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(54) **Title:** TRANSFORMATION METHOD OF SUGAR BEET PROTOPLASTS BY TALEN PLATFORM TECHNOLOGY

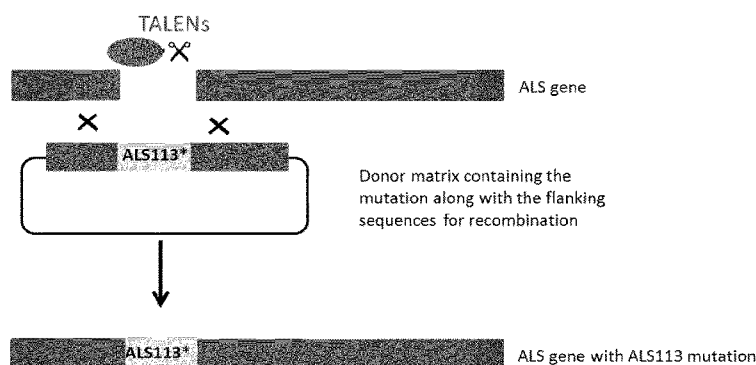


FIG.1

(57) **Abstract:** Method for the transformation of sugar beet protoplasts comprising the step of: -obtaining protoplasts from stomatal guard cells isolated from a sugar beet plant, -transforming said protoplasts with a nucleic acid construct comprising a nucleotide sequence of interest and Transcription Activator-Like Effector Nucleases (TALEN) or one or more vectors comprising sequences encoding these Transcription Activator-Like Effector Nucleases (TALEN) sequences, wherein these TALEN target and process a target sequence and replace this target sequence through homologous recombination with the nucleic acid construct comprising the nucleotide sequence of interest, -possibly applying to an in vitro culture of these protoplasts, a medium that is toxic, preferably lethal to the in vitro culture of the protoplasts, and -regenerating sugar beet plants from the cell culture, preferably from the surviving protoplasts having integrated the nucleic acid construct comprising the sequence of interest that possibly renders the transformed cell resistant to the toxic activity of the applied medium.



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**TRANSFORMATION METHOD OF SUGAR BEET PROTOPLASTS BY TALEN
PLATFORM TECHNOLOGY**

10 Field of the invention

[0001] The present invention is in the field of plant biotechnology, especially related to a method and means (or tools) for the efficient transformation of sugar beet protoplasts by exogenous sequences.

15

Background of the invention

[0002] The ability to modify chromosomes through homologous recombination (gene targeting) is needed for a long time in the field of plant genetics, because gene
20 targeting can target specific sequences or nucleotides resulting into modifications of specific plant functions and opening new possibilities for crops improvement, for increased production of new seeds with required carbohydrate profile or with enhanced nutritional qualities, or for
25 increasing resistance to diseases and stress.

[0003] Transcription Activator-Like Effector Nucleases (TALEN) sequences originating from plant pathogen bacteria of the genus xanthomonas, play important role in diseases or trigger defence, by binding host DNA and
30 activating effector-specific host genes. TALEN's are specific transcription activator-like effector nucleases (artificial restriction enzymes) that can be engineered to bind and cut the specific DNA sequences in the genome. Once

these TALEN's are introduced into cells, they can be used for genome editing.

[0004] These nucleases sequences and their derived protein allow to efficiently target and/or process double
5 stranded nucleic acid sequences.

[0005] Their derived proteins are specific chimeric protein monomers composed of a core scaffold comprising Repeat Variable Dipeptide regions (RVDs) having binding specificity to a target sequence, to which is fused a
10 catalytic domain to its N-terminal portion. This catalytic domain, which can be a monomer of a nuclease, is placed at a position to possibly interact with another catalytic domain fused to another TAL monomer, such that, when both monomers are binding to their respective target DNA sequence, both
15 catalytic domains will form catalytic entity likely to process DNA in the proximity of this target sequence.

[0006] The international patent application WO2011/072246 describes a method for modifying the genetic material of a cell with the use of this Transcription
20 Activator-Like Effector nuclease (also called DNA enzyme) nucleotide sequence or its derived protein, wherein the TAL Effector Nuclease will bind to and will process the target DNA.

[0007] This method will comprise also the step of
25 providing to the cell, a nucleic acid sequence comprising a sequence being homologous to at least a portion of the target DNA sequence, such that a homologous recombination could occur between the target sequence portion(s) and its corresponding nucleic acid portion(s) disposed between the
30 sequence of the gene of interest.

[0008] A transformation of a cell could be obtained either by using one or more vectors comprising the sequences encoding the TALEN protein or by introducing directly the corresponding derived protein into the cell through a

mechanic injection, through the means of a bacterial secretion system or through electroporation.

[0009] This method has been already used for the transformation of various cells through the use of
5 Transcription Activator-Like proteins, especially type II restriction endonuclease (such as FokI).

[0010] However, in the field of plants genetics, transformation methods are complicate, not always efficient, and time consuming. This is particularly true for several
10 plant species, such as sugar beet cells and plants which are reluctant to genetic transformation.

[0011] Therefore, it exists a clear need for the improvement of genetic methods dedicated to transformation especially gene targeting of sugar beet plants.

15 [0012] Therefore, the present invention aims to provide a new method and tools for gene targeting of sugar beet cells and plants which allow its genetic transformation, especially genetic targeting and editing of sugar beet protoplasts.

