% identical to the B. vulgaris ALS nucleotide sequence of SEQ ID NO: 1 or 3, wherein these 60, 70, 80, 90, 95, 97, 98, or 99% identical nucleotide sequences comprise at a position corresponding to position 1705-1707 of the nucleotide sequence of SEQ ID NO: 1 a codon encoding an amino acid different from tryptophan.

Likewise, these at least 90, 95, 97, 98, or 99% identical nucleotide sequences encode an ALS polypeptide comprising at a position corresponding to position 569 of SEQ ID NO: 2 an amino acid different from tryptophan. Said identical nucleotide sequences encode an ALS protein which retains the activity as described herein, more preferably the thus-encoded ALS polypeptide is tolerant to one or more ALS inhibitor herbicides as described herein. Said term also includes allelic variants and homologs encoding an ALS polypeptide which is preferably tolerant to one or more ALS inhibitor herbicides as described herein.

In order to determine whether a nucleic acid sequence has a certain degree of identity to the nucleotide sequences of the present invention, the skilled person can use means and methods well-known in the art, e.g., alignments, either manually or

by using computer programs such as those mentioned further down below in connection with the definition of the term "hybridization" and degrees of homology.

For example, BLAST, which stands for Basic Local Alignment Search Tool (Altschul, Nucl. Acids Res. 25 (1997), 3389-3402; Altschul, J. Mol. Evol. 36 (1993), 290-300; Altschul, J. Mol. Biol. 215 (1990), 403-410), can be used to search for local sequence alignments. BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying similar sequences. The fundamental unit of BLAST algorithm output is the High-scoring Segment Pair (HSP). An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches. E is interpreted as the upper bound of the expected frequency of chance occurrence of an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

Analogous computer techniques using BLAST (Altschul (1997), loc. cit.; Altschul (1993), loc. cit.; Altschul (1990), loc. cit.) are used to search for identical or related molecules in nucleotide databases such as GenBank or EMBL. This analysis is much faster than multiple membrane-based hybridizations. In addition, the sensitivity of the computer search can be modified to determine whether any particular match is categorized as exact or similar. The basis of the search is the product score which is defined as:

$$\frac{\% \text{ sequence identity} \times \% \text{ maximum BLAST score}}{100}$$

and it takes into account both the degree of similarity between two sequences and the length of the sequence match. For example, with a product score of 40, the match will be exact within a 1-2% error; and at 70, the match will be exact. Similar molecules are usually identified by selecting those which show product scores  
5 between 15 and 40, although lower scores may identify related molecules.

The term *B. vulgaris* "ALS" or "AHAS" polypeptide also includes amino acid sequences which are at least 90, 95, 97, 98, or 99% identical to the ALS amino acid sequence of SEQ ID NO: 2 or 4, wherein these at least 90, 95, 97, 98, or 99%  
10 identical amino acid sequences comprising at a position corresponding to position 569 of SEQ ID NO: 2 an amino acid different from tryptophan. Said identical amino acid sequences retain the activity of ALS as described herein, more preferably the ALS polypeptide is tolerant to ALS inhibitor herbicides as described herein.

15 ALS activity, if required, can be measured in accordance with the assay described in Singh (1991), Proc. Natl. Acad. Sci. 88:4572-4576.

However, the ALS nucleotide sequences referred to herein encoding an ALS polypeptide preferably confer tolerance to one or more ALS inhibitor herbicides (or,  
20 vice versa, less sensitivity to an ALS inhibitor herbicide) as described herein. This is because of the point mutation leading to an amino acid substitution as described herein.

Accordingly, tolerance to an ALS inhibitor herbicide (or, vice versa, less sensitivity to an ALS inhibitor herbicide) can be measured by comparison of ALS activity obtained  
25 from cell extracts from plants containing the mutated ALS sequence and from plants lacking the mutated ALS sequence in the presence of an ALS-inhibitor herbicide, like it is described in Singh et al (1988) [J. Chromatogr., 444, 251-261].

However, a more preferred activity assay for the ALS polypeptide encoded by a  
30 nucleotide sequence comprising a codon encoding an amino acid different from tryptophan at a position corresponding to position 1705-1707 of the nucleotide

sequence of the *B. vulgaris* ALS gene shown in SEQ ID NO: 1 can be done as follows:

5 The coding sequence of a *Beta vulgaris* wild-type and a mutant *B. vulgaris* plant is cloned into, for example, Novagen pET-32a(+) vectors and the vectors are transformed into, for example, *Escherichia coli* AD494 according to the instructions of the manufacturer. Bacteria are preferably grown at 37°C in medium under selection pressure such as in LB-medium containing 100 mg/l carbenicillin and 25 mg/l kanamycin, are induced with, for example, 1 mM isopropyl- $\beta$ -D-  
10 thiogalactopyranoside at an OD<sub>600</sub> of preferably about 0.6, cultivated for about 16 hours at preferably 18°C and harvested by centrifugation. Bacterial pellets are resuspended in 100 mM sodium phosphate buffer pH 7.0 containing 0.1 mM thiamine-pyrophosphate, 1 mM MgCl<sub>2</sub>, and 1  $\mu$ M FAD at a concentration of 1 gram wet weight per 25 ml of buffer and disrupted by sonification. The crude protein  
15 extract obtained after centrifugation is used for ALS activity measurements.

ALS assays are then carried out in, for example, 96-well microtiter plates using a modification of the procedure described by Ray (1984), *Plant Physiol.*, 75, 827-831. The reaction mixture contains preferably 20 mM potassium phosphate buffer pH 7.0,  
20 20 mM sodium pyruvate, 0.45 mM thiamine-pyrophosphate, 0.45 mM MgCl<sub>2</sub>, 9  $\mu$ M FAD, ALS enzyme and various concentrations of ALS inhibitors in a final volume of about 90  $\mu$ l.

Assays are initiated by adding enzyme and terminated after preferably 75 min  
25 incubation at 30°C by the addition of 40  $\mu$ l 0.5 M H<sub>2</sub>SO<sub>4</sub>. After about 60 min at room temperature about 80  $\mu$ l of a solution of 1.4%  $\alpha$ -naphthol and 0.14% creatine in 0.7 M NaOH is added and after an additional about 45 min incubation at room temperature the absorbance is determined at 540 nm. pI50-values for inhibition of  
ALS were determined as described by Ray (1984) ), *Plant Physiol.*, 75, 827-831,  
30 using the XLFit Excel add-in version 4.3.1 curve fitting program of ID Business Solutions Limited, Guildford, UK.

When plants are used, ALS activity is preferably determined in cell extracts or leaf extracts of wild type and *B. vulgaris* cell extracts or leaf extracts of the obtained mutant in the presence of various concentrations of ALS-inhibitor herbicides, preferably sulfonylurea herbicides or sulfonylamino-carbonyltriazolinone herbicides, more preferably in the presence of various concentrations of the ALS inhibitor herbicide "foramsulfuron". ALS is thus preferably extracted from sugar beet leaves or sugar beet tissue cultures as described by Ray (1984) in Plant Physiol 75:827-831.

It is preferred that the *B. vulgaris* plants of the present invention are less sensitive to an ALS inhibitor, more preferably it is at least 100 times less sensitive, more preferably, 500 times, even more preferably 1000 times and most preferably less than 2000 times. Less sensitive when used herein may, vice versa, be seen as "more tolerable" or "more resistant". Similarly, "more tolerable" or "more resistant" may, vice versa, be seen as "less sensitive".

