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(54) TARGETED GENE INTEGRATION IN PLANTS

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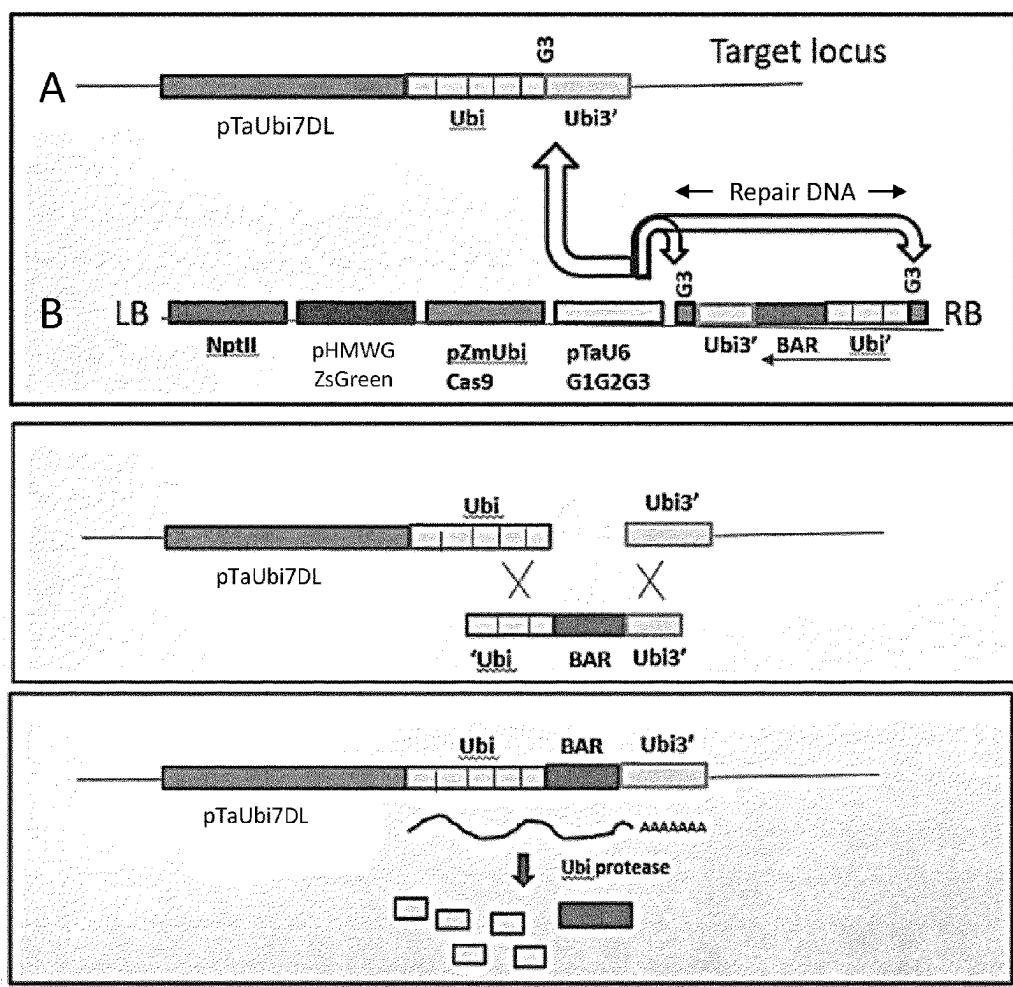
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ABSTRACT

The present invention relates to a vector suitable for a targeted integration of at least one gene of interest in 5' or 3' of a polyubiquitin gene in a plant. The present invention also relates to the use of said vector in a method for targeted insertion of at least one gene of interest in a plant genome and to a plant cell or plant tissue obtained by transformation with said vector. The present invention further relates to a method of identifying a plant having at least one gene of interest inserted in 5' or 3' of a polyubiquitin gene.

Specification includes a Sequence Listing.