20

Summary of the invention

[0013] The present invention is related to the transformation method of sugar beet protoplasts comprising the steps of:

- 25 - obtaining protoplasts from stomate guard cells isolated from a sugar beet (*Beta vulgaris*) plant,
- transforming these protoplasts with a nucleic acid construct comprising a nucleotide sequence of interest and Transcription Activator-Like Effector Nucleases
30 (TALEN) sequences or one or more vectors comprising sequences encoding these Transcription Activator-Like Effector Nucleases (TALEN) sequences, wherein these Transcription Activator-Like Effector Nucleases targeting and processing a target (DNA) sequence of the

protoplast and replace this target (DNA) sequence through homologous recombination with the nucleic acid construct comprising the nucleotide sequence of interest,

- 5 - possibly applying to an *in vitro* culture of these protoplasts, a medium (such as one or more herbicide(s)) that is toxic, preferably lethal to the *in vitro* culture of the protoplasts, and
- 10 - regenerating sugar beet plants from the cell culture, preferably from the surviving protoplasts having integrated the nucleic acid construct comprising the sequence of interest that possibly renders the transformed cell resistant to the toxic, preferably lethal activity of the applied medium (such as one or
- 15 more herbicide(s)).

[0014] Preferably, in the method according to the invention, the nucleic acid construct further comprises one or more regulatory sequences for expression of the nucleotide sequence of interest in sugar beet protoplasts, cells,

20 tissues (calli) and/or plants.

[0015] Preferably, in the method according to the invention, the applied medium to the *in vitro* culture of protoplasts comprises one or more ALS inhibitors (herbicide(s)) selected from the group consisting of

25 sulfonylurea herbicides, sulfonylaminocarbonyltriazolinone herbicides, imidazolinone herbicides, triazolopyrimidine herbicides, pyrimidinyl(thio)benzoate herbicides or a mixture thereof.

[0016] More preferably, these sulfonylurea herbicides

30 are selected from the group consisting of foramsulfuron, iodosulfuron, amidosulfuron, ethoxysulfuron, chloramsulfuron or a mixture thereof. More preferably, in the method according to the invention the ALS inhibitor(s) (herbicide(s)) is (are) applied at a concentration comprised

between (about) $5 \times 10^{-9} \text{M}$ and (about) $1 \times 10^{-6} \text{M}$ for foramsulfuron, and between (about) $5 \times 10^{-11} \text{M}$ and (about) $5 \times 10^{-10} \text{M}$ for ethoxysulfuron.

[0017] In the method according to the invention, the
5 sequence of interest encodes a peptide or protein conferring (or involved in) a resistance to one or more herbicide(s), resistance to insects, resistance to nematodes, resistance to plant diseases, resistance to viral infections, resistance to stress (such hydric or saline stress), may
10 encode one or more enzymes and/or may encode a peptide or protein having antibacterial or antifungal properties.

[0018] The nucleic acid construct used in the method according to the invention may also use or be present in a vector that comprises one or more regulatory sequence(s)
15 for expression of the nucleic sequence of interest into a sugar beet protoplast, cell, tissue and/or plant. These regulatory sequences are preferably selected from the group consisting of promoter(s) or transcription termination and/or poly-A signal sequence(s), more preferably the
20 CAMV35S promoter sequence and the Nos terminator sequence obtained from *agrobacterium tumefaciens*.

[0019] When the nucleic sequence of interest is the BVALS 113 sequence carrying a sequence mutation at amino acid 113 position from an L-Alanine (Ala) to an L-tyrosine
25 (Tyr) compared to its corresponding wild-type ALS sequence, the preferred and suitable ALS inhibitor(s) (herbicides) is (are) one of the above mentioned herbicides, more preferably foramsulfuron and/or ethoxysulfuron.

[0020] Furthermore, the person skilled in the art may
30 select other adequate herbicides which interact with the above mentioned ALS sequence and wherein the above mentioned mutation (at amino acid 113 position from L-Alanine (Ala) to L-tyrosine (Tyr) compared to its wild type corresponding ALS sequence) may render protoplast cell tissue and plant

resistance to this ALS inhibitor (herbicide) or mixture of ALS inhibitors (herbicides).

[0021] In the method according to the invention, the preferred protoplasts are stomate guard cell protoplasts which have the ability to divide (grow) and to originate viral sugar beet callus when grown in suitable culture media. A callus refers to a mass of undifferentiated cells which can be obtained from explants, such as embryos or parenchyma-derived explants from leaves or a cotyledon. According to the invention, the callus is preferably the result of the growth of well-regenerating (stomatate) guard cell protoplasts, having the capacity to develop shoots or to regenerate into viable sugar beet plants when grown in appropriate culture media, such as polymer-containing medium (preferably an alginate or an agarose containing medium).

[0022] The present invention also relates to the protoplast(s), cell(s), tissue(s) (calli) or plant(s) obtained by the transformation method according to the invention, more particularly to a sugar beet plant integrating in its genome the nucleotide sequence of interest, preferably one or more of the sequences above described.

[0023] The present invention will now be described in detail in the following detailed description of the invention in reference to the enclosed figures presented as non-limited embodiment of the present invention.

Short description of the drawings

[0024] Fig. 1 represents the introduction of ALS113 mutation in sugar beet protoplasts using specific TALEN sequence and donor matrix containing the mutation along with the flanking sequences for recombination according to the invention.

[0025] Fig. 2 represents the T7E1 assay for sugar beet BvALS TALEN activity validation according to the invention.

[0026] Fig. 3 represents two plant expression vectors with specific Transcription Activator-Like Effector Nucleases (TALEN) sequence expressed in protoplasts.

[0027] Fig. 4 to 6 represent the sequences of donor matrix sequences according to the invention.

Detailed description of the invention

10

[0028] **The introduction of specific mutation in sugar beet ALS gene for providing resistance against ALS-inhibitors using TALEN technology is illustrated in the figure 1.**

15 [0029] CPS developed TALENs (TALEN sequences) based on their yeast screening platform and provided SV with 3 engineered TALEN couples directed against 3 different target sites in the ALS sequence (target sites in close proximity to ALS113 mutation site). All three TALEN pairs came in plant
20 expression vectors along with the three specific donor matrix for each TALEN. When both the TALEN pair and the donor matrix were co-transformed together, the TALEN pair making a double stranded cleavage at specific site and then the donor matrix contacting the specific mutation (ALS113) is inserted into
25 the cleavage site based on the homologous recombination of flanking sequences.