For example, the *B. vulgaris* plants of the present invention and in particular the *B. vulgaris* plant described in the appended Examples are/is at least 2000 times less sensitive to the ALS inhibitor herbicide foramsulfuron (a member of the ALS inhibitor subclass "sulfonylurea herbicides") compared to the wild type enzyme.

Preferably, the *B. vulgaris* plants of the present invention are less sensitive to various members of ALS inhibitor herbicides, like sulfonylurea herbicides, sulfonylamino-carbonyltriazolinone herbicides, and imidazolinone herbicides. Sulfonylurea herbicides and sulfonylamino-carbonyltriazolinone herbicides against which said plants are less sensitive are preferably selected. In a particular preferred embodiment, the *B. vulgaris* plants of the present invention are less sensitive to the ALS inhibitor herbicide foramsulfuron (sulfonylurea herbicide) either alone or in combination with one or more further ALS inhibitor herbicides either from the subclass of the sulfonylurea-herbicides or any other sub-class of the ALS inhibitor herbicides.

Hence, the *B. vulgaris* plants of the present invention which are preferably less sensitive to an ALS inhibitor herbicide can likewise also be characterized to be "more tolerant" to an ALS inhibitor" (i.e. an ALS inhibitor tolerant plant).

5 Thus, an "ALS inhibitor tolerant" plant refers to a plant, in particular a *B. vulgaris* plant that is more tolerant to at least one ALS inhibitor herbicide at a level that would normally inhibit the growth of a normal or wild-type plant, preferably the ALS inhibitor herbicide controls a normal or wild-type plant. Said normal or wild-type plant does not comprise in the nucleotide sequence of any allele of the endogenous ALS gene,  
10 a codon encoding an amino acid different from tryptophan at a position corresponding to position 1705-1707 of the nucleotide sequence of the *B. vulgaris* ALS gene shown in SEQ ID NO: 1.

Said nucleotide sequence may generally also be characterized to be an "ALS  
15 inhibitor herbicide tolerant" nucleotide sequence. By "ALS inhibitor herbicide tolerant nucleotide sequence" is intended a nucleic acid molecule comprising a nucleotide sequence comprising at least the mutation that results in a codon encoding an amino acid different from tryptophan relative to an ALS protein which does not have at a position corresponding to position 569 of the amino acid sequence of the *B. vulgaris*  
20 ALS protein shown in SEQ ID NO: 2 an amino acid different from tryptophan, wherein said at least one mutation results in the expression of a less sensitive to an ALS inhibitor herbicide ALS protein.

By "herbicide-tolerant ALS protein", it is intended that such an ALS protein displays higher ALS activity, relative to the ALS activity of a wild-type ALS protein, in the  
25 presence of at least one ALS inhibitor herbicide that is known to interfere with ALS activity and at a concentration or level of said herbicide that is known to inhibit the ALS activity of the wild-type ALS protein.

Similarly, the terms "ALS-inhibitor herbicide(s)" or simply "ALS-inhibitor(s)" are used  
30 interchangeably. As used herein, an "ALS -inhibitor herbicide" or an "ALS inhibitor" is not meant to be limited to single herbicide that interferes with the activity of the ALS enzyme. Thus, unless otherwise stated or evident from the context, an "ALS-inhibitor

herbicide" or an "ALS inhibitor" can be a one herbicide or a mixture of two, three, four, or more herbicides known in the art, preferably as specified herein, each of which interferes with the activity of the ALS enzyme.

- 5 Surprisingly, it was found that even the single point mutation according to the present invention confers agronomically useful and stable levels of ALS inhibitor herbicide tolerance in *B. vulgaris* plants as well as in their offsprings, particularly, if homozygosity is established. Compared to herbicide tolerant *Beta vulgaris* plants of the same genetic background in which such mutation is only heterozygously  
10 present, the herbicide tolerant *Beta vulgaris* plants which are homozygous for the mutation revealed a better agronomical level of ALS inhibitor herbicide tolerance.

Therefore, present invention relates to an ALS inhibitor herbicide tolerant *Beta vulgaris* plant having a mutation of the endogenous acetolactate synthase (ALS)  
15 gene, wherein the ALS gene encodes an ALS polypeptide containing an amino acid different from tryptophan at a position 569 of the ALS polypeptide. The respective mutation can be heterozygously present, and can preferably be the sole mutation of the ALS gene. More preferably, the respective mutation can be homozygously present, and most preferably, the respective mutation is homozygously present as  
20 the sole mutation of the endogenous ALS gene.

It could also not be expected that only one single mutation of an ALS gene in *Beta vulgaris* would be sufficient, since, for example, WO 2010/037061 teaches that  
25 double or triple mutants in the ALS gene are necessary to confer the agronomically useful ALS-inhibitor herbicide tolerance.

Therefore, *B. vulgaris* plants and parts thereof which are heterozygous for the mutation are less preferred, but are still covered by the present invention and may be  
30 sufficient for certain application schemes and/or certain environment conditions. Also covered by the present invention are plants containing at least in one allele of the endogenous ALS gene, a codon encoding an amino acid different from

tryptophan, preferably leucine at a position corresponding to position 1705-1707 of the nucleotide sequence of the *B. vulgaris* ALS gene shown in SEQ ID NO: 1, and containing one (in case of diploidy) or more further alleles (in case of polyploidy) having one or more further mutations in the endogenous ALS gene.

5

Accordingly, when used herein the term "heterozygous" or "heterozygously" means that a plant of the present invention has different alleles at a particular locus, in particular at the ALS gene locus.

10 "Homozygous" or "homozygously" indicates that a plant of the present invention has two copies of the same allele on different DNA strands, in particular at the ALS gene locus.

As used herein unless clearly indicated otherwise, the term "plant" intended to mean  
15 a plant at any developmental stage.

It is preferred that the *Beta vulgaris* plant of the present invention is orthoploid or anorthoploid. An orthoploid plant may preferably be haploid, diploid, tetraploid, hexaploid, octaploid, decaploid or dodecaploid, while an anorthoploid plant may  
20 preferably be triploid or pentaploid.

Parts of plants may be attached to or separate from a whole intact plant. Such parts of a plant include, but are not limited to, organs, tissues, and cells of a plant, and preferably seeds.

25

Accordingly, the *B. vulgaris* plant of the present invention is non-transgenic as regards an endogenous ALS gene. Of course, foreign genes can be transferred to the plant either by genetic engineering or by conventional methods such as crossing. Said genes can be genes conferring herbicide tolerances, preferably conferring  
30 herbicide tolerances different from ALS inhibitor herbicide tolerances, genes improving yield, genes improving resistances to biological organisms, and/or genes concerning content modifications.



In a further aspect, the present invention relates to a method for the manufacture of the *Beta vulgaris* plant and the parts thereof, comprising the following steps:

- 5 (a) exposing calli, preferably from sugar beet, to about  $10^{-7}$  M -  $10^{-9}$  M of an ALS inhibitor herbicide, preferably foramsulfuron;
- (b) selecting cell colonies which can grow in the presence of up to  $3 \times 10^{-6}$  M of an ALS inhibitor herbicide, preferably foramsulfuron [CAS RN 173159-57-4];
- (c) regenerating shoots in presence of an ALS inhibitor herbicide, preferably foramsulfuron;
- 10 (d) selecting regenerated plantlets with an ALS inhibitor herbicide, preferably foramsulfuron, iodosulfuron-methyl-sodium [CAS RN 144550-36-7] and/or a mixture of both, wherein the dose of foramsulfuron is preferably equivalent to 7-70 g a.i./ha and the dose of iodosulfuron-methyl-sodium is preferably equivalent to 1-10 g a.i./ha.