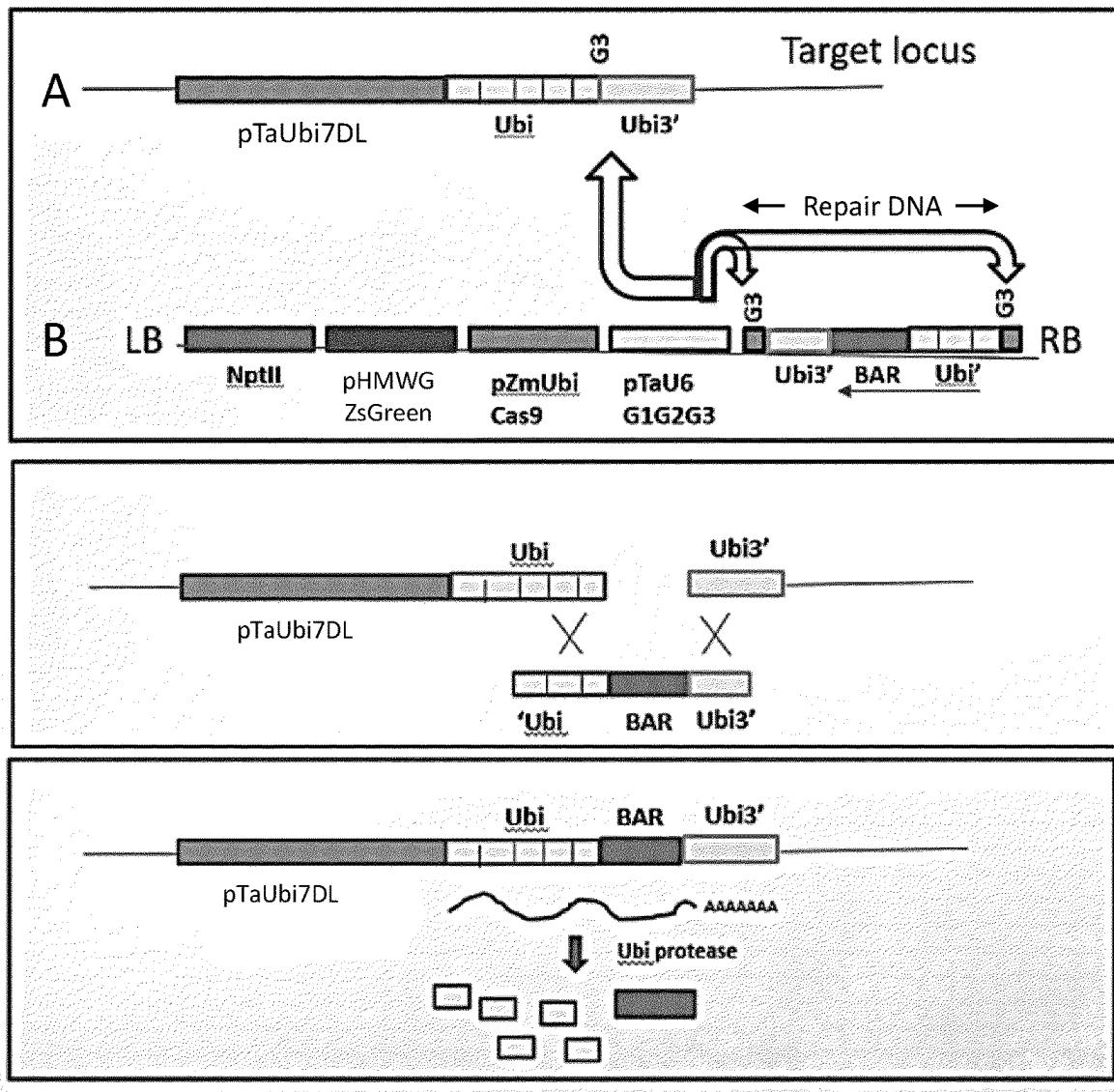


FIG.1

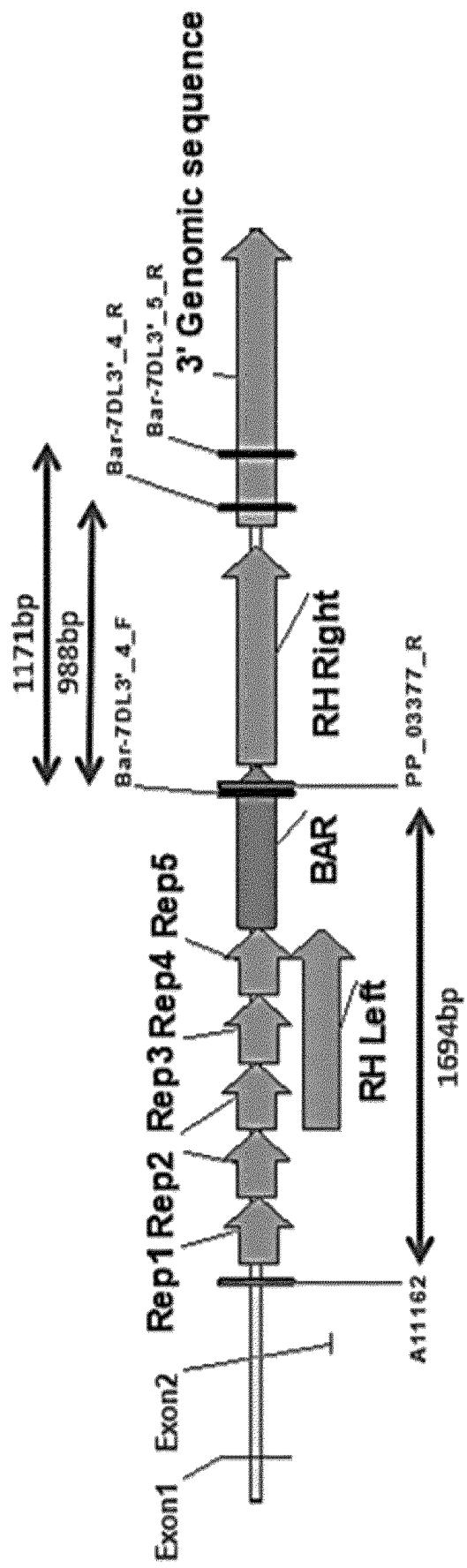