[0030] Three TALENS (TALEN sequences) were designed and produced for introducing a point mutation (A113Y, GCA to TAT) into Sugar Beet 3' ALS gene. Two ALS homologous loci
30 were identified from Sugar Beet (Beta vulgaris) genome and either of them can be used as the target site.

TALEN pairs	Plasmid name	gal37 (sd)	gal30 (sd)
BvALS_T01.1	pCLS24852-pCLS24854	0.85 (0.02)	0.87 (0.06)
BvALS_T02.1	pCLS24856-pCLS24858	0.83 (0.03)	0.84 (0.06)
BvALS_T03.1	pCLS24860-pCLS24862	0.91 (0.03)	0.90 (0.06)

Table 1: List of TALEN produced and validated by yeast assay

[0031] Three TALEN pairs were synthesized for making the double strand break in the ALS gene near the mutation site. The objective was to measure the efficiency of these three TALEN's by using deep sequencing method to look for the NHEJ (Non-Homologous End Joining) activities and select the one with higher efficiency for carrying sugar beet transformation along with the donor matrix.

TALEN sequences:

BvALS_T01: TATTGAAGATTCATCTTTTCGTTTCTCGATTTGGCCCTGATGAACCCAGA
 BvALS_T02: TCTTGAGCGTGAAGGTGTTACCAATGTGTTTGCTTACCCTGGTGGTGCA
 BvALS_T03: TGAACAAGGCGGGGTTTTCGCCGCCGAGGGATATGCTAGAGCTACTGGA

[0032] The efficiency in cutting the double stranded DNA for each TALEN pair was assessed by Sugar Beet protoplast transformation using standard direct gene transformation protocol. After the transformation process, the protoplast were incubated for 24 hours at a temperature of about 26°C. After the incubation, the resulting protoplast were frozen and the DNA isolation was performed according to the same standard protocol. The sequences flanking TALENs Recognition Sites were amplified by PCR. Purified PCR products were then sent for 454 deep sequencing. The sequences were analyzed by bioinformatics to identify the presence of targeted deletions or insertions resulting from NHEJ (Non-Homologous End Joining) events.

[0033] Based on the limited 454 sequencing results, TALEN BvALS_T03 cleavage activity (~2.2% NHEJ mutagenesis rate, see Table 2) were identified. Another approach using

the cloning of the PCR fragments and run SANGER sequencing which is semi-quantitative, was used to identify that both BvALS_T02 and BvALS_T03 had good cleavage activities (~5%, Table 2)

Sample	TALEN	# 454 sequence	# seq with In/Del	% of In/Del	Sanger seq	# seq with Del	% of Del
1	BvALS_T01	190	1	0.53	94	0	0
2	BvALS_T02	123	0	0	94	4	4.26
3	BvALS_T03	2883	63	2.19	94	6	6.38
4	N/A	3244	0	0	n/a	n/a	n/a

5

Table 2. Sequencing results of PCR products amplified from TALEN transformed sugar beet protoplast DNA.

[0034] T7E1 is an endonuclease that recognizes mismatched double strand DNA and makes cleavage on the mismatched sites. When the PCR products (amplified sequences) were denatured and re-annealed, single strand DNA (sequences) with and without deletions could make double strand DNA (sequence) that has mismatches. Those DNA (sequences) were target for T7E1. Compare to 454 sequencing assay, T7E1 assay was simple and not expensive, but the skilled person can detect TALEN activities that exceed 5%.

[0035] Figure 2 shows the T7E1 assay results. Since there were SNPs between the two ALS genes in sugar beet genome, T7E1 assay indicated some default cuttings even in wild-type material. Those banding pattern may cover the cuttings from BvALS_T01 and BvALS_T03 cleavages. The extra was cutting from BvALS_T02 samples (pointed in figure 2), indicating BvALS_T02 had significant cutting activity.

[0036] Based on the results from different validation analysis, both BvALS_T02 and BvALS_T03 were good TALEN sequences. Given the fact that the donor for BvALS_T02 did not contain any extra mutation other than the three planned mutation, the BvALS_T02 and its donor were selected for

transformation in sugar beet to introduce ALS113 mutation in sugar beet ALS gene.

Sugar beet transformation with TALEN and donor matrix

5 combination

[0037] The inventors had initiated the transformation experiments with TALEN BvALS_T02 (pCLS24856-pCLS24858) and its corresponding donor matrix BvALST2 (pCLS26201) to test the efficiency of targeted insertion carrying the mutated
10 ALS. Once the efficiency was determined, the transformation experiments were initiated to regenerate the plants carrying the mutation using the killing curve concentration determined for the selected ALS inhibitor herbicide(s), preferably foramsulfuron and ethoxysulfuron.

CLAIMS

1. Method for the transformation of sugar beet protoplasts comprising the step of:
- obtaining protoplasts from stomate guard cells isolated
5 from a sugar beet plant,
 - transforming said protoplasts with a nucleic acid construct comprising a nucleotide sequence of interest and Transcription Activator-Like Effector Nucleases (TALEN) sequence or one or more vectors comprising sequences encoding
10 said Transcription Activator-Like Effector Nucleases (TALEN) sequences, wherein the said Transcription Activator-Like Effector Nucleases targeting and processing a target sequence of the protoplast and replacing this target sequence through homologous recombination with the nucleic acid
15 construct comprising the nucleotide sequence of interest,
 - possibly applying to an *in vitro* culture of the said protoplasts, a medium that is toxic to the *in vitro* culture of the protoplasts, and
 - regenerating sugar beet plants from the said protoplasts
20 culture, preferably from the surviving protoplasts having integrated the nucleic acid construct comprising the sequence of interest that possibly renders the transformed protoplast resistant to the toxic activity of the applied medium.
- 25 2. The method according to the claim 1, wherein the sequence of interest comprises or consist of a mutated BVALS 113 sequence carrying a sequence mutation at amino acid 113 position from an L-Alanine (Ala) to an L-tyrosine (Tyr) and wherein the medium that is toxic for the
30 *in vitro* culture of protoplasts comprises one or more ALS inhibitors.
3. The method according to claim 2, wherein the one or more ALS inhibitor(s) is selected from the group consisting of sulfonylurea herbicides,

sulfonylaminocarbonyltriazolinone herbicides, imidazolinone herbicides, triazolopyrimidine herbicides, pyrimidinyl(thio)benzoate herbicides or a mixture thereof.

