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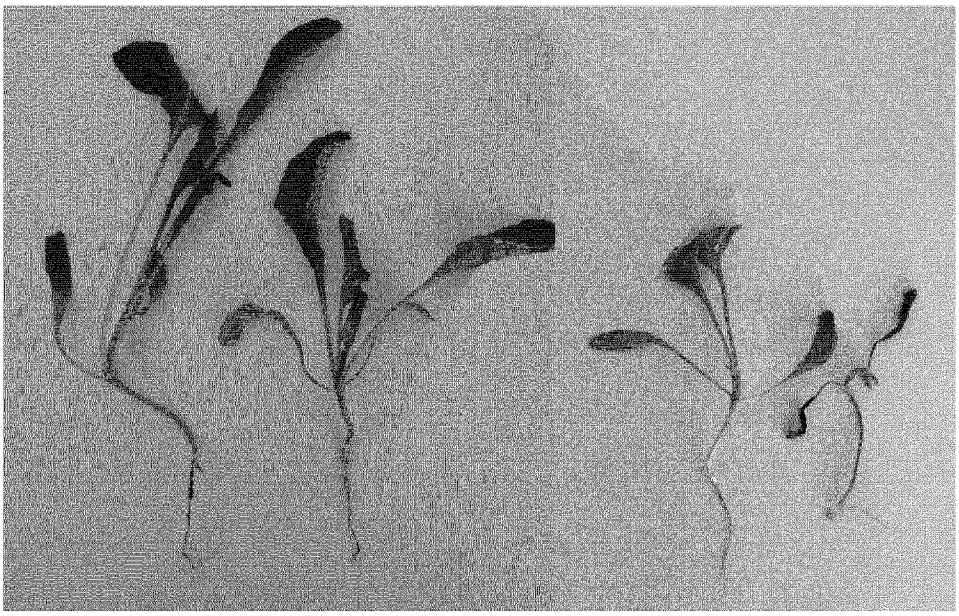


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A method for producing herbicide-resistant sugar beet plant comprising the steps of: -obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant; -applying to the cellsa composition comprising an ALS herbicide at a concentration which is lethal to the said cells; and -regenerating sugar beet plants from the surviving cells.

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*[Continued on next page]*

(54) Title: METHOD TO DEVELOP HERBICIDE-RESISTANT SUGAR BEET PLANTS

Fig. 1


(57) Abstract: A method for producing herbicide-resistant sugar beet plant comprising the steps of: -obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant; -applying to the cellsa composition comprising an ALS herbicide at a concentration which is lethal to the said cells; and -regenerating sugar beet plants from the surviving cells.

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# Method to develop herbicide-resistant sugar beet plants

## Field of the invention

**[0001]** The present invention relates to a method to generate sugar beet plants resistant to herbicides, for instance inhibitor(s) of the acetohydroxyacid synthase enzyme (ALS).

**[0002]** The present invention further relates to the plants that are obtained by this method.

**[0003]** Sugar beet is an important agricultural crop in temperate and subtropical regions.

**[0004]** In modern agriculture, herbicides are widely used to manage weeds proliferation.

**[0005]** The development of sugar beet plants resistant towards herbicide(s) such as ALS inhibitors can be undertaken by using transgenic approaches.

**[0006]** Indeed, the introduction of foreign DNA carrying a gene conferring resistance towards an herbicide has been successfully performed in a variety of field crops, including in sugar beet.

**[0007]** WO 95/10178 discloses a transgenic-induced resistance to the herbicide Bialaphos. The gene encoding the resistance is introduced in protoplasts of guard cells from sugar beet, then these protoplasts are regenerated into sugar beet plants. Those plants are further resistant to the chemical phosphinothricin and its derivative glufosinate. Non transformed plants did not acquire resistance to Bialaphos.

**[0008]** At best, 28 transformed calli were regenerated from 83000 protoplasts; at worst, 1 callus was regenerated from 190 000 protoplasts. Subsequently, some of these calli can show somatic embryogenesis and can regenerate sugar beet plantlets, with an efficiency of 1% and, a characteristic found very advantageous, up to 30%.

**[0009]** This approach, is nevertheless not commonly used in the art, which widely relies on the more direct transformation of calli from explants obtained from sugar beet organs such as embryos and/or from leaf discs.

**[0010]** There still exists an important need to develop herbicide-resistant plants such as in the sugar beet crop and that without relying on DNA vectors and/or on the insertion of foreign genes.

**[0011]** On the other hand, the development of a sugar beet resistant plant towards herbicide(s), such as ALS inhibitors can theoretically be undertaken via classical breeding with a sugar beet plant which would contain a naturally-occurring resistance gene. However, such approach is time consuming and, to the applicants' best knowledge, did not result into success, despite the fact that naturally-occurring ALS inhibitor-resistant plants were documented, at least for species other than sugar beet including a variety of weed species. Especially a double mutant (i.e a plant having two mutations in the same ALS gene) is very unlikely to occur in nature.

**[0012]** The patent application WO 98/02527 discloses a method for the manufacture of sugar beet plant resistant to some ALS inhibitors, such as sulfonylurea herbicides, which comprises the steps of exposing to sulfonylurea calli obtained from explants of *B. vulgaris*, and regenerating plants from the few spontaneous mutants that can grow in the presence of this herbicide.

**[0013]** This method yielded a plant having a mutation in the ALS gene, where the proline at position 188 of the encoded ALS enzyme (corresponding to the 197 position of the *Arabidopsis thaliana* ALS enzyme) is substituted by a serine. However, this mutant is not commercially used because treatments with the preferred modern sulfonylureas ALS herbicides (e.g. foramsulfuron) exhibit some phytotoxicity in field trials at the necessary dose rate.

**[0014]** The patent application WO 2012/049268 relies on the same method, except that calli obtained from explants of *B. vulgaris* are exposed to foramsulfuron, and thus resulted into sugar beet plant resistant to several ALS inhibitors, including to foramsulfuron.

**[0015]** This method yielded a plant having a mutation in the ALS gene, where the tryptophan at position 569 of the encoded ALS enzyme (corresponding to the 574 position of the *Arabidopsis thaliana* ALS enzyme) is substituted by a leucine.

**[0016]** Field trials of this homozygote 569/569 mutant showed good resistance to foramsulfuron, to iodosulfuron (another ALS inhibitor), as well as to mixtures of different ALS inhibitors.

**[0017]** Both these published methods take benefit from the well-established steps of isolation of calli from individual explants such as embryos. However, this is a time-consuming

approach involving (i) isolation of large number of fresh embryos, (ii) their repeated culture on agar-solidified culture medium, and (iii) the selection of the regenerable calli using morphological selection approaches.

**[0018]** Other strategies to transfer genetic traits to sugar beet have been developed and rely on mesophyll protoplasts (which are different of stomatal guard cell protoplasts) as starting material (Krens et al., 1990, Theor. appl. Genet., vol 79, pages 390-396).

Gurel et al. 2008, Critical reviews in Plant Science, 27, 108-140, discloses biotechnology applications for sugar beet. Seven different *in vitro* culture techniques are disclosed. Among them is the culture of protoplasts either from stomatal guard cells for transformation purposes, or from friable callus from etiolated hypocotyl explants, the latter being much more efficient. This approach using protoplasts is however associated to major difficulties.

Hall et al. 1997, J. Exp. Botany, 48, 255-263 have used a culture of 500000 stomatal guard cell protoplasts for carrying a transformation experiment with foreign DNA. The transformation efficiency was higher than 2%. On the other hand, *in vitro* culture of plants is reported to be associated to stomatal failure, pointing to problems in the corresponding cells and thus to the production of very high amount of stomatal guard cell protoplasts, such as amounts needed for carrying a method involving a mutational event.

Summary of the invention

**[0019]** In a broad aspect, the present invention discloses a method for producing a mutant sugar beet plant being resistant to an herbicide comprising the steps of:

- obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant;

- applying to an in vitro culture of these protoplasts a composition comprising this herbicide at a concentration that is lethal to more than 99% of the in vitro cultured cells; and

- regenerating sugar beet plants from the surviving cells of these in vitro cultured cells,

- possibly selecting regenerated sugar beet plants having a mutation in the gene encoding the peptide(s) targeted by this herbicide, wherein these stomatal guard cells protoplasts are pre selected for their capacity to regenerate into a sugar beet plant, and wherein this herbicide is applied to more than 20 000 000 of these protoplasts.

**[0019a]** In another broad aspect, the present invention discloses a method for producing a mutant sugar beet plant being resistant to one or more inhibitor(s) of the acetohydroxyacid synthase (ALS) enzyme comprising the steps of:

- obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant;

- applying to an *in vitro* culture of the said protoplasts a composition comprising one or more ALS inhibitor(s) at a concentration that is lethal to more than 99% of the *in vitro* cultured cells; and
- regenerating sugar beet plants from the surviving cells of the said *in vitro* cultured cells,

wherein the said stomatal guard cell protoplasts are pre selected for their capacity to regenerate into a sugar beet plant, wherein the said pre selected protoplasts have a probability of more than 10% (number of growing protoplasts:total protoplast number put into culture) to divide and regenerate into a viable callus, wherein calli obtained by the said protoplasts have more than 10% (number of calli producing shoots:total calli number) of capacity to develop shoots and wherein the said ALS inhibitor(s) is/are applied to more than 20 000 000 of the said protoplasts.