FIG.2

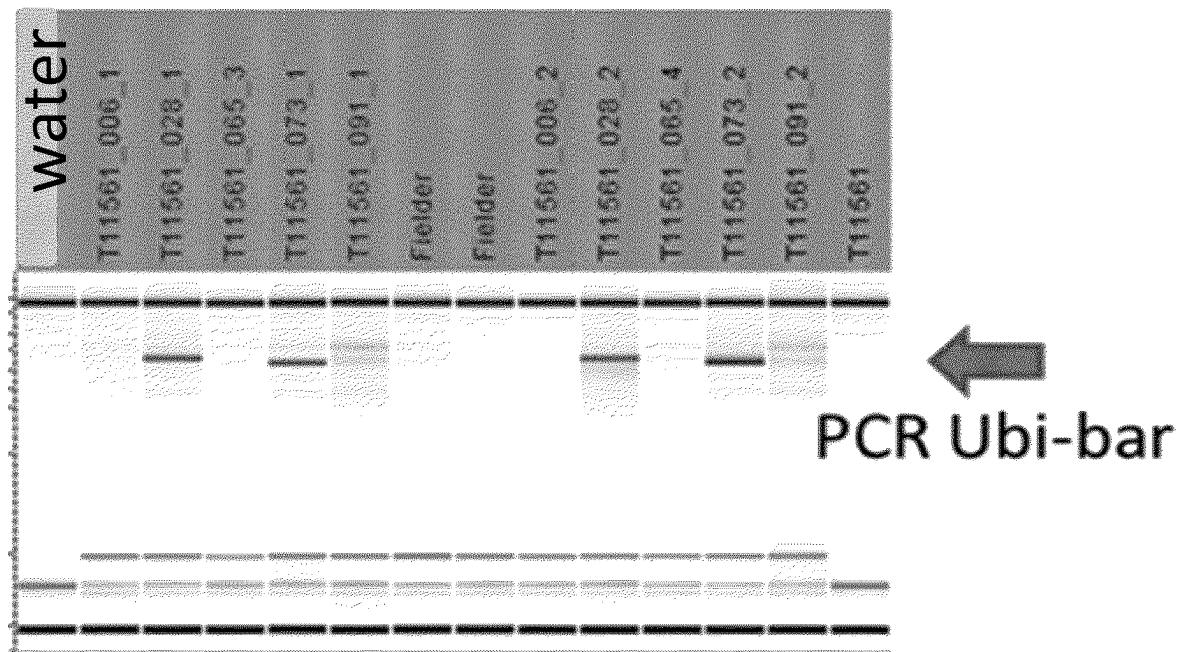
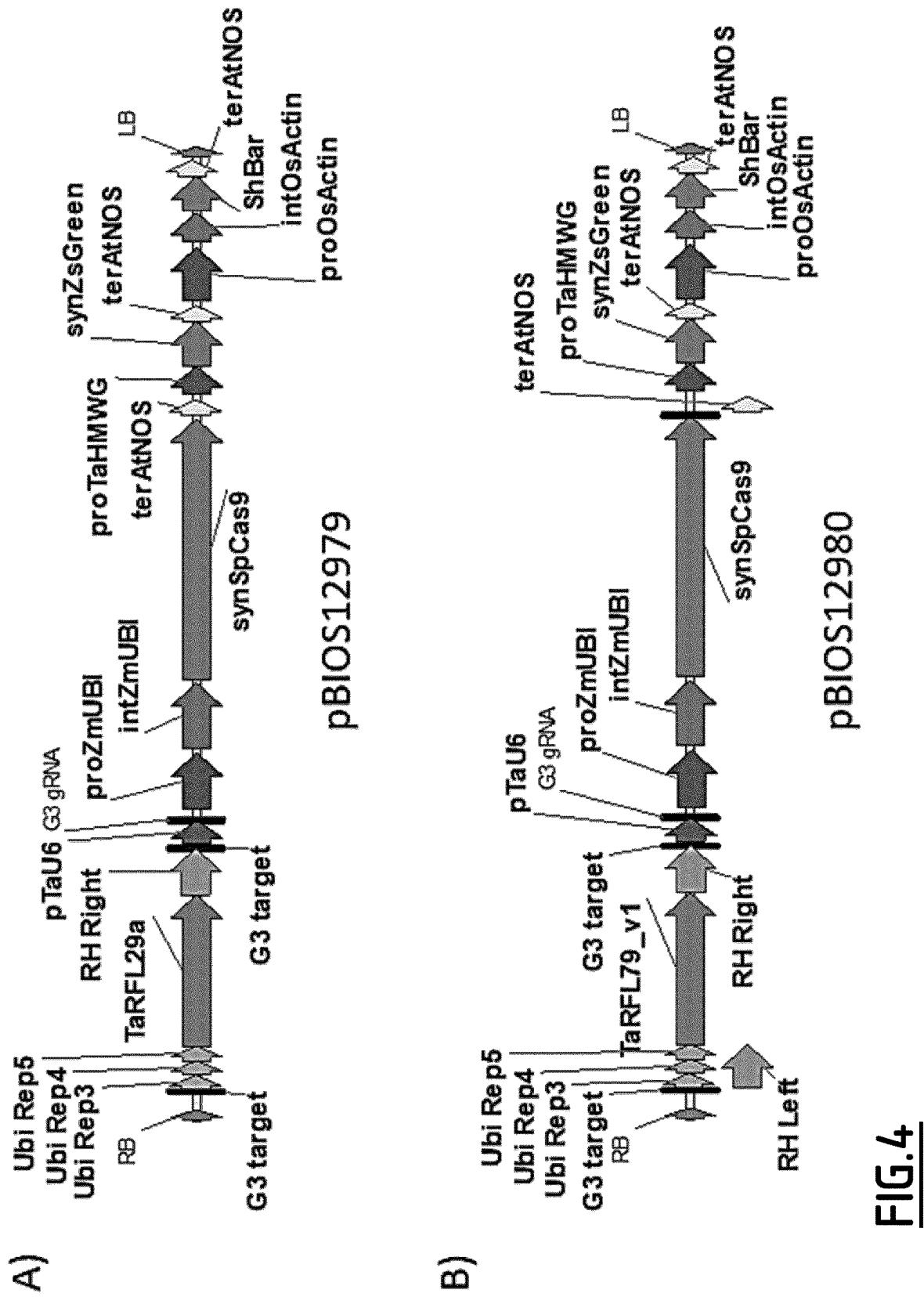