4. The method according to claim 3, wherein
5 the sulfonylurea herbicides are selected from the group consisting of foramsulfuron, iodosulfuron, amidosulfuron, ethoxysulfuron, chloramsulfuron or a mixture thereof.

5. The method according to the claim 4,
wherein the ALS inhibitor is applied at a concentration
10 comprised between $5 \times 10^{-9} \text{M}$ and $1 \times 10^{-6} \text{M}$ for foramsulfuron, and between $5 \times 10^{-11} \text{M}$ and $5 \times 10^{-10} \text{M}$ for ethoxysulfuron.

6. The method according to any one of the preceding claims, wherein the sequence of interest encodes a peptide or protein conferring resistance to one or more
15 herbicides, resistance to insects, resistance to nematodes, resistance to plant diseases, resistance to viral infections, resistance to stress, or encodes one or more enzymes and/or encodes a peptide or protein having antibacterial or antifungal properties.

20 7. The method according to any one of the preceding claims, wherein the nucleic acid construct comprises one or more regulatory sequences allowing expression of the nucleotide sequence of interest into a sugar beet plant protoplasts, cells, tissues and/or plants.

25 8. The method of claim 7, wherein the one or more regulatory sequences are selected from the group consisting of promoter transcription termination sequence(s) and poly-A signal sequence(s).

9. A sugar beet protoplast, cell, tissue or
30 plant obtained by the transformation method according to any one of the preceding claims.

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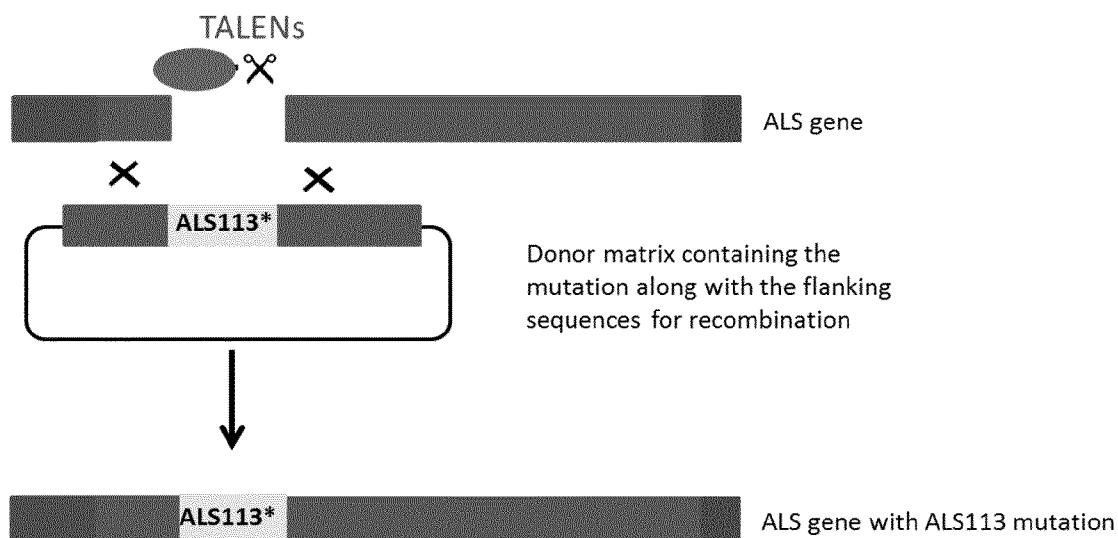