**[0019b]** In yet another broad aspect, the present invention relates to a method for producing a mutant sugar beet plant being resistant to an herbicide comprising the steps of:

- obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant;
- applying to an in vitro culture of the said protoplasts a composition comprising the said herbicide at a concentration that is lethal to more than 99% of the in vitro cultured cells;
- regenerating sugar beet plants from the surviving cells of the said in vitro cultured cells; and
- selecting regenerated sugar beet plants having a mutation in the gene encoding the peptide targeted by the said herbicide,

wherein the said stomatal guard cell protoplasts are pre selected for their capacity to regenerate into a sugar beet plant, wherein the said pre selected protoplasts have a probability of more than 10% (number of growing protoplasts:total protoplast number put into culture) to divide and regenerate into a viable callus, wherein calli obtained by the said protoplasts have more than 10% (number of calli producing shoots: total calli number) of capacity to develop shoots and wherein the said herbicide is applied to more than 20 000 000 of the said protoplasts.

**[0019c]** In yet another broad aspect, the present invention relates to a mutated sugar beet plant cell comprising SEQ ID NO:3 on one allele of the acetohydroxyacid synthase (ALS) gene and SEQ ID NO:5 on the second allele of the ALS gene.

**[0020]** The herbicide used in the present method can be an herbicide not targeting ALS gene.

**[0021]** The preferred herbicides (not targeting ALS) are selected from the group consisting of:

- 4-HPPD inhibitors (such as mesotrione, isoxaflutole, pyrasulfotole, benzobicyclon, benzofenap, pyrazolynate, pyrazoxyfen, tembotrione, topramezone, sulcotrione and sulcotrion),
- inhibitors of the carotenoid biosynthesis (such as flurtamone, fluridone, flurochloridone, beflubutamid, norflurazon, picolinafen and diflufenican);
- inhibitors of EPSP synthase (such as glyphosate or glyphosate-trimesium);
- inhibitors of phosphosystem II (such as Phenyl-carbamates (for instance phenmedipham or desmedipham), Pyridazinones (for instance chloridazon = pyrazon), Triazines (for instance cyanazine, remtal, eglinazine-ethyl, proglinazine-ethyl, ametryn, atrazine, desmetryn, dimethametryn, prometon, prometryn, propazine, simazine, simetryn, terbumeton, terbuthylazine, terbutryn, methoprotyn), Triazinones (for instance metamitron, metribuzin, hexazinone, metribuzin), Uracils (bromacil, lenacil, terbacil), Ureas (dimefuron, isoproturon, linuron, monolinuron, ethidimuron, methabenzthiazuron, tebuthiuron, diuron, fenuron, neburon, siduron, isouron, chlorobromuron, chlorotoluron, chloroxuron, fluometuron, metobromuron metoxuron, thiazafluron, monuron, cycluron, monolinuron), or amicarbazone, solan, propanil, bentazon, bromoxynil, ioxynil, bromofenoxim, pyridate, pyridafol;
- inhibitors of phosphosystem I (such as diquat or paraqua);
- inhibitors of cell division (such as carbetamide, chlorpropham, propham, naproanilide, diphenamid, napropamide, butenachlor, metazachlor, diethatyl-ethyl, acetochlor, alachlor, butachlor, propachlor Monsanto, propisochlor, dimethachlor, dimethenamid, metolachlor, pretilachlor, S-metolachlor, pethoxamid, thenylchlor, anilofos, cafenstrole, indanofan, bromobutide, piperophos, flufenacet, mefenacet, fentrazamide);
- inhibitors of microtubule assembly (such as propyzamide = pronamide, tebutam, chlorthal-dimethyl = DCPA, fluchloralin, pendimethalin, butralin, benefin=benfluralin, ethalfluralin, oryzalin, trifluralin, prodiamine, dinitramine, butamifos, dithiopyr, thiazopyr);
- inhibitors of protoporphyrinogen oxidase (such as Diphenylethers (acifluorfen-sodium, bifenox, ethoxyfen-ethyl, chlornitrofen, fluoroglycofen-ethyl, oxyfluorfen, chlomethoxyfen, fluordifen, fomesafen, lactofen, nitrofen, aclonifen), N-phenylphthalimides (cinidon-ethyl, flumiclorac-pentyl, flumioxazin, flumiclorac-pentyl), Oxadiazoles (oxadiargyl, oxadiazon) Oxazolidinediones, (pentoxazone), Phenylpyrazoles (fluazolate, fluazolate, pyraflufen-ethyl), Pyrimidindiones (saflufenacil, benzfendizone, butafenacil), Thiadiazoles (thidiazimin, fluthiacet-methyl),

Triazolinones (azafenidin, carfentrazone-ethyl sulfentrazone), pyraclonil, profluazol, flufenpyr-ethyl);

- inhibitors of Acetyl CoA carboxylase (such as Aryloxyphenoxypropionates (such as clodinafop-propargyl, cyhalofop-butyl, diclofop-methyl, fenoxaprop-P-ethyl, fluazifop-P-butyl, haloxyfop-etotyl, haloxyfop-methyl, haloxyfop-P-methyl, propaquizafop, quizalofop-P-ethyl or quizalofop-P-tefuryl), Cyclohexanediones (such as alloxydim, butroxydim, clethodim, cycloxydim, profoxydim, sethoxydim, tepraloxydim or tralkoxydim) or Phenylpyrazoline (pinoxaden));

- cell wall synthesis inhibitors (such as indaziflam, isoxaben, chlorthiamid, dichlobenil, quinclorac or flupoxam);

- inhibitor of glutamine synth(et)ase (glufosinate-ammonium or bialaphos=bilanaphos) and

- synthetic auxin (such as TBA, dicamba, chloramben, benazolin-ethyl 4, dichlorprop-P, mecoprop-P, 2,4,5-T (Weedar) 2,4-D (Weedar), 2,4-DB (Butyrol), dichlorprop, MCPB, mecoprop, MCPA-thioethyl, clomeprop, Agroxone 4, aminopyralid, clopyralid, fluroxypyr, halauxifen-methyl, picloram, triclopyr, quinclorac or quinmerac),

more preferably from the group consisting of 4-HPPD inhibitors, inhibitors of the carotenoid biosynthesis, inhibitors of EPSP synthase, inhibitors of phosphosystem II, inhibitors of phosphosystem I, inhibitors of microtubule assembly, inhibitors of protoporphyrinogen oxidase and synthetic auxin.

**[0022]** Another highly preferred herbicide is an ALS inhibitor.

**[0023]** Therefore, the present invention relates to a method for producing a mutant sugar beet plant being resistant to one or more inhibitor(s) of the acetohydroxyacid synthase enzyme (ALS) comprising the steps of:

- obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant;

- applying to an *in vitro* culture of the said protoplasts a composition comprising one or more ALS inhibitor(s) at a concentration that is lethal to (more than 99% (preferably more than 99.9% or even more than 99.99%) of) the *in vitro* cultured cells (yet allowing some mutants to escape); and

- regenerating sugar beet plants from the surviving cells of the said *in vitro* cultured cells,

wherein the said stomatal guard cells protoplasts are pre selected for their capacity to regenerate into a sugar beet plant and/or wherein the said ALS inhibitor(s) is/are applied to more than 2 000 000 (preferably more than 10 000 000, more preferably to more than 20 000 000 or even more than 50 000 000) of the said protoplasts.

**[0024]** Preferably this method comprises the sub-steps of isolating stomatal guard cells protoplasts from sugar beet plants of different genotypes and measuring for each genotype the proportion of the said protoplasts that is growing when the said protoplasts are put in culture.

**[0025]** Preferably this method further comprises the step of sequencing the genome of the regenerated plants from the surviving *in vitro* cultured cells, advantageously for identifying a mutation in the ALS gene and/or for selecting regenerated sugar beet plants having one or more, preferably one, two, or more mutations in the ALS gene.

**[0026]** Advantageously, sugar beet having one or several mutation(s) in the ALS gene at positions encoding the amino acids selected from the group consisting of Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372, Methionine 565, Valine 566, Phenylalanine 573, Serine 648 and Glycine 649, preferably selected from the group consisting of Alanine 196, Aspartate 371, Arginine 372, Serine 648 and Glycine 649, are obtained and/or selected by the method of the present invention.

Another preferred sugar beet plant having a mutation of the Proline at position 188 and of the Tryptophan at position 569 is obtained and/or selected by the method of the present invention.

**[0027]** Preferably, sugar beet having two or more mutations has two mutations in one allele, meaning that the encoded peptide harbours two mutations synergizing the resistance towards ALS inhibitors (especially towards compositions comprising several ALS inhibitors). An examples of most preferred sugar beet is a sugar beet having a mutation of the Proline at position 188 and of the Tryptophan at position 569 in one allele (and possibly the same mutations in the second allele; alternatively, the second allele harbours different mutations).

**[0028]** Alternatively, sugar beet having two mutations has one mutations on each allele.

**[0029]** The method of the present invention allows to regenerate a sugar beet plant having one mutation in the ALS gene at positions encoding Proline 188 and one or more mutation(s) in the ALS gene at positions encoding Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372, Methionine 565, Valine 566, Tryptophan 569 (preferably mutated into Glycine), Phenylalanine 573, Serine 648 and Glycine 649. An examples of preferred sugar beet is a sugar beet having a mutation of the Proline at position 188 and of another mutation (possibly not the tryptophan 569 or the Trp569Gly) in the same allele.