FIG.3



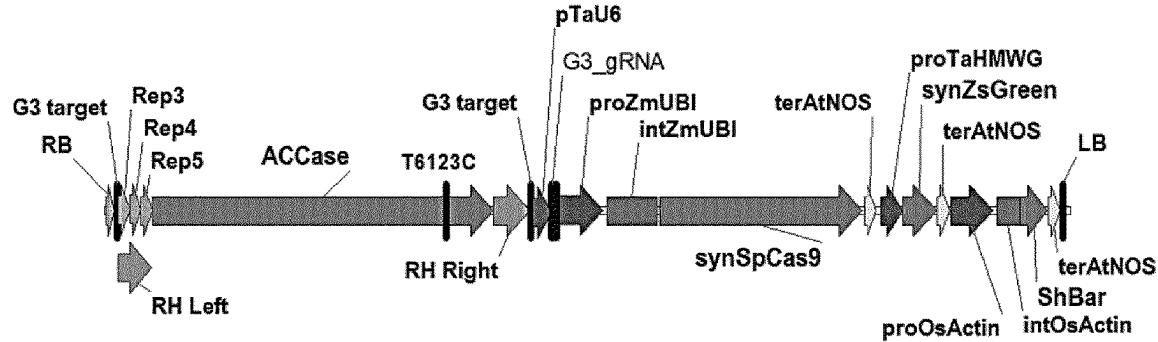


FIG.5

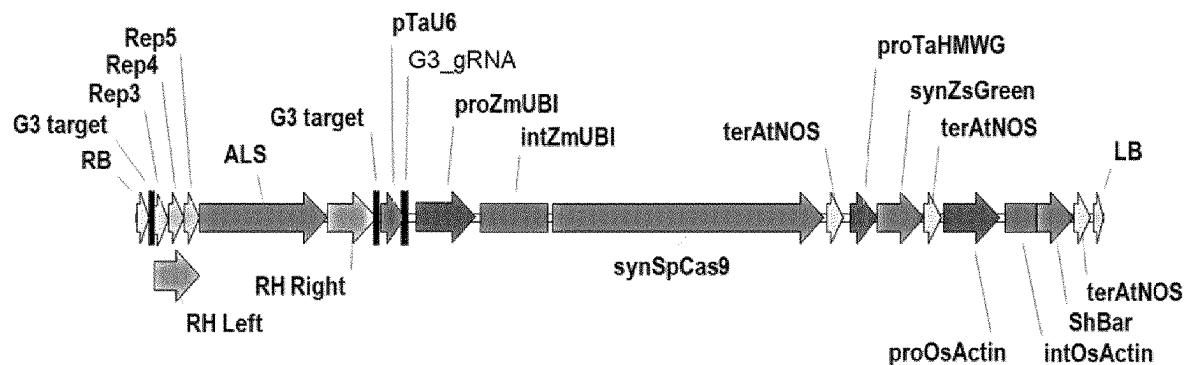


FIG.6