15

In a further aspect, the regenerated plantlets obtained according to the processes (a) to (d) above, can be employed for further manufacture of *Beta vulgaris* plants by applying the following steps (e) to (m):

- 20 (e) vegetative micropropagation of individual plantlets of step (d) to rescue different positive variants by establishing a cell line (clone) of each ALS inhibitor herbicide tolerant plantlet;
- (f) longterm storage of each established clone in the vegetative state;
- (g) transfer of cloned plants of each clone from the long term storage into the  
25 greenhouse;
- (h) vernalisation and adaptation in vernalisation chambers to induce flowering;
- (i) transfer of vernalised plants to growth rooms (controlled temperature and light);
- (j) select best pollen shedding plants of best flowering clones for crossing with  
30 emasculated plants of an elite but ALS inhibitor herbicide sensitive line to overcome the negative impact of somaclonal variation on the generative fertility (male and female) of plantlets of step (d);

- (k) backcross to elite line until fertility is restored and finally self heterozygous plants to reach the homozygous state;
- (l) produce testcrosses with an ALS inhibitor herbicide- sensitive partner and selfed seed of each backcrossed line for field evaluations;
- 5 (m) applying agronomically relevant dose rates of different ALS inhibitor herbicides to select the best performing line, preferably in its homozygous state.

- The lines obtained according to above steps (a) to (m) form the basis for the
- 10 development of commercial varieties following procedures known in the breeding community supported by molecular breeding techniques (like marker assisted breeding or marker assisted selection) for speeding up the processes and to secure the correct selection of plants to either obtain the mutation in its homozygous form or
- 15 in case of containing one or more mutations at various locations of the ALS encoding endogenous gene to perform the correct selection of heterozygous plants that do contain at least at one of the alleles the W569 mutation according to present invention. (For review, see Bertrand C.Y. et al. (2008), Phil. Trans. R. Soc. B., 363, 557-572)
- 20 Calli are obtained by means and methods commonly known in the art, for example, as described in the appended Examples.

Seeds obtained under step (m), above, have been deposited with the NCIMB, Aberdeen, UK, under Number NCIMB 41705.

25

- In a further aspect, the present invention relates to a method for producing an herbicide tolerant *Beta vulgaris* plant and parts thereof comprising (i) a mutation of an endogenous acetolactate synthase (ALS) gene, wherein the ALS gene encodes an ALS polypeptide containing an amino acid different from tryptophan at a position
- 30 569 of the ALS polypeptide, and (ii) an additional mutation in the endogenous ALS gene, comprising the following steps:

- 5 (a) producing an ALS inhibitor herbicide tolerant *Beta vulgaris* plant comprising a mutation of an endogenous acetolactate synthase (ALS) gene, wherein the ALS gene encodes an ALS polypeptide containing an amino acid different from tryptophan at a position 569 of the ALS polypeptide (parent A);
- (b) crossing parent A with a *Beta vulgaris* plant (parent B) containing one or more further mutations in the endogenous ALS gene at positions differing from amino acid position 569;
- 10 (c) obtaining a *Beta vulgaris* progeny that is heterozygous for the ALS gene mutation of amino acid position 569 and to one or more of any further ALS gene mutations encoded by parent B;
- (d) wherein the breeding process is controlled by
- (i) the application of marker assisted breeding and/or microsequencing techniques, and/or
- 15 (ii) the application of agronomically relevant doses of one or more ALS inhibitor herbicides to which the generated progeny according to step (c) are tolerant.

Accordingly, it is envisaged that the present invention also relates to *B. vulgaris* plants obtainable by the aforementioned methods of manufacture.

In a non-limiting example, sugar beet plants of the present invention were obtained by performing the following non-limiting protocol. Without being bound by theory, the same protocol may be used for obtaining *B. vulgaris* plants other than sugar beet.

25

Sugar beet cell cultures were initiated from seedlings of a diploid sugar beet genotype 7T9044 (as, for example, described by Alexander Dovzhenko, PhD Thesis, Title: "Towards plastid transformation in rapeseed (*Brassica napus* L.) and sugarbeet (*Beta vulgaris* L.)", Ludwig-Maximilians-Universität München, Germany, 2001).

30 Sugar beet seeds were immersed for 60 seconds in 70 % ethanol, then rinsed twice in sterile water with 0.01 % detergent and then incubated for 1-4 hours in 1 % NaOCl bleach. Thereafter the seeds were washed 3 times with sterile H<sub>2</sub>O and the seeds

were stored in sterile water overnight at 4°C. The embryos were then isolated using forceps and scalpel.

The freshly prepared embryos were immersed in 0.5 % NaOCl for 30 min and then washed 3 times in sterile water. After the last washing step they were placed on hormone free MS agar medium (Murashige and Skoog (1962), *Physiol. Plantarum*, 15, 473-497). Those embryos which developed into sterile seedlings were used for the initiation of regenerable sugar beet cell cultures.

Cotyledons as well as hypocotyls were cut into 2-5 mm long segments and then cultivated on agar (0.8 %) solidified MS medium containing either 1 mg/l

Benzylaminopurine (BAP) or 0,25 mg/l Thidiazuron (TDZ). 4 weeks later the developing shoot cultures were transferred onto fresh agar medium of the same composition and then sub-cultured in monthly intervals. The cultures were kept at 25°C under dim light at a 12h/12 h light/dark cycle.

After 7-10 subcultures the shoot cultures which were grown on the thidiazuron containing medium formed a distinct callus type, which was fast growing, soft and friable. The colour of this callus type was yellowish to light green. Some of these friable calli consistently produced chlorophyll containing shoot primordia from embryo-like structures. These fast growing regenerable calli were used for the selection of ALS-inhibitor herbicide tolerant sugar beet mutants.

When this callus type was exposed to  $10^{-9}$  M of the sulfonyleurea foramsulfuron (CAS RN 173159-57-4), the cells survived, but produced less than 50 % of the biomass of their siblings on medium devoid of the inhibitor. On medium containing  $3 \times 10^{-8}$  M foramsulfuron no growth was detectable. For large scale mutant selection experiments  $10^{-7}$  M foramsulfuron was chosen. Surviving and growing cell colonies were numbered and transferred after 4-6 weeks onto fresh medium containing  $3 \times 10^{-7}$  M of the inhibitor. One of these cell colonies was able to grow not only at this concentration of the inhibitor but even in presence of  $3 \times 10^{-6}$  M of foramsulfuron.

From this clone (SB574TL), shoots were regenerated in presence of the ALS-inhibitor herbicide and then the shoots were transferred to MS medium containing 0.05 mg/l Naphthalene acetic acid (NAA).