**[0030]** The method of the present invention allows to regenerate a sugar beet plant having one mutation in the ALS gene at position encoding Tryptophan 569 (preferably mutated into Leucine) and one or more mutation(s) in the ALS gene at positions encoding Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Proline 188 (preferably mutated into Threonine, Arginine, Leucine, Glutamine or Alanine), Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372, Methionine 565, Valine 566, Phenylalanine 573, Serine 648 and Glycine 649.

An examples of preferred sugar beet is a sugar beet having a mutation of the tryptophan at position 569 and of another mutation (possibly not the Proline 188 or the Pro188Ser) in the same allele.

**[0031]** The method of the present invention allows to regenerate a sugar beet plant having one or more mutation(s) in the ALS gene, wherein the said one or more mutation is selected from the group consisting of Alanine 113 Proline 188, Alanine 196, Aspartate 371, Arginine 372, Tryptophan 569, Serine 648 and Glycine 649, wherein the said Alanine 113 is mutated into Valine or Threonine, wherein the said Proline 188 is mutated into Threonine, Arginine, Leucine, Glutamine or Alanine, wherein the said Alanine 196 is mutated into Valine, wherein the said Aspartate 371 is mutated into Glutamate, wherein the said Arginine 372 is mutated into Histidine, wherein the said Tryptophan 569 is mutated into Glycine, wherein the said Serine 648 is mutated into Threonine and wherein the said and Glycine 649 is mutated into Aspartate.

**[0032]** Preferably, sugar beet having two or more of these specific mutations has two mutations in one allele, meaning that the encoded peptide harbours two mutations synergizing the resistance towards ALS inhibitors (especially towards compositions comprising several ALS inhibitors).

**[0033]** The method of the present invention allows to regenerate a sugar beet plant having one mutation in the ALS gene at position encoding Proline 188 and one mutation in the ALS gene at position encoding Tryptophan 569.

**[0034]** Advantageously, this method comprises a preliminary step of deducing the concentration of the one or more ALS inhibitor(s) at which the composition (comprising the one or more ALS inhibitor(s)) is lethal for at least 99% (preferably at least 99.9% or even 99.99%) of the *in vitro* cultured cells (yet allowing some mutants to escape).

**[0035]** The preferred ALS inhibitor present in the composition (the said composition comprising in addition another ALS inhibitor, being preferably from another class than sulfonylurea ALS inhibitor, such as thiencarbazone-methyl) to be added to the *in vitro* cultured stomatal guard

cell protoplasts is foramsulfuron, preferably at a concentration of $10^{-9}$ mol/l to (more preferably) $10^{-6}$ mol/l.

**[0036]** Another suitable ALS inhibitor present in the composition to be added to the *in vitro* cultured stomatal guard cell protoplasts is ethoxysulfuron (possibly this composition further comprises other ALS inhibitors).

**[0037]** A related aspect of the present invention is a mutated sugar beet plant obtainable by the method of the present invention, such as a sugar beet having a mutation at tryptophan 569 and/or at proline 188.

**[0038]** Another related aspect of the present invention is a mutated sugar beet plant comprising SEQ.ID.NO:3 (or SEQ.ID.NO:4) and/or SEQ.ID.NO:5 (or SEQ.ID.NO:6).

**[0039]** A preferred sugar beet plant according to the present invention correspond to the deposit under NCIMB 42050 or NCIMB 42051.

**[0040]** The present invention is also related to tissue or plant part (for instance stomatal guard cells or leaf strips) or seeds derived from the mutated plant of the invention, as well as to their use for the introduction of another genetic trait.

**[0041]** The present invention is also related to the use of the (well regenerating) stomatal guard cell protoplasts developed in the present invention for the introduction of another genetic trait than resistance to ALS inhibitors.

## Detailed description of the invention

**[0042]** The inventors have discovered that protoplasts from stomatal guard cell of sugar beet represent a good starting material for inducing resistance towards herbicides such as ALS inhibitors, despite the fact that regeneration of a sugar beet plant from a stomatal guard cell protoplast is very difficult, of low occurrence, and requires longer and more complex procedures than the direct regeneration from calli obtained from explants, as usually used in the art.

**[0043]** Indeed, callus (calli) from explants is (are) a mass of non-differentiated (or dedifferentiated) cells, which, under appropriate culture conditions, will differentiate (or re-differentiate) and regenerate a wholly functional sugar beet plant. In such method, seeds are collected in large amounts, then easily sterilized and explants are thus obtained in large amounts. Such method is convenient as allowing to perform a large part of the work without the difficulties of a sterile environment.

On the other hand, the stomatal guard cells have a well-defined organization in the plant, and their isolation from the plant tissue results into individual cell(s), then after a treatment into individual protoplasts.

In the method of the present invention, plantlets must be grown *in vitro* and maintained under sterile conditions, then stomatal guard cells are isolated from these small plantlets and protoplasts are obtained while maintaining sterile conditions, then these individual protoplasts are induced to produce again their cell wall, to grow firstly into micro-calli, then into a sugar beet plant.

Stomatal guard cells protoplasts of sugar beet were found to be very vulnerable, reducing the scope of their application: for instance addition of a mutagen, instead of favouring the appearance of a desired genetic trait, was found to be lethal to the protoplasts in many cases.

In other words, it was completely unexpected to succeed in using stomatal guard cell protoplasts as a starting material for developing a sugar beet plant having acquired a desired genetic trait through mutation.

**[0044]** Furthermore, the inventors have found that the capacity of stomatal guard cell protoplasts to divide is very dependent on sugar beet genotypes: the stomatal guard cell protoplasts from the majority of the genotypes exhibit a very low (almost zero) capacity to divide (grow), while the protoplasts from some specific genotypes have a sizable capacity to grow *in vitro*.

**[0045]** The inventors have identified that the culture on solid medium (polymer-containing medium, such as alginate or agarose-like containing medium) of very large amounts of these stomatal guard cell protoplasts, then the exposure of the cultured material (normally in the form of regenerated calli) to herbicides such as ALS inhibitor(s) resulted into the selection of some mutated cells being resistant to this herbicide molecule, despite the fact that, in absence of added mutagens, spontaneous mutations are known to be very rare, and that the method cannot take benefit of the genetic diversity within a population, in selecting an already existing mutant plant exhibiting the desired resistance towards one or more ALS inhibitors.

**[0046]** Some of these mutated cells having acquired resistance towards herbicides such as ALS inhibition were further able to regenerate into viable sugar beet plant(s). Although protoplasts are very difficult to use in practice, the present method nevertheless yielded very rapidly stable mutants having acquired resistance towards the herbicide.

**[0047]** The method of the present invention allows the production of herbicide-resisitant (ALS inhibitor-resistant) sugar beet plants having one or several mutations, including one or several mutations in the enzyme being the target of the herbicide, for instance an ALS inhibitor (such as a mutation of the ALS gene, wherein the ALS gene encodes an ALS protein containing

an amino acid different from tryptophan at position 569 of the ALS protein; corresponding to position 574 of the Arabidopsis thaliana ALS ), possibly in addition to at least another mutation in this gene and/or to other mutations in other genes.

**[0048]** An aspect of the present invention is therefore a method for producing sugar beet plant (*Beta vulgaris*; thus also *Beta vulgaris maritima* ssp) resistant to one or more ALS inhibitor(s) comprising the steps of:

- obtaining (protoplasts from) stomatal guard cells isolated from a sugar beet plant (sensitive to ALS inhibitors);
- applying to these *in vitro* cultured cells a composition comprising one or more ALS inhibitors at a concentration that is lethal to these *in vitro* cultured cells (at least 99% of the (wild-type) cells are killed by the ALS inhibitor, but some mutants can escape the treatment); and

regenerating sugar beet plants from the surviving cells.

**[0049]** This method can be seen as an alternative to the introduction of a transgene (for instance disclosed in WO 95/10178). However, a method based on mutation of endogenous gene(s) is more laborious by comparison to a transgenic approach. Indeed, the introduction of a transgenic (mutated) gene is much more rapid, flexible and predictable.

**[0050]** The absence of transgene-induced resistance refers to the fact that the acquired resistance to the ALS inhibitor(s) is not directly caused by the *in vitro* insertion of a (foreign) DNA into the stomatal guard cells protoplasts (or into the cells obtained thereof), such as a (foreign) DNA encoding a protein directly providing resistance to the ALS inhibitor(s), such as a protein locally reducing the toxicity of the ALS inhibitor(s) (e.g. an enzyme degrading the ALS inhibitor(s) or reducing its intracellular concentration), or an ALS protein being resistant to ALS inhibitor(s), such as an ALS mutant enzyme that retains significant activity and functionality even in the presence of the inhibitor(s).

**[0051]** However, the plant obtained by the method of the invention can further be crossed with a transgenic plant in order to stack another genetic trait in the progeny. The plant (or a part thereof) obtained by the present invention can further be used in a subsequent transgenic method to introduce another genetic trait (different from the genetic trait obtained in the method of the present invention).

**[0052]** Possibly, this method further comprises the step of adding a mutagen agent to the culture of the isolated stomatal guard cell protoplasts.

Suitable mutagen agents are physical (such as UV or X-ray) exposure or exposure to chemical agents (such as Ethyl Methane Sulfonate (EMS), for instance at 0.05%, 0.1%, 0.15%, 0.2% or even at 2.5%). However, protoplasts viability was shown to be detrimentally affected by some mutagenesis treatments usually performed, such as treatment of more than 0.2% EMS.

**[0053]** Alternatively, this method thus does not comprises the step of adding a mutagen agent.

**[0054]** In the context of the present invention, an herbicide preferably refers to any molecule, which, when applied at a given dose, is used for weed control.