FIG.1

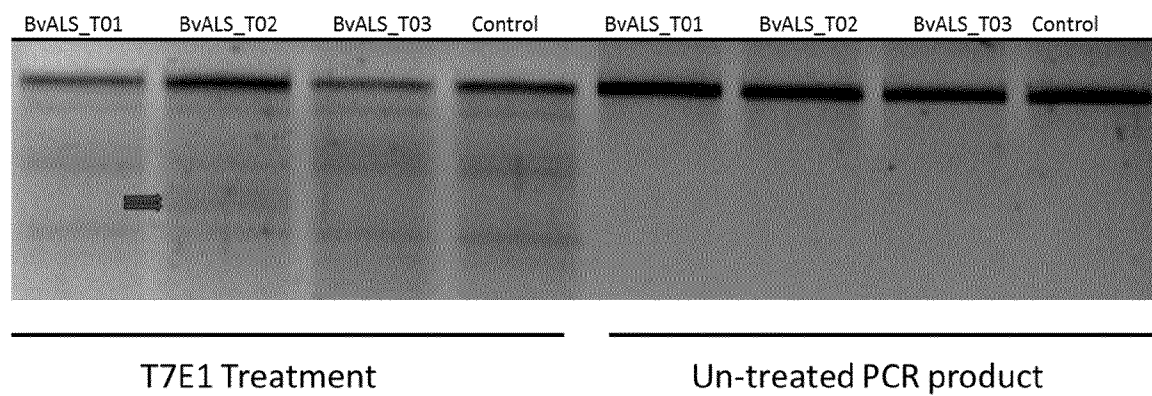
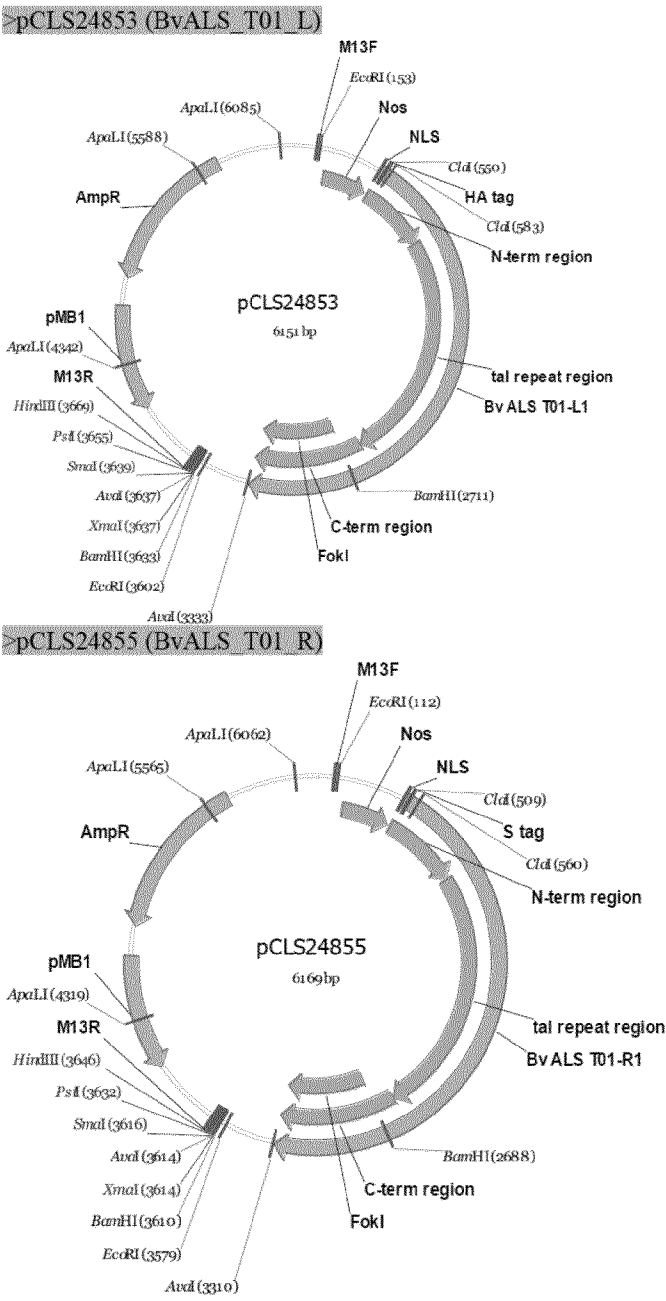
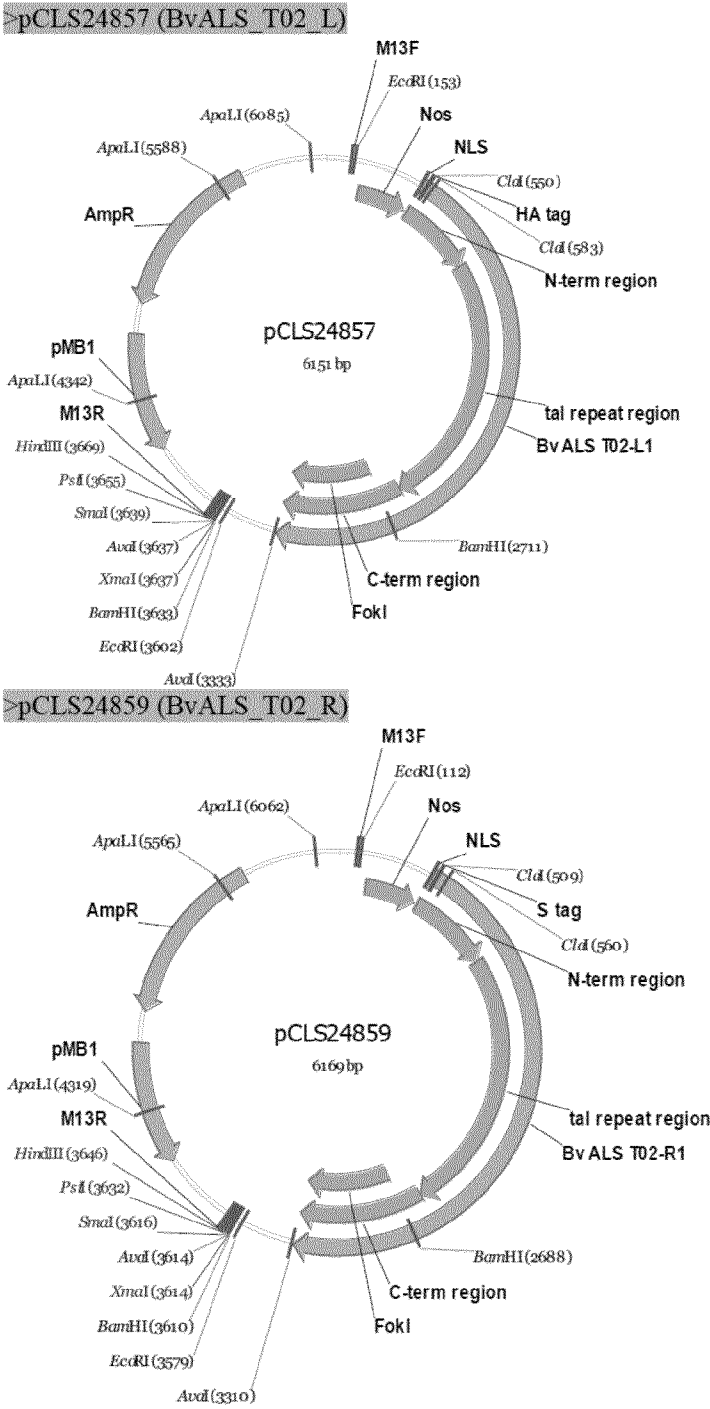