Within 4-12 weeks the shoots formed roots and then they were transferred into sterile plant containers filled with wet, sterilized perlite, watered with half strength MS

inorganic ingredients. Alternatively the plantlets were transferred directly from the agar solidified medium in a perlite containing soil mixture in the greenhouse. During the first 10-15 days after transfer into soil containing substrate the plants were kept in an environment with high air humidity. During and after they were weaned to  
5 normal greenhouse air humidity regimes the plants were kept in the greenhouse under artificial light (12 h) at 20  $\pm$  3°C/ 15 $\pm$  2°C day/night temperatures.

3-5 weeks later, the regenerated plants from the above obtained foramsulfuron tolerant cell culture (SB574TL) as well as from the wild type cell cultures were  
10 treated with foramsulfuron, iodosulfuron-methyl-sodium (CAS RN 144550-3-7) and a mixture of both active ingredients. The herbicide doses tested were equivalent to 7-70 g a.i./ha for foramsulfuron and 1-10 g a.i./ha for iodosulfuron-methyl-sodium. Regenerated plants from this tolerant cell line tolerated even the highest herbicide doses (foramsulfuron, iodosulfuron-methyl-sodium and their mixtures in the ratio 7:1  
15 whereas even the lowest doses killed the wild type plants.

Offsprings were tested as follows (in a non-limiting way):

Based on SB574TL, F2 and F3 seeds of experimental hybrids conferring the resistance allele in the heterozygous state as well as F4–F6 seeds conferring the  
20 mutant allele in the homozygous state were sown in the field and treated with foramsulfuron, iodosulfuron-methyl-sodium as well as with mixtures of both ALS inhibitor herbicides when the plants developed 3-5 rosette leaves. The homozygous seedlings tolerated mixtures of 35 g foramsulfuron/ha + 7 g iodosulfuron-methyl-sodium/ha without growth retardation or any signs of visible damage. In several  
25 cases, heterozygous lines showed signs of retarded growth and some leaf chlorosis at these rates, but they recovered within 3-5 weeks, whereas the conventional sugar beet seedlings were killed by the ALS inhibitor herbicides.

The ALS mutants were characterized as follows:

30 Extraction and nucleic acid sequence analysis of the obtained mutant was performed by LGC Genomics GmbH, Berlin, Germany according to amended standard protocols.

The nucleic acid sequence obtained from the sugar beet mutant SB574TL is shown in SEQ ID NO: 3. SEQ ID NO: 4 represents the corresponding amino acid sequence, whereas SEQ ID NO: 1 was obtained after sequencing the wild type sugar beet plant that was taken as the starting material. SEQ ID NO: 2 represents the corresponding amino acid sequence of the wild type sugar beet.

Comparison of all these sequences shows up that there is only the mutation at position 574 but no other change took place at any other part of this endogenous ALS gene.

10

SEQ ID No1

(1)

ATGGCGGCTACCTTCACAAACCCAACATTTTCCCCTTCCTCAACTCCATTAACCAAACC

SEQ ID No 3

15

(1)

ATGGCGGCTACCTTCACAAACCCAACATTTTCCCCTTCCTCAACTCCATTAACCAAACC

SEQ ID No1

(61)

20

CTAAAATCCCAATCTTCCATCTCTTCAACCCTCCCCTTTTCCACCCCTCCCAAACCCCA

SEQ ID No 3

(61)

CTAAAATCCCAATCTTCCATCTCTTCAACCCTCCCCTTTTCCACCCCTCCCAAACCCCA

25

SEQ ID No1

(121)

ACTCCACTCTTTCACCGTCCCCTCCAAATCTCATCCTCCCAATCCCACAAATCATCCGCC

SEQ ID No 3

(121)

30

ACTCCACTCTTTCACCGTCCCCTCCAAATCTCATCCTCCCAATCCCACAAATCATCCGCC

SEQ ID No1

(181)

ATTAAACACAACTCAAGCACCTTCTTCTCCAGCTATTGAAGATTCATCTTTCGTTTCT

SEQ ID No 3

5 (181)

ATTAAACACAACTCAAGCACCTTCTTCTCCAGCTATTGAAGATTCATCTTTCGTTTCT

SEQ ID No1

(241)

10 CGATTTGGCCCTGATGAACCCAGAAAAGGGTCCGATGTCCTCGTTGAAGCTCTTGAGCGT

SEQ ID No 3

(241)

CGATTTGGCCCTGATGAACCCAGAAAAGGGTCCGATGTCCTCGTTGAAGCTCTTGAGCGT

15 SEQ ID No1

(301)

GAAGGTGTTACCAATGTGTTTGCTTACCCTGGTGGTGCATCTATGGAAATCCACCAAGCT

SEQ ID No 3

(301)

20 GAAGGTGTTACCAATGTGTTTGCTTACCCTGGTGGTGCATCTATGGAAATCCACCAAGCT

SEQ ID No1

(361)

CTCACACGCTCTAAAACCATCCGCAATGTCCTCCCTCGCCATGAACAAGGCGGGGTTTTTC

25 SEQ ID No 3

(361)

CTCACACGCTCTAAAACCATCCGCAATGTCCTCCCTCGCCATGAACAAGGCGGGGTTTTTC

SEQ ID No1

30 (421)

GCCGCCGAGGGATATGCTAGAGCTACTGGAAAGGTTGGTGTCTGCATTGCGACTTCTGGT

SEQ ID No 3

(421)

GCCGCCGAGGGATATGCTAGAGCTACTGGAAAGGTTGGTGTCTGCATTGCGACTTCTGGT

SEQ ID No1

5 (481)

CCTGGTGCTACCAACCTCGTATCAGGTCTTGCTGACGCTCTCCTTGATTCTGTCCCTCTT

SEQ ID No 3

(481)

CCTGGTGCTACCAACCTCGTATCAGGTCTTGCTGACGCTCTCCTTGATTCTGTCCCTCTT

10

SEQ ID No1

(541)

GTTGCCATCACTGGCCAAGTTCCACGCCGTATGATTGGCACTGATGCTTTTCAGGAGACT

SEQ ID No 3

15 (541)

GTTGCCATCACTGGCCAAGTTCCACGCCGTATGATTGGCACTGATGCTTTTCAGGAGACT

SEQ ID No1

(601)

20 CCAATTGTTGAGGTGACAAGGTCTATTACTAAGCATAATTATTTAGTTTTGGATGTAGAG

SEQ ID No 3

(601)

CCAATTGTTGAGGTGACAAGGTCTATTACTAAGCATAATTATTTAGTTTTGGATGTAGAG

25 SEQ ID No1

(661)

GATATTCCTAGAATTGTAAAGGAAGCCTTTTTTTTAGCTAATTCTGGTAGGCCTGGACCT

SEQ ID No 3

(661)

30 GATATTCCTAGAATTGTAAAGGAAGCCTTTTTTTTAGCTAATTCTGGTAGGCCTGGACCT



SEQ ID No1

(721)

GTTTTGATTGATCTTCCTAAAGATATTCAGCAGCAATTGGTTGTTTCCTGATTGGGATAGG

SEQ ID No 3

5 (721)

GTTTTGATTGATCTTCCTAAAGATATTCAGCAGCAATTGGTTGTTTCCTGATTGGGATAGG

SEQ ID No1

(781)

10 CCTTTTAAGTTGGGTGGGTATATGTCTAGGCTGCCAAAGTCCAAGTTTTCGACGAATGAG

SEQ ID No 3

(781)