**[0055]** Preferred herbicides used in the present method (for developing sugar beet plants resistant to this herbicide) have a specific known activity on one peptide (so that a single mutation in the corresponding gene can confer resistance towards this herbicide). In other words, the preferred herbicides are specific for one peptide target (usually inhibiting specifically the activity of one plant enzyme) and/or their target peptide is known (so as to be in a position to screen the resistant sugar beet for a mutation in the target gene)

**[0056]** In the context of the present invention, an ALS inhibitor refers to any molecule inhibiting the function of the ALS gene.

**[0057]** Preferably, the ALS inhibitor(s) used in the present invention (substantially) do not inhibit other (sugar beet) enzymes than ALS.

**[0058]** Advantageously, the ALS inhibitor(s) are selected and used in the present invention at a concentration where more than 90% (more than 95%, more than 99%, more than 99.9%) of the function of the wild-type ALS enzyme is inhibited, but substantially not affecting the function of non-related enzymes.

**[0059]** Suitable ALS inhibitors (for carrying the method) are selected from the group consisting of sulfonylurea herbicides, sulfonylaminocarbonyltriazolinone herbicides, imidazolinone herbicides, triazolopyrimidine herbicides and pyrimidinyl(thio)benzoate herbicides. Advantageously, the herbicide composition comprises at least one sulfonylurea herbicides and at least one triazolopyrimidine.

The preferred ALS inhibitors (for carrying the method) are foramsulfuron, amidosulfuron, thiencarbazone-methyl, ethoxysulfuron and mixture thereof (especially a composition comprising thiencarbazone-methyl and either foramsulfuron or amidosulfuron); however, the regenerated sugar beet mutant is advantageously resistant towards several ALS inhibitors.

**[0060]** Other ALS inhibitors (including mixtures of ALS inhibitors) can be used, and the skilled person knows which mutation provides strong resistance to a given ALS inhibitor (e.g. the mutation of the tryptophan at position 569 is known to be associated to foramsulfuron resistance); hence, given the flexibility and the efficacy of the present method, the skilled person can design sugar beet plants having acquired several mutations and thus broader resistance towards herbicides such as ALS inhibitors (e.g. mixtures of ALS inhibitors).

**[0061]** More preferably, this method comprises a preliminary step of selecting a sugar beet plant genotype (line) for its capacity of the stomatal guard cells protoplasts to regenerate into a fully functional sugar beet plant, and/or the method of the present invention is carried on stomatal guard cells protoplasts isolated from well-regenerating sugar beet genotypes (lines).

**[0062]** A suitable preliminary step (of selecting sugar beet plant genotypes for the ability of their stomatal guard cells protoplasts cells to regenerate into a sugar beet plant) involves the comparison (independently of their possible advantageous features such as yield or resistance towards parasitic infections) of at least 10 different sugar beet plant genotypes (from different genotype backgrounds), preferably at least 15 different genotypes, or even at least 30 different genotypes, for the capacity of their stomatal guard cells protoplasts to regenerate into a sugar beet plant (much more preferably their capacity to grow *in vitro* and/or to form calli), and the selection of a well-regenerating genotype (line) for carrying the method of the present invention.

**[0063]** In the context of the present invention, well-regenerating stomatal guard cells protoplasts refer to protoplasts having a probability of more than 0.25% (number of growing protoplasts : total protoplast number put into culture; growing:total), preferably more than 1% (growing:total), more preferably more than 5% (growing:total), still more preferably more than 10% (growing:total) or even more than 20% (growing:total) or 50% (growing:total) to divide and/or to grow and/or regenerate into viable sugar beet callus (when grown in the suitable culture media and without exogenous selection pressure such as the toxic molecule/herbicide to be applied in the method of the present invention).

**[0064]** Callus (calli) refers to a mass of undifferentiated cells. In the art, calli can be obtained from explants such as embryos, or parenchyme-derived explants from leaves or cotyledon. However, in the context of the present invention, calli are the result of the growth of (well-regenerating) stomatal guard cells protoplasts.

Advantageously, the calli obtained by these well-regenerating protoplasts have more than 10% (number of calli producing shoots : total calli number; shoots:total), preferably more than 20% (shoots:total) or even more than 30% (shoots:total) of capacity to develop shoots.

**[0065]** Preferably, well-regenerating sugar beet stomatal guard cells protoplasts refer to protoplasts having more than 0.1% (sugar beet plant : total protoplast number) (more preferably more than 1%) of capacity to regenerate into a viable sugar beet plant.

**[0066]** Also preferably, in this method, the composition comprising the herbicide (e.g. one or more ALS inhibitor(s)) is applied to an *in vitro* culture of more than 2 000 000 of these (well-regenerating) sugar beet stomatal guard cells protoplasts.

**[0067]** Alternatively, or more preferably in addition to the pre-selection step, the composition comprising the herbicide (e.g. one or more ALS inhibitor(s)) is applied to an *in vitro* culture of more than 5 000 000, or even more than 10 000 000, 20 000 000, or 50 000 000, of these (well-regenerating) sugar beet stomatal guard cells protoplasts.

**[0068]** Preferably, at least 50 000, such as about 100 000, (well-regenerating) stomatal guard cells protoplasts per millilitre were grown onto polymer-containing medium (such as alginate- or agarose-containing medium).

**[0069]** Possibly, these, (well-regenerating) sugar beet stomatal guard cells protoplasts are grown for at least about one week (preferably about 3 weeks and/or of less than 4 weeks) on polymer (alginate)-containing medium, before the application of the composition comprising the herbicide (e.g. one or more) ALS inhibitor(s) such as foramsulfuron and possibly thiencarbazone-methyl).

**[0070]** Preferably, this method further comprises the step of comparing the growth of mutated stomatal guard cell and the growth of wild-type stomatal guard cell (and/or naive and/or not yet herbicide-treated) on a medium that does not comprise ALS inhibitor, and possibly of selecting mutant(s) keeping at least 75% of the growth, preferably at least 90% of the growth of the corresponding wild-type cell.

**[0071]** Preferably, or in addition this method further comprises the step of comparing the growth and/or the yield of the regenerated sugar beet from the mutated cell and the growth and/or the yield of the wild-type (and/or naive and/or not yet treated by the ALS inhibitor) sugar beet in greenhouse assays and in agronomic conditions without any ALS inhibitor, and possibly of selecting ALS inhibitor-resistant mutant(s) sugar beet plants keeping at least 75% of the growth and/or of the yield, preferably at least 90% of the growth and/or of the yield of the wild-type sugar beet.

**[0072]** Preferably, this method further comprises the step of sequencing the regenerated plants from the surviving protoplasts and/or of identifying one or several mutation(s) that are (can be) associated to the resistance to the herbicide (e.g. one or more ALS inhibitor(s)).

**[0073]** In the context of the present invention, the term 'mutation' preferably refers to one (one single) change in the nucleotide sequence encoding the peptide targeted by the herbicide (e.g. the ALS protein) that causes one change in the corresponding amino acid, such that the resulting plant has acquired some resistance towards the herbicide such as ALS inhibitors. In other words, in the context of the present invention, 'mutation' is preferably understood as equivalent to a 'point mutation' that allows some resistance to the herbicide (the ALS inhibitor). Accordingly, "several mutations" preferably refers, in the present invention, to (a stack of) multiple point mutations, each point mutation causing a change of the encoded amino acid so as to provide some resistance to an herbicide (e.g. an ALS inhibitor and/or an herbicide not an ALS inhibitor). Therefore, preferably, in the context of the present invention 'mutation' does not comprise change in the nucleotide sequence that do not modify the encoded protein (such as a change in the third amino acid of a coding triplet), change in amino acids that are not associated to herbicide (such as ALS inhibitor) resistance, nor multiple simultaneous changes in the nucleotide sequence.

**[0074]** Advantageously, this identification step of the mutation(s) associated to the resistance towards the (one or more) ALS inhibitor(s) (preferably one or two mutation(s) in the ALS gene) is coupled to the development of oligonucleotide primers spanning over this mutation.

**[0075]** Advantageously, this identification step of the mutation(s) in the ALS gene is followed by (*in vitro*) enzymatic activity measurements of the protein encoded by the wild-type and by the mutated ALS genes.

**[0076]** Preferably, these enzymatic measurements of the wild-type ALS enzyme and of the mutated ALS enzyme are performed in the presence of one or more ALS inhibitors (at one or at several concentrations in order to derive an inhibition curve).

**[0077]** Possibly, these enzymatic measurement of the wild-type enzyme and of the mutated enzyme are (further) performed in absence of ALS inhibitors (to compare the enzymatic activity of the mutated enzyme; preferably, in absence of the ALS inhibitor, the mutated enzyme keeps at least 50% of the activity of the wild-type enzyme, more preferably at least 75%, still more preferably at least 90%, at least 95% or even at least 99%).

**[0078]** Preferably, the method of the present invention comprises a step of comparing compositions comprising one or more ALS inhibitors at different concentrations, and deducing the concentration at which the ALS inhibitor, and/or ALS inhibitor in a special formulation

within this composition, is /are lethal for an *in vitro* culture of the stomatal guard cell protoplast isolated from the sugar beet plant (such as stomatal guard cell protoplasts grown on alginate for at least one week).

**[0079]** For instance, this step of deducing the concentration at which the (one or more) ALS inhibitor(s) is/are lethal for stomatal guard cell protoplast isolated from the sugar beet plant is performed on an *in vitro* culture of the stomatal guard cell protoplasts isolated from the wild-type sugar beet plant (and/or naive and/or not yet treated by the ALS inhibitor).