FIG.2





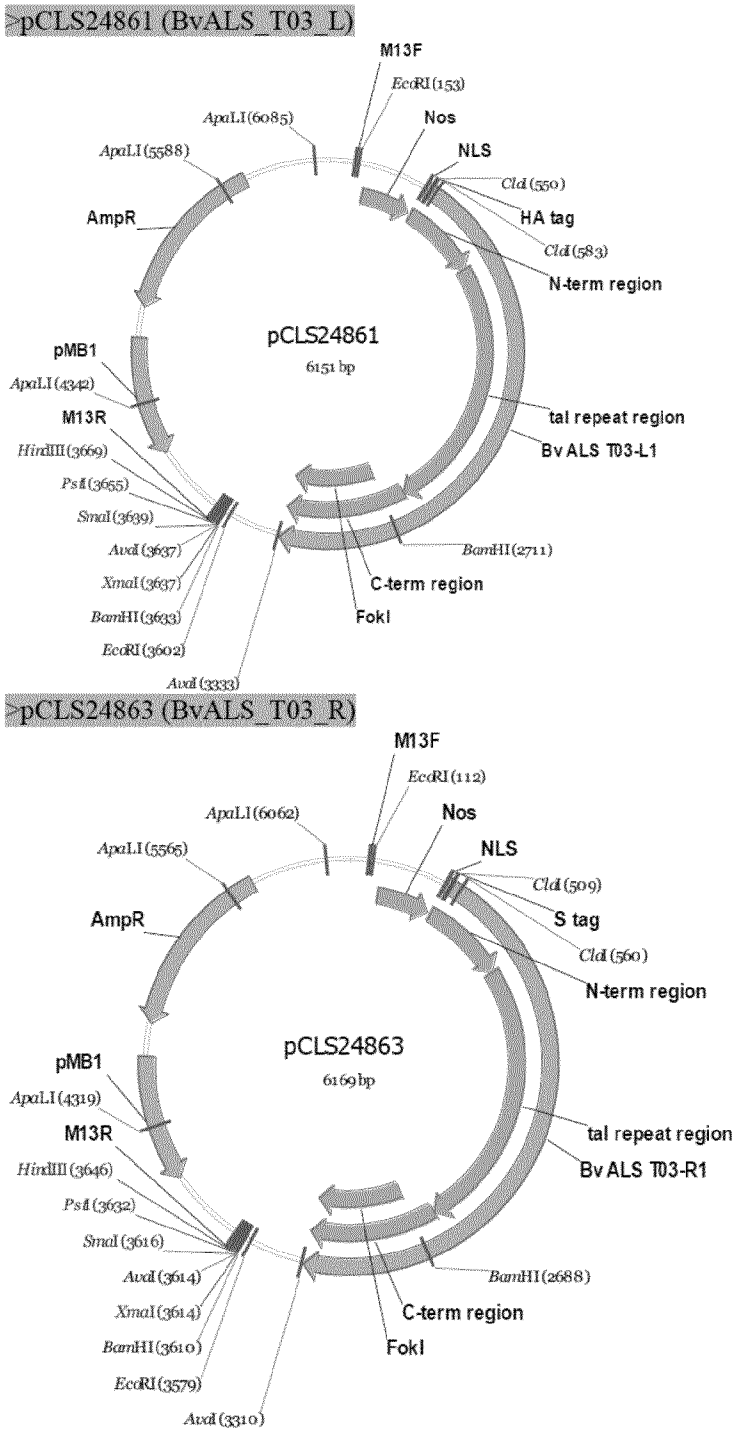


FIG.3C

Donor designed based on "4D6834_47C_1731C" sequence

>Original sequence

TCTTCAACCCCTCCCCTTTTCCACCCCTCCCAAAACCCCAACTCCACTCTTTCACCGTCCCCCTCCAAATCT
CATCCTCCCAATCCCACAAATCATCCGCCATTAAACACAAACTCAAGCACCTTCTTCTCCAGCT**TATTGA**
AGATTCATCTTTCGTTTCTCGATTGGCCCTGATGA**ACCCAGAAAAGGGTCCGATGTCCTCGTTGAAGCT**
CTTGAGCGTGAAGGTGTTACCAATGTGTTTGCTTACCCTGGTGGTGCATCTATGGAAATCCACCAAGCTC
TCACACGCTCTAAAACCATCCGCAATGTCCTCCCTCGCCAT**TGAACAAGGCGGGGTTTTCGCCGCCGAGGG**
ATATGCTAGAGCTACTGGAAAGGTGGTGTCTGCATTGCGACTTCTGGTCTGGTGCTACCAACCTCGTA
TCAGGTCTTGCTGACGCTCTCCTTGATTCTGTCCCTCTTGTTGCCATCACTGGCCAAGTTCACGCCGTA
TGATTGGCACTGATGCTTTTCAGGAGACTCC

>BvALST1 donor

TCTTCAACCCCTCCCCTTTTCCACCCCTCCCAAAACCCCAACTCCACTCTTTCACCGTCCCCCTCCAAATCT
CATCCTCCCAATCCCACAAATCATCCGCCATTAAACACAAACTCAAGCACCTTCTTCTCCAGC**GATCGA**
AGATTCATCTTTCGTTTCTCGATTGGCCCTGATGA**ACCCAGAAAAGGGTCCGATGTCCTCGTTGAAGCT**
CTTGAGCGTGAAGGTGTTACCAATGTGTTTGCTTACCCTGGTGGTTATTCTATGGAAATCCACCAAGCTC
TCACACGCTCTAAAACCATCCGCAATGTCCTCCCTCGCCAT**TGAACAAGGCGGGGTTTTCGCCGCCGAGGG**
ATATGCTAGAGCTACTGGAAAGGTGGTGTCTGCATTGCGACTTCTGGTCTGGTGCTACCAACCTCGTA
TCAGGTCTTGCTGACGCTCTCCTTGATTCTGTCCCTCTTGTTGCCATCACTGGCCAAGTTCACGCCGTA
TGATTGGCACTGATGCTTTTCAGGAGACTCC