CCTTTTAAGTTGGGTGGGTATATGTCTAGGCTGCCAAAGTCCAAGTTTTCGACGAATGAG

15 SEQ ID No1

(841)

GTTGGACTTCTTGAGCAGATTGTGAGGTTGATGAGTGAGTCGAAGAAGCCTGTCTTGTAT

SEQ ID No 3

(841)

20 GTTGGACTTCTTGAGCAGATTGTGAGGTTGATGAGTGAGTCGAAGAAGCCTGTCTTGTAT

SEQ ID No1

(901)

GTGGGAGGTGGGTGTTTGAATTCTAGTGAGGAGTTGAGGAGATTTGTTGAGTTGACAGGG

25 SEQ ID No 3

(901)

GTGGGAGGTGGGTGTTTGAATTCTAGTGAGGAGTTGAGGAGATTTGTTGAGTTGACAGGG

SEQ ID No1

30 (961)

ATTCGGTGGCTAGTACTTTGATGGGGTTGGGGTCTTACCCTTGTAATGATGAACTGTCT

SEQ ID No 3

(961)

ATTCGGTGGCTAGTACTTTGATGGGGTTGGGGTCTTACCCTTGTAATGATGAACTGTCT

SEQ ID No1

5 (1021)

CTTCATATGTTGGGGATGCACGGGACTGTTTATGCCAATTATGCGGTGGATAAGGCGGAT

SEQ ID No 3

(1021)

CTTCATATGTTGGGGATGCACGGGACTGTTTATGCCAATTATGCGGTGGATAAGGCGGAT

10

SEQ ID No1

(1081)

TTGTTGCTTGCTTTCGGGGTTAGGTTTGATGATCGTGTGACCGGGAAGCTCGAGGCGTTT

SEQ ID No 3

15 (1081)

TTGTTGCTTGCTTTCGGGGTTAGGTTTGATGATCGTGTGACCGGGAAGCTCGAGGCGTTT

SEQ ID No1

(1141)

20 GCTAGCCGTGCTAAGATTGTGCATATTGATATTGACTCTGCTGAGATTGGGAAGAACAAG

SEQ ID No 3

(1141)

GCTAGCCGTGCTAAGATTGTGCATATTGATATTGACTCTGCTGAGATTGGGAAGAACAAG

25 SEQ ID No1 (1201)

CAGCCCCATGTGTCCATTTGTGCTGATGTTAAATTGGCATTGCGGGGTATGAATAAGATT

SEQ ID No 3 (1201)

CAGCCCCATGTGTCCATTTGTGCTGATGTTAAATTGGCATTGCGGGGTATGAATAAGATT

30 SEQ ID No1

(1261)

CTGGAGTCTAGAATAGGGAAGCTGAATTTGGATTTCTCCAAGTGGAGAGAAGAATTAGGT

SEQ ID No 3

(1261)

CTGGAGTCTAGAATAGGGAAGCTGAATTTGGATTTCTCCAAGTGGAGAGAAGAATTAGGT

SEQ ID No1

5 (1321)

GAGCAGAAGAAGGAATTCCCCTGAGTTTTAAGACATTTGGGGATGCAATTCCTCCACAA

SEQ ID No 3

(1321)

GAGCAGAAGAAGGAATTCCCCTGAGTTTTAAGACATTTGGGGATGCAATTCCTCCACAA

10

SEQ ID No1 (1381)

TATGCCATTCAGGTGCTTGATGAGTTGACCAATGGTAATGCTATTATAAGTACTGGTGTT

SEQ ID No 3 (1381)

TATGCCATTCAGGTGCTTGATGAGTTGACCAATGGTAATGCTATTATAAGTACTGGTGTT

15

SEQ ID No1

(1441)

GGGCAGCACCAAATGTGGGCTGCGCAGCATTACAAGTACAGAAACCCTCGCCAATGGCTG

SEQ ID No 3

20

(1441)

GGGCAGCACCAAATGTGGGCTGCGCAGCATTACAAGTACAGAAACCCTCGCCAATGGCTG

SEQ ID No1

(1501)

25 ACCTCTGGTGGGTTGGGGGCTATGGGGTTTGGGCTACCAGCCGCCATTGGAGCTGCAGTT

SEQ ID No 3

(1501)

ACCTCTGGTGGGTTGGGGGCTATGGGGTTTGGGCTACCAGCCGCCATTGGAGCTGCAGTT

30

SEQ ID No1

(1561)

GCTCGACCAGATGCAGTGGTTGTCGATATTGATGGGGATGGCAGTTTTATTATGAATGTT

SEQ ID No 3

(1561)

GCTCGACCAGATGCAGTGGTTGTCGATATTGATGGGGATGGCAGTTTTATTATGAATGTT

SEQ ID No1

5 (1621)

CAAGAGTTGGCTACAATTAGGGTGGAAAATCTCCCAGTTAAGATAATGCTGCTAAACAAT

SEQ ID No 3

(1621)

CAAGAGTTGGCTACAATTAGGGTGGAAAATCTCCCAGTTAAGATAATGCTGCTAAACAAT

10

SEQ ID No1

(1681)

CAACATTTAGGTATGGTTGTCCAATGGGAAGATAGGTTCTATAAAGCTAACCGGGCACAT

SEQ ID No 3

15 (1681)

CAACATTTAGGTATGGTTGTCCAATTGGAAGATAGGTTCTATAAAGCTAACCGGGCACAT

SEQ ID No1

(1741)

20 ACATACCTTGGAACCCTTCCAAATCTGCTGATATCTTCCCTGATATGCTCAAATTCGCT

SEQ ID No 3

(1741)

ACATACCTTGGAACCCTTCCAAATCTGCTGATATCTTCCCTGATATGCTCAAATTCGCT

25 SEQ ID No1

(1801)

GAGGCATGTGATATTCCTTCTGCCCCGTGTTAGCAACGTGGCTGATTTGAGGGCCGCCATT

SEQ ID No 3

(1801)

30 GAGGCATGTGATATTCCTTCTGCCCCGTGTTAGCAACGTGGCTGATTTGAGGGCCGCCATT

SEQ ID No1

(1861)

CAAACAATGTTGGATACTCCAGGGCCGTACCTGCTCGATGTGATTGTACCGCATCAAGAG

SEQ ID No 3

5 (1861)

CAAACAATGTTGGATACTCCAGGGCCGTACCTGCTCGATGTGATTGTACCGCATCAAGAG

SEQ ID No1

(1921)

10 CATGTGTTGCCTATGATTCCAAGTGGTGCCGGTTTCAAGGATACCATTACAGAGGGTGAT

SEQ ID No 3

(1921)

CATGTGTTGCCTATGATTCCAAGTGGTGCCGGTTTCAAGGATACCATTACAGAGGGTGAT

15 SEQ ID No1

(1981) GGAAGAACCTCTTATTGA

SEQ ID No 3

(1981) GGAAGAACCTCTTATTGA

20 SEQ ID No. 2

(1)

MAATFTNPTFSPSSTPLTKTLKSQSSISSTLPFSTPPKTPTPLFHRPLQISSSQSHKSSA

SEQ ID No.4

(1)

25 MAATFTNPTFSPSSTPLTKTLKSQSSISSTLPFSTPPKTPTPLFHRPLQISSSQSHKSSA

SEQ ID No. 2

(61)