**[0080]** In the context of the present invention, the lethal concentration of the composition comprising (one or more) ALS inhibititor(s) refers to a concentration sufficient to kill at least 99%, preferably at least 99.9%, more preferably at least 99.99% of the cultured cells (yet allowing some mutants to escape this treatment).

**[0081]** Alternatively, or in addition, this step of deducing the concentration at which the (one or more) ALS inhibitor(s) is lethal for an *in vitro* culture of the stomatal guard cell protoplasts isolated from the sugar beet plant is (further) performed on an *in vitro* culture of mutated stomatal guard cells (on cells having acquired a mutation in the ALS gene and being resistant to ALS inhibitor(s)).

**[0082]** The comparison of the lethal concentration of the ALS inhibitor (in a composition comprising this ALS inhibitor) on the naive and on the mutated cells is advantageously expressed as a ratio (or as several ratios, one ratio per ALS inhibitor tested).

**[0083]** Preferably, for one ALS inhibitor, the lethal concentration on naive cell(s) is 50-fold lower than the lethal concentration on mutated cell(s), more preferably, the lethal concentration on naive cell(s) is 200-fold lower than the lethal concentration on mutated cell(s), still more preferably, the lethal concentration on naive cell(s) is 1000-fold lower than the lethal concentration on mutated cell(s).

**[0084]** The herbicide (used in the method of the present invention) can be a mixture (of inhibitors) comprising at least one ALS inhibitor, such as foramsulfuron.

**[0085]** Possibly, the ALS inhibitor used in the method of the invention is a mixture of ALS inhibitors, such as a sulfonylurea (e.g. foramsulfuron) and another ALS inhibitor selected from the group consisting of iodosulfuron, amidosulfuron and thiencarbazone-methyl.

**[0086]** Preferably, the ALS inhibitor used in the method of the invention is (or comprises) foramsulfuron, such as foramsulfuron applied to a one-week old (or to a three-weeks old) *in vitro* culture of protoplasts (more particularly to the *in vitro* culture comprising calli

regenerated from these cultured protoplasts) on alginate-containing medium and maintained during the *in vitro* culture of the cells at a concentration of $10^{-9}$-$10^{-6}$ mol/l (or $10^{-9}$-$10^{-6}$ mol/l).

**[0087]** A related aspect of the present invention is a mutated sugar beet plant obtainable by this method, (for instance when this methods comprises the use of one or more herbicide (not ALS inhibitors) or the use of one or more ALS inhibitors, or comprises the use of one ALS inhibitor and of one herbicide being not an ALS inhibitor).

**[0088]** Therefore, one aspect of the present invention is a sugar beet (obtainable by the method of the present invention) having one or several mutation(s) in the ALS gene at positions encoding the amino acids selected from the group consisting of Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372, Methionine 565, Valine 566, Phenylalanine 573, Serine 648 and Glycine 649.

**[0089]** A preferred sugar beet (obtainable by the method of the present invention) has one or several mutation(s) in the ALS gene at positions encoding the amino acids selected from the group consisting of Alanine 113 (e.g. mutated into Valine or Threonine) Proline 188 mutated into Threonine, Arginine, Leucine, Glutamine or Alanine, Alanine 196 (e.g. mutated into Valine), Aspartate 371 (e.g. mutated into glutamate), Arginine 372 (e.g. mutated into Histidine), Tryptophan 569 mutated into Glycine, Serine 648 (e.g. mutated into Threonine) and Glycine 649 (e.g. mutated into Aspartate).

**[0090]** A related aspect of the present invention is a mutated sugar beet plant (or a mutated sugar beet plant cell such as a mutated stomatal guard cell isolated from sugar beet) comprising a mutation in the ALS gene where the tryptophan at position 569 in the encoded ALS enzyme (corresponding to position 574 in the Arabidopsis thaliana ALS enzyme) is substituted by another amino acid (such as a leucine), and possibly another (one or several) mutation, preferably another (one or several) mutation in the ALS gene, such as a mutation causing a further amino acid substitution in the ALS gene.

**[0091]** Another preferred a sugar beet plant has a mutation of the Tryptophan into Leucine at position 569 and one or several mutation(s) in the ALS gene at positions encoding the amino acids selected from the group consisting of Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372, Methionine 565, Valine 566, Phenylalanine 573, Serine 648 and Glycine 649.

**[0092]** A preferred sugar beet (obtainable by the method of the present invention) has one mutation Tryptophan into Leucine mutation at position 569 and one or several mutation(s) in the ALS gene at positions encoding the amino acids selected from the group consisting of Alanine 113 (e.g. mutated into Valine or Threonine) Proline 188 mutated into Threonine, Arginine, Leucine, Glutamine or Alanine, Alanine 196 (e.g. mutated into Valine), Aspartate 371 (e.g. mutated into Glutamate), Arginine 372 (e.g. mutated into Histidine), Serine 648 (e.g. mutated into Threonine) and Glycine 649 (e.g. mutated into Aspartate).

**[0093]** This mutated sugar beet plant is resistant to one or several ALS inhibitor(s) used, such as a sulfonylurea (e.g. foramsulfuron) and advantageously to other ALS inhibitor(s), preferably selected from the group consisting of iodosulfuron, amidosulfuron and thiencarbazone-methyl.

**[0094]** A related aspect of the present invention is a mutated sugar beet plant (or a mutated sugar beet plant cell such as a mutated stomatal guard cell isolated from sugar beet) comprising a mutation in the ALS gene where the proline at position 188 in the encoded ALS enzyme (corresponding to position 197 in *Arabidopsis thaliana* ALS enzyme) is substituted by another amino acid (such as a serine).

**[0095]** Alternatively, a preferred sugar beet plant (obtainable by the method of the present invention) has a mutation of the Proline into Serine at position 188 and one or several mutation(s) in the ALS gene at positions encoding the amino acids selected from the group consisting of Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372, Methionine 565, Valine 566, Phenylalanine 573, Serine 648 and Glycine 649.

**[0096]** A preferred sugar beet (obtainable by the method of the present invention) has one Proline into Serine mutation in the ALS gene at position 188 and one or more mutation(s) of the ALS gene at positions encoding Alanine 113 (e.g. mutated into Valine or Threonine), Aspartate 371 (e.g. mutated into glutamate), Arginine 372 (e.g. mutated into Histidine), Tryptophan 569 mutated into Glycine, Serine 648 (e.g. mutated into Threonine) and Glycine 649 (e.g. mutated into Aspartate).

**[0097]** Another related aspect of the present invention is a mutated sugar beet plant (or a mutated sugar beet plant cell such as a mutated stomatal guard cell isolated from sugar beet) comprising a mutation of tryptophan at position 569 in the ALS enzyme and a mutation of proline

at position 188 in the ALS enzyme, as well as possibly another (one or several) mutation, preferably another (one or several) mutation in the ALS gene.

**[0098]** Preferably, (one allele of) the ALS gene of this mutated sugar beet plant corresponds to SEQ.ID.NO:3 or SEQ.ID.NO:5.

**[0099]** Advantageously, the mutated sugar beet plant of the present invention comprises SEQ.ID.NO:3 (in one allele) and SEQ.ID.NO:5 (in the second allele).

**[0100]** Possibly, the mutated sugar beet plant of the present invention comprises SEQ.ID.NO:3 (in one allele) and either SEQ.ID.NO:1, or SEQ.ID.NO:7 (in the second allele).

**[0101]** Another related aspect of the present invention is a nucleotide fragment (of at least 20 or at least 25 consecutive nucleotides, but of less than 200 consecutive nucleotides, preferably of less than 50 consecutive nucleotides) covering the one or more mutations; possibly this fragment is for use as a primer or a probe (including a nucleotide probe being further labelled e.g. by a non-nucleotidic moiety or using radioactivity, or a probe labelled with a nucleic acid sequence foreign to the ALS gene of sugar beet).

**[0102]** Still another related aspect of the present invention is the use of this nucleotide fragment spanning over the mutation for marker-assisted selection of sugar beet plants having a resistance towards the toxic molecule (herbicide).

## Examples

**[0103]** Comparative example

Because mutated sugar beet were successfully generated in the art (e.g. in WO 98/02527) upon the addition of ALS herbicide to calli being explants from wild-type sugar beet, the inventors firstly selected the sugar beet genotype (line) derived from the line of WO 98/02527 and isolated protoplasts from their stomatal guard cells.

**[0104]** Several millions of these protoplasts (on average about 2 to about 5 millions, and up to 11 millions by experiment; in total, ALS herbicide was applied to about 150 millions of protoplasts) were isolated as in WO 95/10178, placed in culture medium comprising alginate, and treated with MS culture medium comprising $10^{-9}$ to $10^{-6}$ mol/l foramsulfuron.

**[0105]** The inventors have then regenerated the sugar beet, following the protocol as described in WO 95/10178, and observed only a few calli surviving to the ALS inhibitor. However, to one exception, none of these regenerated calli were able to develop into a sugar beet plant. The only regenerated sugar beet plant showed no mutation in the ALS gene (encoding the target enzyme of foramsulfuron).

**[0106]** Therefore this sugar beet line, whose parental line was shown, on the basis of the direct exposition of calli (from an explant) to an herbicide, to acquire mutation-induced resistance to this herbicide, was not useful for the same purpose, when the method involves stomatal guard cells protoplasts.

**[0107]** **Example 1** Selection of sugar beet genotypes (lines) for well-regenerating protoplasts

**[0108]** The inventors have then compared several sugar beet plant genotypes for their capacity of regeneration from stomatal guard cells protoplasts.