Complete sequence of donor matrix pCLS26200

1 tgcagctctg gcccggtgtct caaaatctct gatgttacat tgcacaagat aaaaatatat
61 catcatgaac aataaaactg tctgcttaca taaacagtaa tacaaggggt gttatgagcc
121 atattcaacg ggaaacgtcg aggccgcgat taaattcaa catggatgct gatttatatg
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2341 atgtcctcgt tgaagctctt gagcgtgaag gtgttaccaa tgtgtttgct taccctggtg
2401 gttattctat ggaaatccac caagctctca cacgctctaa aaccatccgc aatgtcctcc
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2521 ttggtgtctg cattgcgact tctggctctg gtgctaccaa cctcgtatca ggtcttgctg
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 2881 attttctcct tacgcatctg tgcggtatctt cacaccgcat atggtgcact ctcagtacaa
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 3001 cctgacgggc ttgtctgctc ccggcatccg cttacagaca agctgtgacc gtctccggga
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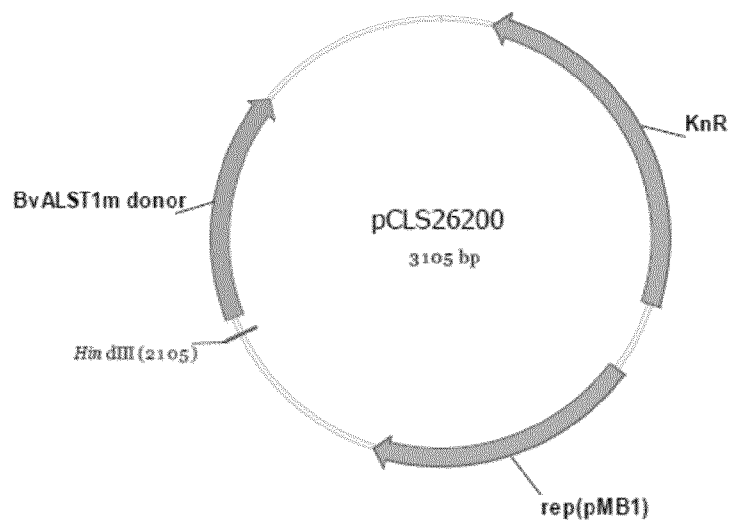


FIG.4

>BvALST2 donor

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AGATTTCATCTTTTCGTTTCTCGATTGGCCCTGATGAACCCAGAAAAGGGTCCGATGTCTCGTTGAAGCT
CTTGAGCGTGAAGGTGTTACCAATGTGTTTGCTTACCCTGGTGGTTATTCTATGGAAATCCACCAAGCTC
 TCACACGCTCTAAAACCATCCGCAATGTCTCCCTCGCCATGAACAAGGCGGGGTTTTCGCCGCCGAGGG
ATATGCTAGAGCTACTGGAAAGGTGTTGTTGCTGTCATTGCGACTTCTGGTCTGGTGTACCAACCTCGTA
 TCAGGTCTTGCTGACGCTCTCCTTGATTCTGTCCCTCTTGTGGCCATCACTGGCCAAGTTCCACGCCGTA
 TGATTGGCACTGATGCTTTTCAGGAGACTCC

Complete sequence of donor matrix pCLS26201

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1 tgcagctctg gcccggtgtct caaaatctct gatgttacat tgcacaagat aaaaatatat
61 catcatgaac aataaaactg tctgcttaca taaacagtaa tacaaggggt gttatgagcc
121 atattcaacg ggaaacgtcg aggccgcgat taaattccaa catggatgct gatttatatg
181 ggtataaatg ggctcgcgat aatgtcgggc aatcagggtgc gacaatctat cgcttgtatg
241 ggaagcccga tgcgccagag ttgtttctga aacatggcaa aggtagcgtt gccaatgatg
301 ttacagatga gatggtcaga ctaaaactggc tgacggaatt tatgcctctt ccgaccatca
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421 cagcattcca ggtattagaa gaatacctg attcagggtga aaatattgtt gatgcgctgg
481 cagtgttcct gcgcgggtg cattcgattc ctgtttgtaa ttgtcctttt aacagcgatc
541 gcgtatttcg tctcgtcag gcgcaatcac gaatgaataa cggtttggtt gatgcgagtg
601 attttgatga cgagcgtaat ggctggcctg ttgaacaagt ctggaaagaa atgcataaac
661 ttttgccatt ctcaccggat tcagtcgtca tcatggtga tttctcactt gataacctta
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901 tgatgctcga tgagttttt taatcagaat tggttaattg gttgtaacat tattcagatt
961 gggttgatt taaaacttca tttttaattt aaaaggatct aggtgaagat cctttttgat
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1081 gaaaagatca aaggatcttc ttgagatctt tttttctgc gcgtaatctg ctgcttgcaa
1141 acaaaaaaac caccgctacc agcgggtggtt tgtttgccgg atcaagagct accaactctt
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1261 ccgtagttag gccaccactt caagaactct gtgacaccgc ctacatacct cgctctgcta
1321 atcctgttac cagtggctgc tgccagtggc gataagtcgt gtcttaccgg gttggactca
1381 agacgatagt taccggataa ggccgacgg tcgggctgaa cgggggggtt cgtcacacag
1441 cccagcttgg agcgaacgac ctacaccgaa ctgagatacc tacagcgtga gctatgagaa
1501 agcggccacgc ttcccgaagg gagaaaggcg gacagggtatc cggtaagcgg cagggtcgga
1561 acaggagagc gcacgaggga gcttccaggg ggaacgcct ggtatcttta tagtcctgtc
1621 gggtttcgcc acctctgact tgagcgtcga tttttgtgat gctcgtcagg ggggaggagc
1681 ctatgaaaaa acgccagcaa cgcggccttt ttacggttcc tggccttttg ctggcctttt
1741 gctcacatgt tcttctcgc gttatccctt gattctgtgg ataaccgtat taccgccttt
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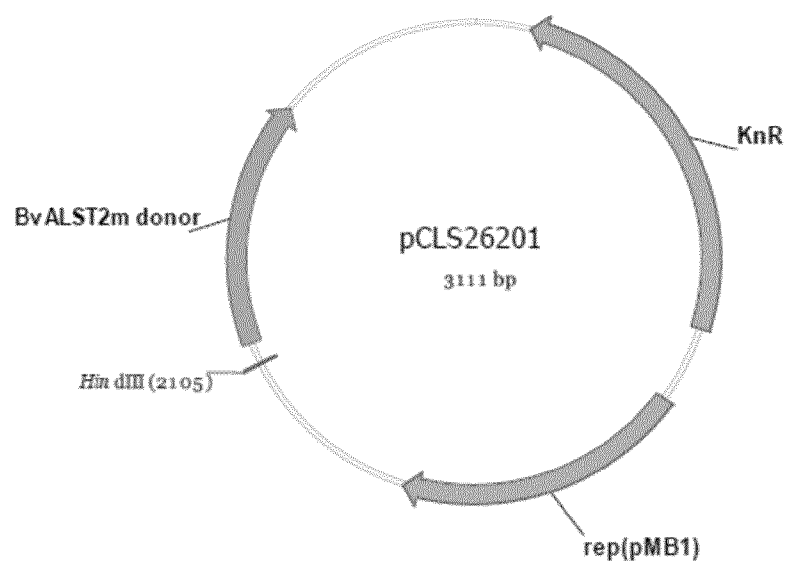