IKTQTQAPSSPAIEDSSFVSRFGPDEPRKGSVDLVEALEREGVTNVFAYPGGASMEIHQA

30 SEQ ID No.4

(61)

IKTQTQAPSSPAIEDSSFVSRFGPDEPRKGSVDLVEALEREGVTNVFAYPGGASMEIHQA

SEQ ID No. 2

(121)

LTRSKTIRNVLP RHEQGGVFAAEGYARATGKVGVC IATSGPGATNLV SGLADALLDSVPL

SEQ ID No. 4

5 (121)

LTRSKTIRNVLP RHEQGGVFAAEGYARATGKVGVC IATSGPGATNLV SGLADALLDSVPL

SEQ ID No. 2

(181)

10 VAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRIVKEAFFLANSGRPGP

SEQ ID No. 4

(181)

VAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVLDVEDIPRIVKEAFFLANSGRPGP

15 SEQ ID No. 2

(241)

VLIDLPKDIQQQLVVPDWDRPFKLGGYMSRLPKSKFSTNEVGLLEQIVRLMSESCKPVLY

SEQ ID No. 4

(241)

20 VLIDLPKDIQQQLVVPDWDRPFKLGGYMSRLPKSKFSTNEVGLLEQIVRLMSESCKPVLY

SEQ ID No. 2

(301)

VGGGCLNSSEELRRFVELTGIPVASTLMGLGSYPCNDELSLHMLGMHGT VYANYAVDKAD

25 SEQ ID No. 4

(301)

VGGGCLNSSEELRRFVELTGIPVASTLMGLGSYPCNDELSLHMLGMHGT VYANYAVDKAD

SEQ ID No. 2

30 (361)

LLLAFGVRFDDRVTGKLEAFASRAKIVHIDIDSAEIGKNKQPHVSICADV KLALRGMNKI

SEQ ID No. 4

(361)

LLLAFGVRFDDRVTGKLEAFASRAKIVHIDIDSAEIGKNKQPHVSI CADVKLALRGMNKI

SEQ ID No. 2

5 (421)

LESRIGKLNLD FSKWREELGEQKKEFPLSFKTFGDAIPPQYAIQVLDEL TNGNAIISTGV

SEQ ID No. 4

(421)

LESRIGKLNLD FSKWREELGEQKKEFPLSFKTFGDAIPPQYAIQVLDEL TNGNAIISTGV

10

SEQ ID No. 2

(481)

GQHQMWA AQHYKYRNPRQWLTS GGLGAMGFGLPAAIGA AAVARPD AVVVDIDGDGSFIMNV

SEQ ID No. 4

15 (481)

GQHQMWA AQHYKYRNPRQWLTS GGLGAMGFGLPAAIGA AAVARPD AVVVDIDGDGSFIMNV

SEQ ID No. 2

(541)

20 QELATIRVENLPVKIMLLNNQHLGMVVQWEDRFYKANRAHTYLG NPSKSADIFPDMLKFA

SEQ ID No. 4

(541)

QELATIRVENLPVKIMLLNNQHLGMVVQLEDRFYKANRAHTYLG NPSKSADIFPDMLKFA

25 SEQ ID No. 2

(601)

EACDIPSARVSNVADLR AAIQTMLDTPGPYLLDVIVPHQEHVLP MIPSGAGFKDTITEGD

SEQ ID No. 4

(601)

30 EACDIPSARVSNVADLR AAIQTMLDTPGPYLLDVIVPHQEHVLP MIPSGAGFKDTITEGD

SEQ ID No. 2

(661) GRTSY-

SEQ ID No. 4

(661) GRTSY-

5

Yet, it is generally preferred that the *B. vulgaris* plants of the present invention and parts thereof are agronomically exploitable. "Agronomically exploitable" means that the *B. vulgaris* plants and parts thereof are useful for agronomical purposes. For example, the *B. vulgaris* plants should serve for the purpose of being useful for sugar production, bio fuel production (such as biogas, biobutanol), ethanol production, betaine and/or uridine production. The term "agronomically exploitable" when used herein also includes that the *B. vulgaris* plants of the present invention are preferably less sensitive against an ALS-inhibitor herbicide, more preferably it is at least 100 times less sensitive, more preferably, 500 times, even more preferably 1000 times and most preferably less than 2000 times. The ALS inhibitor herbicide is one or more described herein, preferably it is foramsulfuron either alone or in combination with one or more further ALS-inhibitor herbicide(s) either from the sub-class of the sulfonyurea herbicides or any other sub-class of the ALS-inhibitor herbicides, most preferably it is foramsulfuron in combination with a further sulfonylurea herbicide and/or an ALS-inhibitor of the sulfonylaminocarbonyltriazolinone herbicide sub-class.

Preferably, agronomically exploitable *B. vulgaris* plants, most preferably sugar beet plants, of the present invention are fully fertile, more preferably have wild-type fertility. Fertility is of utmost importance for a *B. vulgaris* plant of the present invention in order to be agronomically exploitable.

An example for an agronomically exploitable *B. vulgaris* plant is sugar beet. A sugar beet plant of the present invention when cultivated in an area of one hectare yields (about 80,000 to 90,000 sugar beets) should preferably serve for the production of at least 4 tons of sugar.



Alternatively, a sugar beet plant of the present invention should preferably contain a sugar content between 15-20%, preferably at least 17% so as to be agronomically exploitable. Thus, sugar beet plants that contain a sugar content between 15-20%, preferably at least 17% are a preferred embodiment of the present invention.

5

Plants of the present invention can be identified using any genotypic analysis method. Genotypic evaluation of the plants includes using techniques such as Isozyme Electrophoresis, Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Arbitrarily Primed Polymerase  
10 Chain Reaction (AP-PCR), Allele-specific PCR (AS-PCR), DNA Amplification Fingerprinting (DAF), Sequence Characterized Amplified Regions (SCARs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) which are also referred to as "Microsatellites". Additional compositions and methods for analyzing the genotype of the plants provided herein include those  
15 methods disclosed in U.S. Publication No. 2004/0171027, U.S. Publication No. 2005/02080506, and U.S. Publication No. 2005/0283858.

Another aspect of the present invention is the use of the Beta vulgaris plant described herein and/or the harvestable parts or propagation material described  
20 herein for the manufacture/breeding of Beta vulgaris plants. Methods for the manufacture/breeding of B. vulgaris plants are described herein elsewhere. Such manufacture/breeding methods may be used to generate B. vulgaris plants of the present invention further comprising novel plant traits such as stress-resistance, like but not limited to drought, heat, cold, or salt stress and the like.

25

In a still further aspect, the present invention envisages the use of the herbicide tolerant Beta vulgaris plant described herein and/or harvestable parts or propagation material derived thereof in a screening method for the selection of ALS inhibitor herbicides.

30

A better understanding of the present invention and of its many advantages will be had from the following examples, offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

5

#### Example 1: Mutant isolation

Sugar beet cell cultures were initiated from seedlings of a diploid sugar beet genotype 7T9044 (as, for example, described by Alexander Dovzhenko, PhD Thesis,  
10 Title: "Towards plastid transformation in rapeseed (*Brassica napus* L.) and sugarbeet (*Beta vulgaris* L.)", Ludwig-Maximilians-Universität München, Germany, 2001).