**[0109]** The inventors have found genotypes having about 0.01% (or even less) of capacity to regenerate and several genotypes having (much) more than 0.1% of capacity to regenerate.

**[0110]** The inventors have further established a distinction between the growth of the protoplasts (their capacity to growth and divide *in vitro*), the capacity of the grown calli to form shoots and the proportion of growing calli to regenerate a plant.

| Genotype | protoplasts/gram | Percentage growing cells | Percentage shoot formation | Percentage obtained plants |
|---|---|---|---|---|
| F06R38309 | 1500000 | 0,02 | 55,67 | 3,43 |
| F06R38313 | 500000 | 0,04 | 0,14 | 0,00 |
| F06R38323 | 1000000 | 0,19 | 0,49 | 10,81 |
| F07R38836 | 500000 | 0,26 | 10,51 | 0,73 |
| REL1 | 1000000 | 0,07 | 70,00 | 44,00 |

Table 1: comparison of some sugar beet genotypes

**[0111]** Although the values reflecting the capacity of a stomatal guard cell protoplast to regenerate into a whole sugar beet plant, when taken as a whole were higher for the cell line "Rel1", this cell line was considered as not useful enough for running the present invention.

**[0112]** The inventors have further concluded that the "percentage of growing cells" parameter is much more important for running the present invention than the other parameters.

**[0113]** The inventors have selected a genotype having more than 0.25% of stomatal guard cells protoplasts that are able to grow *in vitro*.

**[0114]** **Example 2** Herbicide treatment of protoplasts

The inventors have applied the same approach as in the comparative Example, but relying on well growing stomatal guard cells protoplasts (for instance identified as in Example 1; plants deposited

as NCIMB 42050 or NCIMB 42051 can also be used, as well as other sugar beet plants having a high proportion of growing stomatal guard cell protoplasts).

**[0115]** In total, about 68 millions of well-growing stomatal guard cell protoplasts were treated with an ALS herbicide composition comprising up to $10^{-6}$M foramsulfuron.

**[0116]** The inventors obtained 46 calli.

**[0117]** Several regenerated plants are showing a mutation in the target gene, the ALS gene: in each case a mutation in the codon for tryptophan at position 569 (W569L; corresponding to tryptophan at position 574 in *Arabidopsis thaliana*). The two alleles of the ALS genes of this mutant are encoded by SEQ.ID.NO:3 and SEQ.ID.NO:7. Other grown calli were sequenced and have mutations in the ALS gene, (including mutations at other positions) but did not regenerate into a plant.

**[0118]** The inventors therefore conclude that the method of the present invention is very useful to develop plants having evolved mutations causing a resistance to an herbicide, especially since this method does not involve the use of foreign DNA and/or the introduction of DNA vectors encoding genetic elements already known to confer resistance to ALS inhibitors, and yielded positive results in only a few months.

**[0119]** The inventors then repeated this method and further applied a mutagen (0.05% to 0.2% EMS) to the protoplasts in order to increase the number of mutations.

**[0120]** **Example 3** ALS inhibitor treatment of sugar beets

The inventors compared the behaviour of regenerated sugar beets having the mutated SEQ.ID.NO:3 (heterozygote for this mutation) and a wild-type (naive) sugar beet commercial variety.

The (heterozygote) mutated variety showed good resistance to Foramsulfuron (12.5 g/ha; up to 3 applications), even when the herbicide has been combined with an organic compound (25 g/ha rapeseed oil methyl ester) to boost its effect.

As expected, the wild-type (naive) plant was very sensitive to Foramsulfuron, even after the first application.

**[0121]** The same experiment was performed using amidosulfuron (15 g /ha), and yielded the same level of resistance in the mutated plants.

On the other hand, the wild-type (naive) plants were very sensitive to amidosulfuron, especially when combined with the organic compound, and/or after several applications of amidosulfuron.

**[0122]** The same experiment was performed using iodosulfuron (3.5 g/ha), and demonstrated a good level of resistance in the mutated plants when iodosulfuron was added, but this resistance declined when iodosulfuron is applied together with the organic compound.

As expected, the wild-type (naive) plant was very sensitive to iodosulfuron even after one application and without the organic compound.

**[0123]** The same experiment was performed using 7.5 g/ha thiencarbazone-methyl, and yielded about the same level of resistance as for iodosulfuron in the mutated plants.

The wild-type (naive) plant was very sensitive to thiencarbazone-methyl at all the tested concentrations and regardless of the addition of the organic compound.

**[0124]** The inventors conclude that, by comparison to the wild-type, the mutated sugar beet plant comprising SEQ.ID.NO:3 (deposited under the Budapest Treaty NCIMB 42051) offers the best resistance against foramsulfuron.

**[0125]** The inventors further conclude that this (heterozygote) mutated plant has further acquired some (although partial) resistance towards other ALS inhibitors, including towards inhibitors belonging to other chemical classes.

**[0126]** **Example 4** ALS inhibitor treatment of sugar beets having further mutations in the ALS gene

**[0127]** The inventors then developed a mutated sugar beet plant comprising SEQ.ID.NO:3 and SEQ.ID.NO:5 (on two different alleles). Such resulting dual mutant has been deposited under the Budapest Treaty under NCIMB 42050. A plant comprising both SEQ.ID.NO:3 and SEQ.ID.NO:5 can be generated by relying on several techniques, including, for instance, a subsequent mutagenesis step applied to the single mutant NCIMB 42051.

**[0128]** The inventors then compared the resistance of this dual mutant plant (a mutation in one allele at amino acid 569 and a mutation in the other allele at amino acid 188) with the single mutant (a mutation at position 569) sugar beet.

**[0129]** The dual mutant plant line at least keeps all the resistance features as in Example 3, and has also acquired a good resistance (compatible with field application) towards thiencarbazone-methyl and towards amidosulfuron treatments, even when put in composition with organic compounds.

Therefore, this dual mutant plant displays improved, synergistic, resistance towards several ALS inhibitors by comparison to the resistance attributed to the single mutant plant (at position 569 in the ALS gene).

**[0130]** **Example 5** Greenhouse trials: ALS inhibitor treatment of different sugar beets in direct comparison

**[0131]** Mutated sugar beet plants comprising SEQ.ID.NO:3 and SEQ.ID.NO:5 (on two different alleles) according to the present invention (as described in Example 4 above, "Line A") were treated with different ALS inhibitors in direct comparison with sugar beet plants where the tryptophan at position 569 of the encoded ALS enzyme is substituted by a leucine ("Line B"), sugar beet plants described in WO 98/02527 where the proline at position 188 of the encoded ALS enzyme is substituted by a serine ("Line C"), and traditional variety (wild-type) sugar beet plants not having a mutation at positions 569 and 188 ("Line WT").

**[0132]** Several groups of seeds of the four different mentioned sugar beet plants were sown separately in the greenhouse and grew up to stage BBCH 14 for *Beta vulgaris* L. *ssp. vulgaris* (i.e. 4 leaves (the second pair) unfolded) according to the monographie "Entwicklungsstadien mono- und dikotyler Pflanzen", 2nd edition, 2001, ed. Uwe Meier, Biologische Bundesanstalt für Land und Forstwirtschaft. Subsequently, the resulting separate groups of sugar beet plants were each individually treated with an ALS inhibitor (ALS-in) in the amounts (g/ha) indicated in Table 2.

**[0133]** On day 14 after application of the respective ALS inhibitor, the damage (i.e. the phytotoxicity) for each sugar beet plant was rated on a scale from 0% (i.e. no damage, no phytotoxicity) to 100% (i.e. the plants were completely killed). The average rating for each group of plants is also shown in Table 2.

Table 2:

| ALS-in | ALS-in g/ha | Line A | Line B | Line C | Line WT |
|---|---|---|---|---|---|
| Foramsulfuron | 13 | 26.9% | 45.6% | 77.5% | 80.0% |
| Iodosulfuron-methyl-Na | 3.5 | 22.5% | 38.8% | 80.0% | 82.5% |
| Amidosulfuron | 15 | 6.3% | 37.5% | 51.9% | 73.1% |
| Thiencarbazone-methyl | 7.5 | 8.1% | 35.6% | 37.5% | 84.4% |
| Bisbyribac-Na | 50 | 17.5% | 38.1% | 71.7% | 80.0% |
| Metosulam | 15 | 13.1% | 40.6% | 69.4% | 79.4% |

**[0134]** Additionally, typical early phenotypes of each sugar beet plants were inspected after treatment with a mixture comprising thiencarbazone-methyl and foramsulfuron. A representative early phenotype of each Line is shown in Figure 1 (Fig.1).

**[0135]** Fig. 1 also demonstrates that the sugar beet plants according to the present invention ("Line A") show improved ALS inhibitor resistance, i.e. superior growth and less phytotoxic effects were observed in comparison to the other early phenotypes.