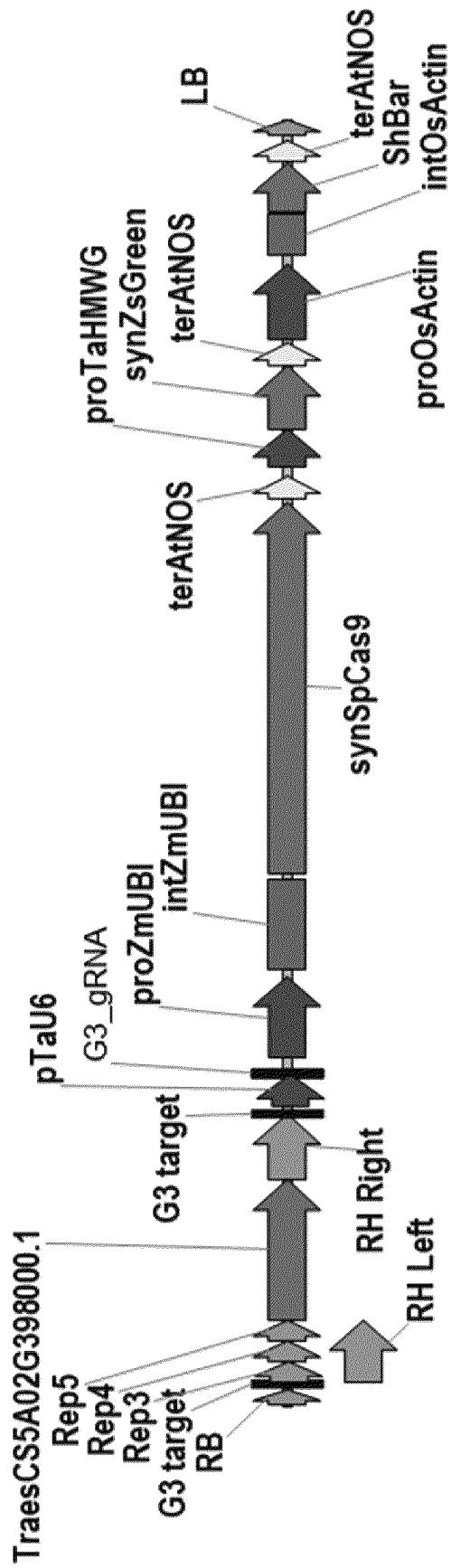


FIG. 7

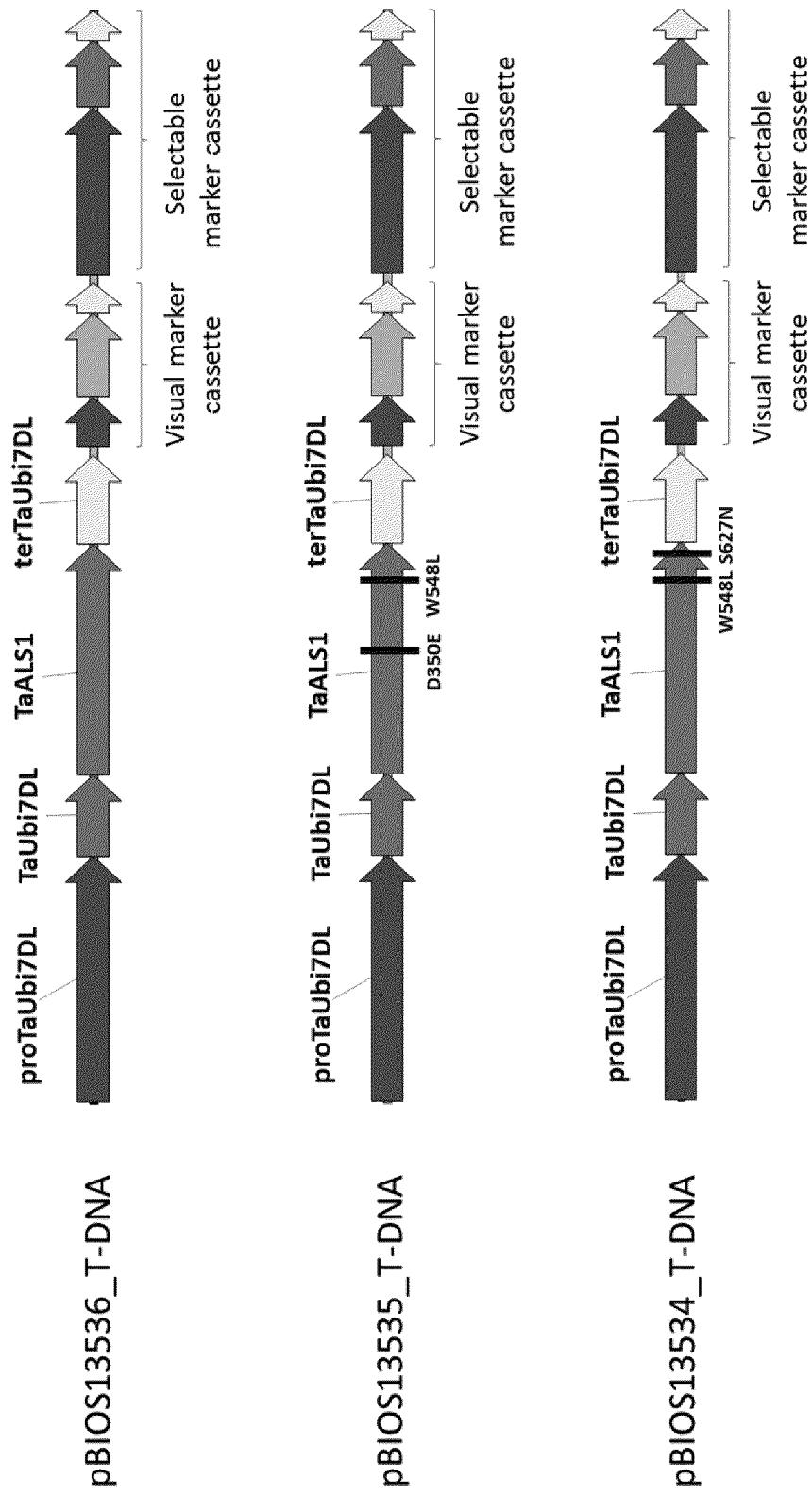
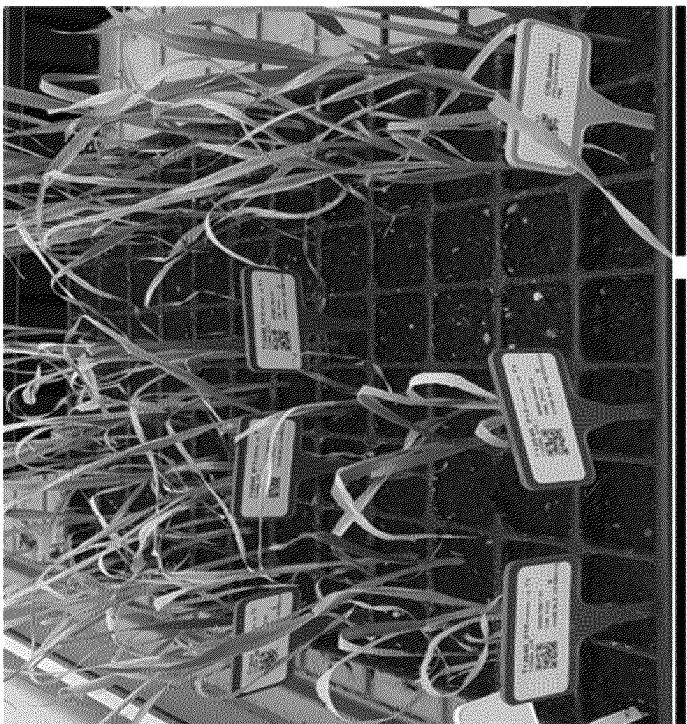