Sugar beet seeds were immersed for 60 seconds in 70% ethanol, then rinsed twice in sterile water with 0,01 % detergent and then incubated for 1-4 hours in 1% NaOCl  
15 bleach. Thereafter the seeds were washed 3 times with sterile H<sub>2</sub>O and the seeds were stored in sterile H<sub>2</sub>O overnight at 4°C. The embryos were then isolated using forceps and scalpel.

The freshly prepared embryos were immersed in 0.5 % NaOCl for 30 min and then washed 3 times in sterile H<sub>2</sub>O. After the last washing step they were placed on  
20 hormone free MS agar medium (Murashige and Skoog (1962), *Physiol. Plantarum*, 15, 473-497). Those embryos which developed into sterile seedlings were used for the initiation of regenerable sugar beet cell cultures.

Cotyledons as well as hypocotyls were cut into 2-5 mm long segments and then  
25 cultivated on agar (0.8 %) solidified MS agar medium containing either 1 mg /l Benzylaminopurin (BAP) or 0.25 mg/l Thidiazuron (TDZ). 4 weeks later the developing shoot cultures were transferred onto fresh MS agar medium of the same composition and then sub-cultured in monthly intervals. The cultures were kept at 25°C under dim light at a 12 h/12 h light/dark cycle.

30 After 7-10 days, subcultures the shoot cultures which were grown on the thidiazuron containing medium formed a distinct callus type, which was fast growing, soft and friable. The colour of this callus type was yellowish to light green. Some of these

friable calli consistently produced chlorophyll containing shoot primordia from embryo-like structures. These fast growing regenerable calli were used for the selection of ALS inhibitor herbicide tolerant sugar beet mutants.

When this callus type was exposed to  $10^{-9}$  M of the ALS inhibitor herbicide

5 foramsulfuron (belonging to the sulfonylurea subclass, see above), the cells survived, but produced less than 50% of the biomass of their siblings on medium devoid of the inhibitor. On medium containing  $3 \times 10^{-8}$  M foramsulfuron no growth was detectable. For large scale mutant selection experiments,  $10^{-7}$  M foramsulfuron was chosen. Surviving and growing cell colonies were numbered and transferred  
10 after 4-6 weeks onto fresh medium containing  $3 \times 10^{-7}$  M of the inhibitor. One of these cell colonies was able to grow not only at this concentration of the inhibitor but even in presence of  $3 \times 10^{-6}$  M of foramsulfuron.

From this clone (SB574TL), shoots were regenerated in presence of the ALS inhibitor herbicide, and then the shoots were transferred to MS medium containing  
15 0.05 mg /l naphthalene acetic acid (NAA).

Within 4-12 weeks the shoots formed roots and then they were transferred into sterile plant containers filled with wet, sterilized perlite, watered with half strength MS inorganic ingredients. Alternatively the plantlets were transferred directly from the  
20 agar solidified medium in a perlite containing soil mixture in the greenhouse. During the first 10-15 days after transfer into soil containing substrate the plants were kept in an environment with high air humidity. During and after they were weaned to normal greenhouse air humidity regimes the plants were kept in the greenhouse under artificial light (12 h) at  $20 \pm 3^{\circ}\text{C}$  /  $15 \pm 2^{\circ}\text{C}$  day/night temperatures.

25

3-5 weeks later, the regenerated plants from the above obtained foramsulfuron tolerant cell culture (SB574TL) as well as from the wild type cell cultures were treated with foramsulfuron, iodosulfuron-methyl-sodium (CAS RN 144550-3-7) and a mixture of both active ingredients. The herbicide doses tested were equivalent to 7-  
30 70 g a.i./ha for foramsulfuron and 1-10 g a.i./ha for iodosulfuron-methyl-sodium. Regenerated plants from this tolerant cell line tolerated even the highest herbicide

doses (foramsulfuron, iodosulfuron-methyl-sodium and their mixtures in the ratio 7:1 whereas even the lowest doses killed the wild type plants.

## 5 Example 2: Test of offsprings

Based on SB574TL, F2 and F3 seeds of experimental hybrids conferring the resistance allele in the heterozygous state as well as F4–F6 seeds conferring the mutant allele in the homozygous state were sown in the field and treated with  
10 foramsulfuron, iodosulfuron-methyl-sodium as well as with mixtures of both ALS inhibitor herbicides when the plants developed 3-5 rosette leaves. The homozygous seedlings tolerated mixtures of 35 g foramsulfuron/ha + 7 g iodosulfuron-methyl-sodium/ha without growth retardation or any signs of visible damage. Heterozygous  
15 lines showed signs of retarded growth and some leaf chlorosis at these rates, but they recovered within 3-5 weeks, whereas the conventional sugar beet seedlings were killed by the ALS inhibitor herbicides.

Example 3: Molecular characterization of the obtained sugar beet mutant (SB574TL)  
20  
Extraction and nucleic acid sequence analysis of the obtained mutant was performed by LGC Genomics GmbH, Berlin, Germany according to amended standard protocols.

25 The nucleic acid sequence obtained from the sugar beet mutant SB574TL is shown under SEQ ID NO: 3 with SEQ ID NO: 4 representing the corresponding amino acid sequence, whereas SEQ ID NO: 1 was obtained after sequencing the wild type sugar beet plant that was taken as the starting material. SEQ ID NO: 2 represents the corresponding amino acid sequence of the wild type sugar beet.

30

Comparison of all these sequences clearly show up that there is only one mutation at position 569 but no other change took place at any other part of this endogenous ALS gene of this sugar beet plant material.

5

#### Example 4: Enzyme activity measurements

The coding sequences of Beta vulgaris wild-type and W574L-mutant (SB574TL) ALS gene were cloned into Novagen pET-32a(+) vectors and the vectors transformed into  
10 Escherichia coli AD494 according to the instructions of the manufacturer. Bacteria were grown at 37 °C in LB-medium (Luria-Broth-medium) containing 100 mg/l carbenicillin and 25 mg/l kanamycin, induced with 1 mM isopropyl-b-D-thiogalactopyranoside at an OD<sub>600</sub> of 0.6, cultivated for 16 hours at 18 °C and harvested by centrifugation. Bacterial pellets were resuspended in 100 mM sodium  
15 phosphate buffer pH 7.0 containing 0.1 mM thiamine-pyrophosphate, 1 mM MgCl<sub>2</sub>, and 1 µM FAD at a concentration of 1 gram wet weight per 25 ml of buffer and disrupted by sonification. The crude protein extract obtained after centrifugation was used for ALS activity measurements.

20 ALS assays were carried out in 96-well microtiter plates using a modification of the procedure described by Ray (1984). The reaction mixture contained 20 mM potassium phosphate buffer pH 7.0, 20 mM sodium pyruvate, 0.45 mM thiamine-pyrophosphate, 0.45 mM MgCl<sub>2</sub>, 9 µM FAD, ALS enzyme and various concentrations of ALS inhibitors in a final volume of 90 µl. Assays were initiated by adding enzyme  
25 and terminated after 75 min incubation at 30°C by the addition of 40 µl 0.5 M H<sub>2</sub>SO<sub>4</sub>. After 60 min at room temperature 80 µl of a solution of 1.4% a-naphtol and 0.14% creatine in 0.7 M NaOH was added and after an additional 45 min incubation at room temperature the absorbance was determined at 540 nm. pI50-values for inhibition of ALS were determined as described by Ray (1984), using the XLFit Excel add-in  
30 version 4.3.1 curve fitting program of ID Business Solutions Limited.