**[0136]** **Example 6** Field trials: ALS inhibitor treatment of different sugar beets in direct comparison

Table 3:

| | | Sensitive | 574 hetero | 574&197 | 574 homo |
|---|---|---|---|---|---|
| 1 | UNTREATED | 0 | 0 | 0 | 0 |
| 2 | AE F 130360 00 WG50 A1 25g/HA (Foramsulfuron) | 97 | 22 | 5 | 0 |
| 3 | BYH18636 15 g/Ha (Thiencarbazone) | 97 | 39 | 5 | 0 |
| 4 | ae f115008 00 wg 10 a2 7g/Ha (iodosulfuron) | 98 | 65 | 23 | 28 |
| 5 | AE F130060 00 WG75 A2 60 g/Ha (mesosulfuron) | 91 | 24 | 18 | 0 |
| 6 | HOESTAR 30 g/Ha (amidosulfuron) | 97 | 34 | 0 | 0 |
| 7 | AEF095404 00 WG60 A2 60 g/Ha (ethoxysulfuron) | 99 | 39 | 0 | 0 |
| 8 | RAPTOR 40 g/Ha (imazamox) | 98 | 44 | 35 | 8 |
| 9 | TACCO 30 g/Ha (metosulam) | 97 | 27 | 0 | 3 |
| 10 | NOMINEE 50 g/Ha (bispyribac) | 98 | 78 | 70 | 28 |
| 11 | MOTIVELL 60 g/Ha (nicosulfuron) | 98 | 53 | 28 | 13 |
| 12 | GROPPER SX 8 g/Ha (metosulfuron) | 100 | 74 | 50 | 35 |
| 13 | LEXUS 50 DF 10 g/Ha (flupyrsulfuron) | 70 | 0 | 0 | 0 |
| 14 | ATTRIBUT 70 g/Ha (propoxycarabazone) | 91 | 25 | 0 | 5 |
| 15 | SIMPLICITY 50 g/Ha (pyroxysulam) | 97 | 45 | 28 | 0 |
| 16 | PRIMUS 10 g/Ha (florasulam) | 99 | 55 | 38 | 0 |
| 17 | POINTER SX 30 g/Ha (tribenuron) | 98 | 74 | 28 | 20 |
| 18 | CATO 13 g/Ha (rimsulfuron) | 68 | 8 | 0 | 0 |
| 19 | MONITOR 80 WG 10 g/Ha (sulfosulfuron) | 93 | 23 | 0 | 0 |
| 20 | DEBUT YX1 15 g/Ha (triflusulfuron) | 0 | 0 | 0 | 0 |
| 21 | EVEREST 40 g/Ha (flucarbazone) | 93 | 18 | 0 | 0 |
| 22 | HARMONY 7.5 g/Ha (thiensulfuron) | 98 | 39 | 0 | 0 |
| 23 | # 2 & #3 (Foramsulfuron+Thiencarbazone) 1 L/Ha | 100 | 65 | 35 | 5 |

Table 3: effect of ALS inhibitors on sugar beet plant. The values represent the average percentage of measured damage.

**[0137]** The inventors have tested commercial compositions at the dose allowing destruction of the weeds.

**[0001]** The sensitive control (i.e. a sugar beet having no mutation in the ALS gene) was killed by all the herbicides but one. The inventors have measured small damages to control (untreated) plant, reaching sometimes 35% or even 40%. These 'damages' reflect the agronomic conditions of this field trial.

**[0002]** On the other hand, sugar beet plant being heterozygote at position 569 (574) have become partially resistant towards several herbicidal compositions. The plant having incorporated the 569 mutation (574) on both alleles and thus being (569/569) homozygote has a further increased resistance: only 7 herbicide composition are moderately toxic (from 5% to 35%).

**[0003]** A sugar beet plant having incorporated the mutation at position 569 (574) in one allele of the ALS gene and a mutation at position 188 (197) in the second allele of the ALS gene have also acquired improved resistance, since 9 herbicide compositions are moderately toxic, and only 3 are quite toxic. Surprisingly, such plant, where a mutation providing a strong resistance (569) has been lost and a mutation providing only a weak resistance (188) towards ALS inhibitor has been added, provides even better resistance than the homozygote (569/569) plant in 3 different conditions of this field test.

## SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with section 111(1) of the *Patent Rules*, this description contains a sequence listing in electronic form in ASCII text format (file: 11700-33 SEQ 30-APR-15 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

**CLAIMS**

**1.** A method for producing a mutant sugar beet plant being resistant to one or more inhibitor(s) of the acetohydroxyacid synthase (ALS) enzyme comprising the steps of:

- obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant;
- applying to an *in vitro* culture of the said protoplasts a composition comprising one or more ALS inhibitor(s) at a concentration that is lethal to more than 99% of the *in vitro* cultured cells; and
- regenerating sugar beet plants from the surviving cells of the said *in vitro* cultured cells,

wherein the said stomatal guard cell protoplasts are pre selected for their capacity to regenerate into a sugar beet plant, wherein the said pre selected protoplasts have a probability of more than 10% (number of growing protoplasts:total protoplast number put into culture) to divide and regenerate into a viable callus, wherein calli obtained by the said protoplasts have more than 10% (number of calli producing shoots:total calli number) of capacity to develop shoots and wherein the said ALS inhibitor(s) is/are applied to more than 20 000 000 of the said protoplasts.

**2.** The method of claim 1, wherein the step of selecting stomatal guard cell protoplasts able to regenerate into a sugar beet plant comprises the sub-steps of isolating stomatal guard cell protoplasts from sugar beet plants of different genotypes and measuring for each genotype the proportion of the said protoplasts that is growing when the said protoplasts are put into *in vitro* culture.

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**3.** The method of claim 1 or 2 further comprising the step of sequencing the genome of the regenerated plants from the surviving *in vitro* cultured cells.

**4.** The method according to any one of claims 1 to 3, wherein the regenerated sugar beet comprises an ALS gene corresponding to SEQ ID NO:1 or to SEQ ID NO:7 and further comprising the step of sequencing the said ALS gene for identifying a mutation in the ALS gene.

**5.** The method according to any one of claims 1 to 4 further comprising the step of selecting regenerated sugar beet plants having a mutation in the ALS gene corresponding to SEQ ID NO:1 or to SEQ ID NO:7.

**6.** The method according to any one of claims 3 to 5 wherein the regenerated sugar beet plant has one or several mutation(s) in the ALS gene corresponding to SEQ ID NO:1 or to SEQ ID NO:7 at positions encoding the amino acids selected from the group consisting of Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372, Methionine 565, Valine 566, Phenylalanine 573, Serine 648 and Glycine 649.

**7.** The method according to any one of claims 3 to 5, wherein the regenerated sugar beet plant has one mutation in the ALS gene corresponding to SEQ ID NO:1 or to SEQ ID NO:7 at positions encoding proline 188 and one or more mutation(s) in the ALS gene at positions encoding Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372,

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Methionine 565, Valine 566, Tryptophan 569, Phenylalanine 573, Serine 648 and Glycine 649.

**8.** The method according to any one of claims 3 to 5, wherein the regenerated sugar beet plant has one mutation in the ALS gene corresponding to SEQ ID NO:1 or to SEQ ID NO:7 at positions encoding tryptophan 569 and one or more mutation(s) in the ALS gene at positions encoding Glycine 112, Alanine 113, Methionine 115, Arginine 133, Valine 187, Proline 188, Arginine 190, Alanine 196, Phenylalanine 197, Lysine 247, Methionine 346, Histidine 347, Arginine 368, Aspartate 370, Aspartate 371, Arginine 372, Methionine 565, Valine 566, Phenylalanine 573, Serine 648 and Glycine 649.

**9.** The method according to any one of claims 3 to 5, wherein the regenerated sugar beet plant has one or more mutation(s) in the ALS gene corresponding to SEQ ID NO:1 or to SEQ ID NO:7, wherein the said one or more mutation is selected from the group consisting of Alanine 113, Proline 188, Alanine 196, Aspartate 371, Arginine 372, Tryptophan 569, Serine 648 and Glycine 649, wherein the said Alanine 113 is mutated into Valine or Threonine, wherein the said Proline 188 is mutated into Threonine, Arginine, Leucine, Glutamine or Alanine, wherein the said Alanine 196 is mutated into Valine, wherein the said Aspartate 371 is mutated into Glutamate, wherein the said Arginine 372 is mutated into Histidine, wherein the said Tryptophan 569 is mutated into Glycine, wherein the said Serine 648 is mutated into Threonine and wherein the said and Glycine 649 is mutated into Aspartate.

**10.** The method according to any one of claims 3 to 5, wherein the regenerated sugar beet plant has one mutation in the ALS gene corresponding to SEQ ID NO:1 or to SEQ ID NO:7 at a

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position encoding Proline 188 and one mutation in the ALS gene at a position encoding Tryptophan 569.

**11.** The method according to any one of claims 1 to 10 comprising a preliminary step of determining the concentration at which the composition comprising the one or more ALS inhibitor(s) is/are lethal for at least 99% of the *in vitro* cultured cells.

**12.** The method according to any one of claims 1 to 11 wherein the one or more ALS inhibitor(s) is/are applied to an *in vitro* culture of more than 50 000 000 stomatal guard cell protoplasts.

**13.** The method according to any one of claims 1 to 12, wherein the composition comprising one or more ALS inhibitor(s) comprises foramsulfuron.

**14.** The method of claim 13, wherein the foramsulfuron is applied at a concentration in the range of $10^{-9}$ mol/l to $10^{-6}$ mol/l.

**15.** The method according to any one of claims 1 to 14, wherein the composition comprising one or more ALS inhibitor(s) comprises ethoxysulfuron.

**16.** A method for producing a mutant sugar beet plant being resistant to an herbicide comprising the steps of:

- obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant;
- applying to an in vitro culture of the said protoplasts a composition comprising the said herbicide at a concentration that is lethal to more than 99% of the in vitro cultured cells;
- regenerating sugar beet plants from the surviving cells of the said in vitro cultured cells; and

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- selecting regenerated sugar beet plants having a mutation in the gene encoding the peptide targeted by the said herbicide,

wherein the said stomatal guard cell protoplasts are pre selected for their capacity to regenerate into a sugar beet plant, wherein the said pre selected protoplasts have a probability of more than 10% (number of growing protoplasts:total protoplast number put into culture) to divide and regenerate into a viable callus, wherein calli obtained by the said protoplasts have more than 10% (number of calli producing shoots: total calli number) of capacity to develop shoots and wherein the said herbicide is applied to more than 20 000 000 of the said protoplasts.