FIG.8



**polyUbi::Mutated ALS1
(W548L_S627N)**

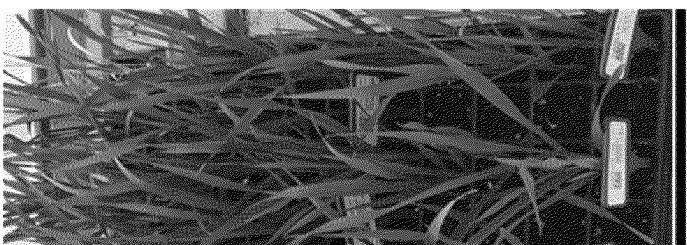


FIG. 9

TARGETED GENE INTEGRATION IN PLANTS

FIELD OF THE INVENTION

[0001] The present invention concerns the targeted integration of genes of interest in plants.

BACKGROUND OF THE INVENTION

[0002] Genetically modified plants often require constitutive and high-level transgene expression in order to possess a new desired agronomic trait. This is most often achieved by the transformation of the plant with one or several transgene cassette(s) comprising of a constitutive promoter linked to the gene of interest (GOI) causing the new trait, and a plant transcript polyadenylation sequence. Transgenic transformation events, created using biolistic or *Agrobacterium* transgene delivery, are usually randomly integrated into the plant genome.

[0003] However, this procedure is not optimal for several reasons. First, the transgene can integrate into endogenous genes, potentially causing a loss of function or altered functionality of these endogenous genes, leading to undesirable phenotypes, sometimes pleiotropic. Secondly, the level of expression of the transgene can be modulated by the surrounding genomic environment. Each transgenic event may have a different level of expression and spatial-temporal expression profile. Thirdly, often transgene events have multiple insertions of the transgene; these are usually discarded. Fourthly, random transgene insertions might create novel open reading frames that may prevent or slow deregulation of the transgenic event. Together, these factors result in the need to make 1000 or more primary plant transformants in order to arrive at a commercially and agronomically acceptable genetically modified event.

[0004] Positioning of a transgene at a defined place in the genome (named a Landing Pad) addresses the concerns outlined above, however an ideal genome location for transgene insertion needs to be identified and tested. Positioning a transgene outside of an endogenous gene is not ideal since the performance of the transgene in terms of expression and stability of expression is hard to predict. Positioning within an endogenous gene would reduce this uncertainty, but as described above might disrupt the function of the endogenous gene. WO2013169/802 for example discloses methods of nuclease-mediated integration of transgenes.

[0005] WO2018/005589 discloses different methods of insertion of a gene of interest in a plant genome. When the insertion of a gene of interest is to be performed in the 3' region of a gene sequence comprising a stop codon, this document discloses that the insertion must occur before the stop codon. A T2A sequence has to be introduced alongside the gene of interest in order to liberate the protein of interest from the fusion protein obtained after the insertion.

[0006] Hondred et al. (1999, Plant Physiol. 119:713-24. doi: 10.1104/pp. 119.2.713.) have demonstrated via transgenesis in tobacco that β-glucuronidase (GUS), fused translationally to the 3' end of polyubiquitin, is highly expressed and processed by an endogenous protease liberating GUS.

[0007] There is still a need for improved methods allowing targeted gene integrations in plants, wherein the gene of interest is efficiently expressed.

DESCRIPTION OF THE INVENTION

[0008] The Inventors have surprisingly found that inserting a gene of interest (GOI) in 5' or in 3' of an endogenous polyubiquitin gene in a plant, such that the GOI is expressed as a polyubiquitin: GOI encoded protein fusion, allows an efficient targeted gene insertion. The fusion protein is then processed by endogenous ubiquitin proteases to release the protein encoded by the GOI and ubiquitin monomers.

[0009] Advantageously, GOI insertion according to the invention has no effect on the functionality of the polyubiquitin gene and allows an efficient and stable expression of the GOI.

[0010] Besides, the advantages of a polyubiquitin gene as a Landing Pad are that the expression of the GOI is directed by a strong and constitutive endogenous polyubiquitin promoter. The expression of a GOI under this strong and constitutive promoter is also within the scope of the invention. All GOI insertion events at polyubiquitin should have similar levels of expression.

[0011] Interestingly, if the short amino acid target site cleaved by the polyubiquitin protease is introduced between several GOI elements, multiple GOIs can be expressed.

[0012] Furthermore, targeting of the protein of interest to various cellular compartments (cytosol, mitochondria or plastids) is not affected by this method and correct expression of GOI in one or more cellular compartments are under the scope of the invention.