In total, the mutant enzyme was at least 2000 times less sensitive against the ALS inhibitor foramsulfuron than the wild type enzyme.

5    Example 5: Enzyme activity measurements (from plants)

ALS was extracted from sugar beet leaves or sugar beet tissue cultures as described by Ray (1984), Plant Physiol 75:827-831.

- 10    ALS activity was determined in leaf extracts of wild type and sugar beets and leaf extracts of the obtained SB574TL in presence of various concentrations of foramsulfuron as described in Example 4.

15    In total, the mutant enzyme was at least 2000 times less sensitive against the ALS inhibitor foramsulfuron than the wild type enzyme.

Example 6    Field Trials by employing homozygous ALS inhibitor herbicide tolerant sugar beet plants

20

Based on SB574TL, F4–F6 seeds conferring the mutant allele of the endogenous ALS gene in the homozygous state were applied for further testing

- 25    Plant seeds of the homozygous SB574TL mutant plants and those of the traditional variety KLARINA (commonly available ALS inhibitor sensitive reference sugar beet varieties, not having the respective mutation at position 569 in its ALS protein.) were sown in the field and grew up to various growth stages according to the BBCH standard (as defined in the monographie „Entwicklungsstadien mono- und dikotylar Pflanzen“, 2nd edition, 2001, ed. Uwe Meier, Biologische Bundesanstalt für Land und Forstwirtschaft).

- 30    Afterwards the plants were treated with the respective ALS inhibitor herbicides as specified in Tables 1 below and which identical to those being employed during the

selection procedure.

The water quantity applied in the various applications equaled 200 l/ha.

At 8, 14, and 28 days (as indicated in Table 1) after application (DAA) of the respective ALS inhibitor herbicide(s), the damages (phytotoxicity/phyto) on the

5 different sugar beet plants were scored according to the scale from 0% to 100%.

In this context, "0%" means "no phytotoxicity/phyto" and "100%" means plants were completely killed.

10 Table 1

Variety characteristic		KLARINA	SB574TL based sugar beet	KLARINA	SB574TL based sugar beet	KLARINA	SB574TL based sugar beet
Stage of application		BBCH 14	BBCH 14	BBCH 14	BBCH 14	BBCH 14	BBCH 14
Rating		% phyto	% phyto	% phyto	% phyto	% phyto	% phyto
Application - Assessment interval		8 days	8 days	14 days	14 days	28 days	28 days
Active substance	gai/ha						
Foramsulfuron	25 g/ha	85	0	83	0	86	0
Foramsulfuron	50 g/ha	90	0	92	0	94	0
Iodosulfuron-methyl-sodium	7 g/ha	90	0	97	0	100	0

PCT

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(This sheet is not part of and does not count as a sheet of the international application)

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0-1-1	Prepared Using	<b>PCT Online Filing Version 3.5.000.225 MT/FOP 20020701/0.20.5.20</b>
0-2	<b>International Application No.</b>	<b>PCT/EP2011/067925</b>
0-3	<b>Applicant's or agent's file reference</b>	<b>BCS091021-WO</b>

1	<b>The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:</b>	
1-1	<b>page</b>	<b>6, 25</b>
1-2	<b>line</b>	<b>23-24, 23-24</b>
1-3	<b>Identification of deposit</b>	
1-3-1	Name of depositary institution	<b>NCIMB NCIMB Ltd.</b>
1-3-2	Address of depositary institution	<b>Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB21 9YA, United Kingdom</b>
1-3-3	Date of deposit	<b>12 March 2010 (12.03.2010)</b>
1-3-4	Accession Number	<b>NCIMB 41705</b>
1-5	<b>Designated States for Which Indications are Made</b>	<b>All designations</b>

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## CLAIMS

1. An ALS inhibitor herbicide tolerant Beta vulgaris plant and parts thereof comprising a mutation of an endogenous acetolactate synthase (ALS) gene,  
5 wherein the ALS gene encodes an ALS polypeptide containing an amino acid different from tryptophan at a position 569 of the ALS polypeptide.
2. The Beta vulgaris plant and parts thereof according to claim 1, in which the ALS polypeptide has at position 569 the amino acid alanine, glycine,  
10 isoleucine, leucine, methionine, phenylalanine, proline, valine, or arginine instead of the naturally encoded amino acid tryptophan.
3. The Beta vulgaris plant and parts thereof according to claim 1, wherein the ALS gene encodes an ALS polypeptide containing an amino acid different  
15 from tryptophan at a position 569 of the ALS polypeptide sequence as set forth in SEQ ID NO: 2.
4. The Beta vulgaris plant and parts thereof according to claim 1, in which the amino acid is leucine and in which the endogenous ALS gene is identical to  
20 the nucleotide sequence defined by SEQ ID NO: 3.
5. The Beta vulgaris plant and parts thereof according to claim 1, which are tolerant to one or more ALS-inhibitor herbicides belonging to the group consisting of sulfonylurea herbicides, sulfonylaminocarbonyltriazolinone  
25 herbicides, imidazolinone herbicides, triazolopyrimidine herbicides, and pyrimidinyl(thio)benzoate herbicides.
6. The Beta vulgaris plant and parts thereof according to claim 1, which are homozygous for the mutation of the endogenous acetolactate synthase (ALS)  
30 gene.

7. The parts of the Beta vulgaris plant according to claim 1, wherein the parts are organs, tissues, and cells of the plant, and preferably seeds.

8. Method for the manufacture of the Beta vulgaris plant and the parts thereof of claim 1, comprising the following steps:

- (a) exposing calli from B. vulgaris, to about  $10^{-7}$  M -  $10^{-9}$  M of an ALS inhibitor herbicide, preferably foramsulfuron;
- (b) selecting cell colonies which can grow in the presence of up to  $3 \times 10^{-6}$  M of an ALS inhibitor herbicide, preferably foramsulfuron;
- (c) regenerating shoots in presence of an ALS inhibitor herbicide, preferably foramsulfuron;
- (d) selecting regenerated plantlets with an ALS inhibitor herbicide, preferably foramsulfuron, iodosulfuron-methyl-sodium and/or a mixture of both, wherein the dose of foramsulfuron is preferably equivalent to 7-70 g a.i./ha and the dose of iodosulfuron-methyl-sodium is preferably equivalent to 1-10 g a.i./ha.

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2011/067925

A. CLASSIFICATION OF SUBJECT MATTER  
INV. A01H5/00 C07K14/415 C12N15/82  
ADD.

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 C07K C12N

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 practical, search terms used)

EPO-Internal, BIOSIS, EMBL, FSTA, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHARLES AMTZEN ET AL: "MOLECULAR STRATEGIES FOR CROP IMPROVEMENT", JOURNAL OF CELLULAR BIOCHEMISTRY, WILEY-LISS INC, US, vol. 44, no. Supplement, 16 April 1990 (1990-04-16), pages 257-360, XP007916848, ISSN: 0730-2312 [retrieved on 2004-02-19] cited in the application R249; page 310	1-7
X	DE 198 21 613 A1 (HOECHST SCHERING AGREVO GMBH [DE]) 18 November 1999 (1999-11-18) claims 8,9; figure 2 ----- -/-	1-8



Further documents are listed in the continuation of Box C.



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