**17.** The method of claim 16, wherein the herbicide is selected from the group consisting of 4-HPPD inhibitors, inhibitors of the carotenoid biosynthesis, inhibitors of EPSP synthase, inhibitors of photosystem II, inhibitors of photosystem I, inhibitors of cell division, inhibitors of microtubule assembly, inhibitors of protoporphyrinogen oxidase, inhibitors of Acetyl CoA carboxylase, cell wall synthesis inhibitors, inhibitors of glutamine synthetase and synthetic auxin.

**18.** A mutated sugar beet plant cell comprising SEQ ID NO:3 on one allele of the acetohydroxyacid synthase (ALS) gene and SEQ ID NO:5 on the second allele of the ALS gene.

**19.** The mutated sugar beet plant cell according to claim 18 obtained from the seeds deposited under NCIMB 42050.

**20.** The mutated plant cell of claim 18 or 19 being stomatal guard cells, or the protoplasts isolated from the said stomatal guard cells comprising SEQ ID NO:3 on one allele of the ALS gene and SEQ ID NO:5 on the second allele of the ALS gene.

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**21.** Use of the protoplasts of claim 20 for the introduction of one or more further genetic trait to a mutated sugar beet plant comprising said mutated sugar beet plant cell.

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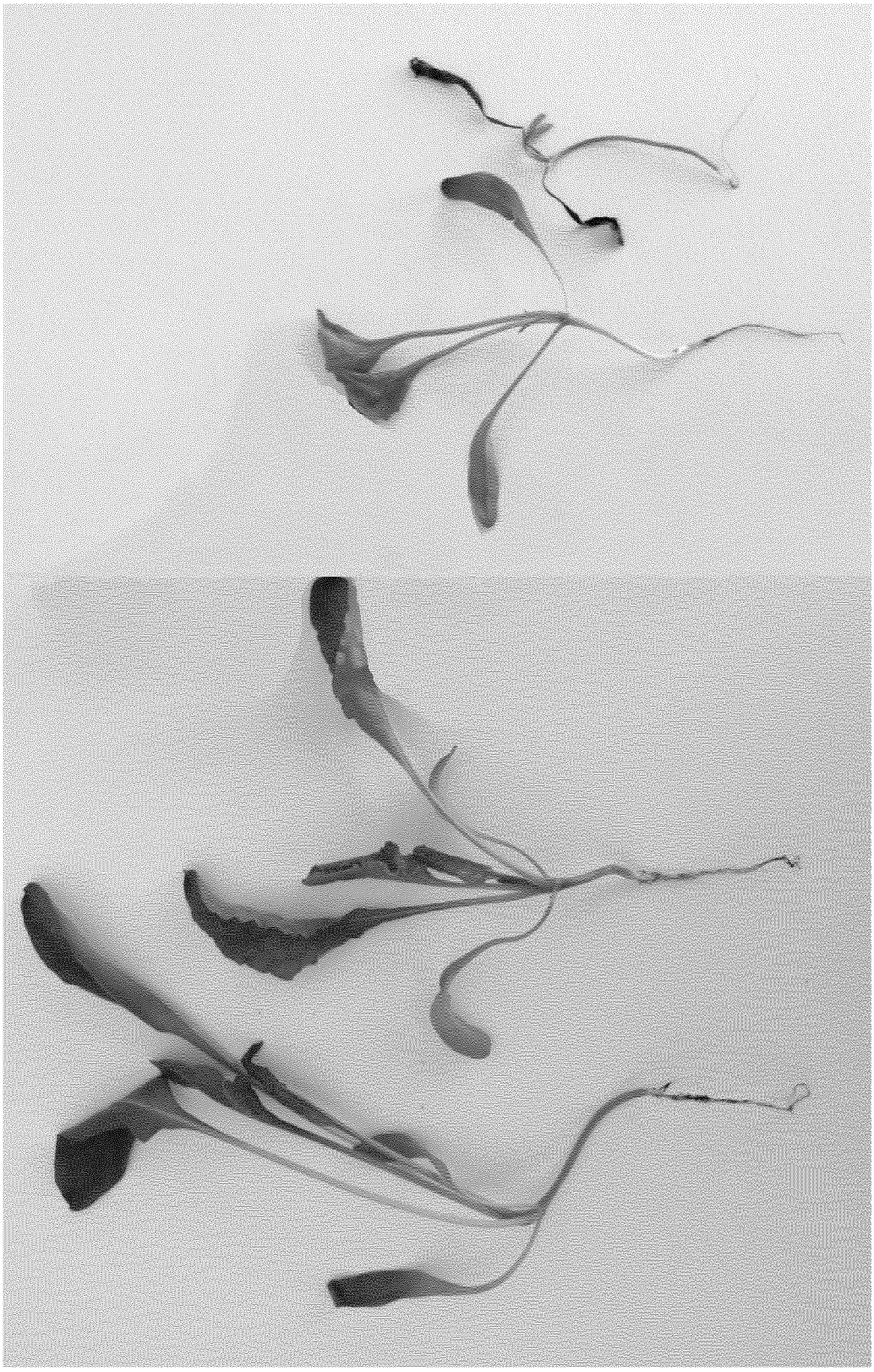
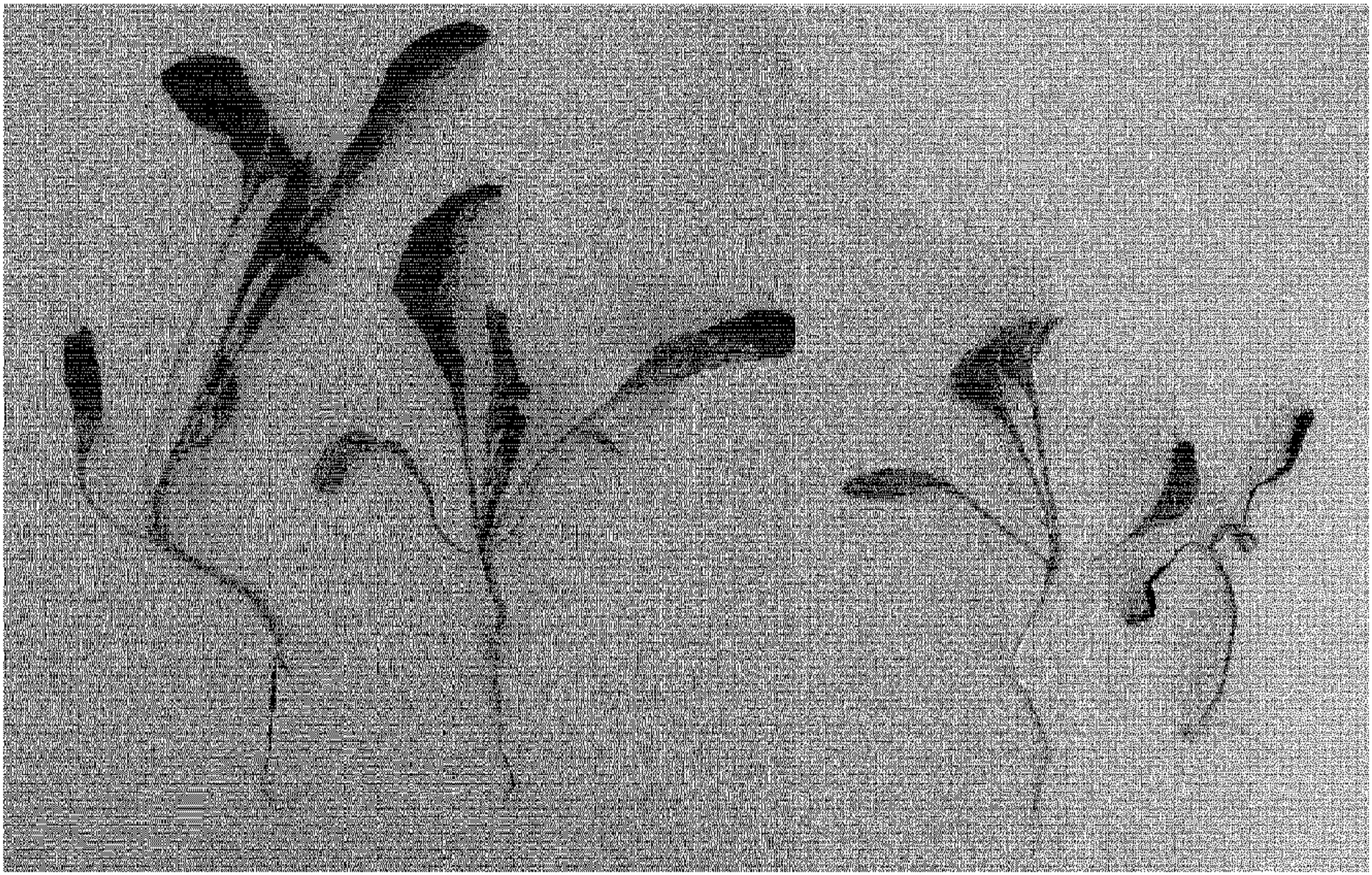
Fig. 1





Line A Line B Line C Line WT