[0013] A first object of the invention is thus a vector

[0014] suitable for a targeted integration of at least one gene of interest in 5' or 3' of a polyubiquitin gene in a plant, wherein said vector comprises a repair DNA comprising from 5' to 3':

[0015] a first gRNA target,

[0016] a left ubiquitin-like region,

[0017] at least one gene of interest,

[0018] a right ubiquitin-like region, and

[0019] a second gRNA target.

[0020] The vector as defined above may further comprises:

[0021] at least one CRISPR-Cas endonuclease expression cassette and/or

[0022] at least one gRNA expression cassette, preferably encoding a gRNA able to recognize a region in 3' or 5' of the polyubiquitin gene, preferably a single gRNA expression cassette.

[0023] These two or three cassettes may be on the same or in different vectors.

[0024] The gene of interest to be integrated may for example be selected from the group consisting of a herbicide tolerance gene, an insect resistance gene, a fungal resistance gene, a bacterial resistance gene, a stress resistance gene, a gene involved in reproductive capability, a gene involved in performance in the fields, a gene involved in performance in an industrial process and a gene involved in nutritional value of a plant.

[0025] Said gene of interest may for example be selected from the group consisting of a BAR gene, ALS gene, GS gene, cyt P450 gene, RFL29a gene, RFL79 gene, Rfo gene, Cry1Ac gene and RCA-Cry1 Ac gene.

[0026] Another object of the invention is a plant cell or plant tissue which is transformed with the vector as defined above.

[0027] Another object of the invention is a plant cell or plant tissue comprising at least one gene of interest inserted

in 5' or in 3' of the polyubiquitin gene, obtained by transformation with the vector as defined above.

[0028] Said plant cell or plant tissue may for example be a protoplast, apical meristem, cotyledon, embryo, pollen and/or microspores.

[0029] Another object of the invention is a plant comprising at least one gene of interest inserted in 5' or in 3' of the polyubiquitin gene, obtained by transformation with the vector as defined above.

[0030] The plant, plant cell or plant tissue comprises at least one polyubiquitin gene.

[0031] Said plant may be a monocotyledon or a dicotyledon.

[0032] Another object of the invention is a progeny plant of a plant as defined above, wherein said progeny plant comprises at least one gene of interest inserted in 5' or in 3' of the polyubiquitin gene.

[0033] Another object of the invention is a method for targeted insertion of at least one gene of interest in 5' or 3' of a polyubiquitin gene in a plant genome, comprising:

[0034] a. the transformation of a plant cell or plant tissue with at least one vector as defined above, to obtain a transformed plant cell or plant tissue, and

[0035] b. the regeneration of the plant from the transformed plant cell or plant tissue.

[0036] In the method as defined above, at least one CRISPR-Cas endonuclease expression cassette may be provided by said vector or in a separate vector and wherein at least one gRNA expression cassette is provided by said vector or in a separate vector.

[0037] Another object of the invention is a method for expressing at least one protein of interest in a plant, comprising the steps of the method as defined above for targeted insertion of at least one gene of interest in 5' or 3' of a polyubiquitin gene in a plant genome, wherein said gene of interest codes for said protein of interest.

[0038] Another object of the invention is the use of the vector as defined above, for expressing at least one gene of interest in a plant, in a plant cell or in a plant tissue.

[0039] Another object of the invention is a method of identifying a plant comprising at least one gene of interest inserted in 5' or 3' of a polyubiquitin gene, wherein said method comprises:

[0040] extracting the DNA and/or RNA of a plant,

[0041] detecting the presence of a DNA comprising at least one gene of interest inserted in 5' or 3' of a polyubiquitin gene and/or the presence of a RNA transcript from said DNA, and

[0042] optionally, detecting the presence of a protein encoded by said at least one gene of interest.

Plant

[0043] The plant for use according to the invention comprises at least one polyubiquitin gene.

[0044] The plant as defined above may be a monocotyledon or a dicotyledon.

[0045] The plant may for example be selected from the group consisting of wheat, maize, rapeseed, rice, oat, barley, sugarcane, sunflower, soybean, cotton, potato and tomato.

[0046] The plant as defined above is preferably an agronomic plant.

[0047] By the term "agronomic plant", it is herein meant a plant suitable for production on a large scale, in particular for human and animal food or for industrial purposes, such as biofuel.

Gene of Interest

[0048] The gene of interest is preferably a gene whose expression in a plant results in at least one phenotype of interest.

[0049] A phenotype of interest is for example:

[0050] an herbicide tolerance,

[0051] a resistance, such as an insect resistance, a fungal resistance, a bacterial resistance, a stress resistance (for example as a water stress resistance),

[0052] a reproductive capability, for example fertility,

[0053] an improved performance in the fields, such as an improved yield, a tolerance to abiotic stress or tolerance to biotic stress,

[0054] an improved performance in an industrial process, for example an improved biofuel production or

[0055] an improved nutritional value, for example an increased oil content.

[0056] Herbicide tolerance may for example be tolerance to PPO (protoporphyrinogen oxidase) inhibitor herbicide (see for example WO201522636, WO201592706) or tolerance to EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) inhibitors, such as resistance to glyphosate-based herbicides.

[0057] The gene of interest may for example be selected from the group consisting of:

[0058] an herbicide tolerance gene,

[0059] an insect resistance gene, a fungal resistance gene, a bacterial resistance gene, a stress resistance gene,

[0060] a gene involved in a reproductive capability (for example a gene involved in fertility restoration, such as a CMS (cytoplasmic male sterility) restorer gene),

[0061] a gene involved in performance in the fields (for example, a gene allowing an improved yield, a tolerance to abiotic stress or a tolerance to biotic stress),

[0062] a gene involved in performance in an industrial process and

[0063] a gene involved in nutritional value of a plant (for example, a gene allowing an increased oil content).