FIG.5

>BvALST3 donor

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TCACACGCTCTAAAACCATCCGCAATGTCTCCCTCGCCATGAACAAGGCGGGGTTTTCGCCGCCGAGGG
ATATGCTAGAGCTACCGGTAAGGTGTTGTTGCTGCTGCACTTCTGGTCTGGTGTACCAACCTCGTA
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Complete sequence of donor matrix pCLS26202

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181 ggtataaatg ggctcgcgat aatgtcgggc aatcagggtgc gacaatctat cgcttgtatg
241 ggaagccccg tgcgccagag ttgtttctga aacatggcaa aggtagcgtt gccaatgatg
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481 cagtgttcct gcgcgggtg cattcgattc ctgtttgtaa ttgtcctttt aacagcgatc
541 gcgtatttcg tctcgtcag gcgcaatcac gaatgaataa cggtttggtt gatgcgagtg
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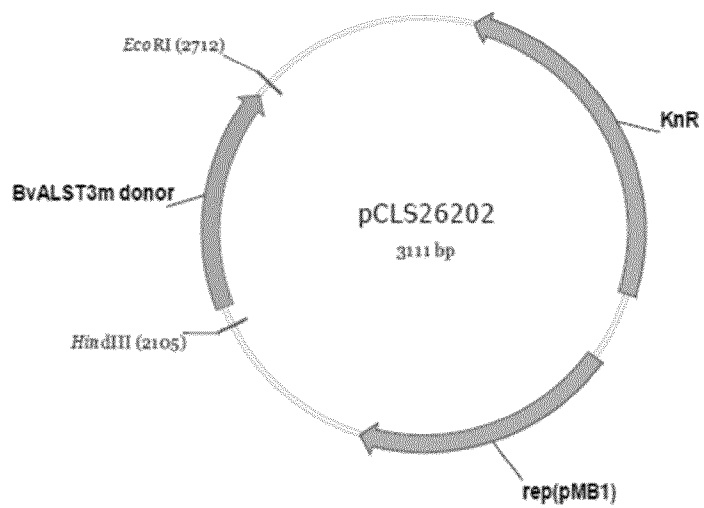


FIG.6

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/063216

A. CLASSIFICATION OF SUBJECT MATTER
 INV. 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)
 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 practicable, 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	Rüdiger Hain: "ALS-inhibitor-Tolerance in Sugar Beet", 18 July 2013 (2013-07-18), XP002742994, Retrieved from the Internet: URL: http://www.ewrs.org/doc/HTV/2013_05_28_ALS-inhibitor_Tolerance_in_Sugar_Beet_KWS_Bayer_for_public.pdf [retrieved on 2015-08-04] Slide 3	9
Y	WO 2014/071006 A1 (COLLECTIS [FR]; MATHIS LUC [FR]; VOYTAS DANIEL [US]; LI JIN [US]; ZHAN) 8 May 2014 (2014-05-08) claims 3,6 ----- -/-	1,3-8



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

12 August 2015

Date of mailing of the international search report

24/08/2015

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 NL - 2280 HV Rijswijk
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 Fax: (+31-70) 340-3016

Authorized officer

Obel, Nicolai

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/063216

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Y. ZHANG ET AL: "Transcription Activator-Like Effector Nucleases Enable Efficient Plant Genome Engineering", PLANT PHYSIOLOGY, vol. 161, no. 1, 2 November 2012 (2012-11-02), pages 20-27, XP055070911, ISSN: 0032-0889, DOI: 10.1104/pp.112.205179 abstract -----	1,3-8
Y	US 2011/201118 A1 (YANG BING [US] ET AL) 18 August 2011 (2011-08-18) paragraph [0150]; claim 1 -----	1-8
Y	WRIGHT T R ET AL: "BIOCHEMICAL MECHANISM AND MOLECULAR BASIS FOR ALS-INHIBITING HERBICIDE RESISTANCE IN SUGARBEET (BETA VULGARIS) SOMATIC CELL SELECTIONS", WEED SCIENCE, WEED SCIENCE SOCIETY OF AMERICA, CHAMPAIGN, IL, US, vol. 46, no. 1, 1 January 1998 (1998-01-01), pages 13-23, XP009002482, ISSN: 0043-1745 abstract -----	2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/063216

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