[0064] The herbicide tolerance gene may for example be selected from the group consisting of:

[0065] a BAR gene, for example the BAR gene of sequence SEQ ID NO: 1,

[0066] a gene encoding a wheat acetyl-CoA Carboxylase (ACCase) (for example ACCase chrA SEQ ID NO: 29 encoding SEQ ID NO: 30, ACCase chrB SEQ ID NO: 31 encoding SEQ ID NO: 32 or ACCase chrD SEQ ID NO: 33 encoding SEQ ID NO: 34) comprising the CoAxium mutation Ala2004Val (U.S. Pat. No. 9,578,880_B2) or other mutations from EP2473022_B1,

[0067] a gene encoding a wheat Acetolactate Synthase (ALS) mutated individually in an amino-acid (numbering according to the reference *Arabidopsis* ALS protein encoded by AT3G48560) A122, P197, A205, D376, W574 or S653, or any of the 4 mutations P197, A205, D376 or W574 in combination between them or with A122 or S653, or the combination of mutations D376 and W574, or W574 and S653. A gene encoding

wild-type wheat ALS comprises or consist of, for example, ALS chr6 genome A of sequence SEQ ID NO: 35 encoding SEQ ID NO: 36, ALS chr6 genome B of sequence SEQ ID NO: 37 encoding SEQ ID NO: 38, or ALS chr6 genome D of sequence SEQ ID NO: 39 encoding SEQ ID NO: 40. Preferred genes encode a mutated wheat ALS that comprises or consists of the polypeptide sequence of SEQ ID NO: 79 (ALS chr6D with amino-acid substitutions D350E and W548L); SEQ ID NO: 80 (ALS chr6D with amino-acid substitutions W548L and S627N), SEQ ID NO: 81 (ALS chr6A with amino-acid substitutions D350E and W548L), SEQ ID NO: 82 (ALS chr6A with amino-acid substitutions W548L and S627N), SEQ ID NO: 83 (ALS chr6B with amino-acid substitutions D350E and W548L), or SEQ ID NO: 84 (ALS chr6B with amino-acid substitutions W548L and S627N); or

[0068] a gene encoding a cytochrome P450 involved in herbicide detoxification. Amongst those cytochrome P450, one can name

[0069] the *Lolium rigidum* CYP81A10v7 described by Han et al. (2021) (SEQ ID NO: 71 encoding SEQ ID NO: 72),

[0070] The maize CYP81A2 (SEQ ID NO: 73 encoding SEQ ID NO: 74) or ZmCYP81A9 (SEQ ID NO: 75 encoding SEQ ID NO: 76) sequences described by Brazier-Hicks et al. (2022)

[0071] a wheat gene (TraesCS5A02G398000) of sequence SEQ ID NO: 41 encoding SEQ ID NO: 42, which is orthologous to the *Lolium rigidum* CYP81A10v7 sequence,

[0072] a wheat CYP71 gene (SEQ ID NO: 77 encoding SEQ ID NO: 78) present under the Su1 QTL for chlorotoluron herbicide tolerance,

[0073] or any wheat cytochrome P450 gene (and orthologous genes from the poaceae clade) involved in herbicide detoxification (Barret 1995; Dimaano and Iwakami, 2020).

[0074] a gene encoding a glutamine synthetase GS1 mutated in amino-acid 59 (numbering according to the *Eleusine indica* GS1-1 protein with GenBank accession number on the NCBI server UJO02307.1, entry from Jan. 29, 2022) as described by Zhang et al. (2022) and/or amino-acid 296 as described in WO2021000870), or a glutamine synthetase GS2 mutated in amino-acid 171 (D171N) (numbering according to the *Lolium rigidum* GS2 protein with GenBank accession number on the NCBI server QEG99483.1, entry from Aug. 28, 2019) as described by Avila-Garcia et al. (2012).

[0075] The fertility restorer gene may for example be selected from the group consisting of a RFL29a gene (for example of sequence SEQ ID NO: 17), a RFL79 gene (for example of sequence SEQ ID NO: 19) and Rfo gene (for example of sequence SEQ ID NO: 55).

[0076] The insect resistance gene may for example be Cry1Ac gene (for example of sequence SEQ ID NO: 50) or a Cry1Ac gene that has an N-terminal chloroplast targeting signal from Rubisco Activase (RCA), also referred to as RCA-Cry1Ac (for example of sequence SEQ ID NO: 52).

[0077] In one preferred embodiment, the gene of interest is for example selected from the group consisting of the BAR gene, ALS gene, GS, cyt P450 gene, RFL29a gene, RFL79 gene, Rfo gene and Cry1Ac gene.

[0078] One or at least two genes of interest (for example two, three or at least four genes of interest) may be integrated in the plant in 5' or in the 3' of a polyubiquitin gene.

[0079] When at least two genes of interest are integrated, these genes may be identical or different. They are preferably different.

[0080] By the expression «gene X», it is herein meant (i) gene X, (ii) the cDNA corresponding to gene X, (iii) a nucleic acid encoding the protein encoded by gene X or (iv) a nucleic acid encoding a protein having at least 90% identity with the protein encoded by gene X, preferably at least 95% identity, more preferably at least 98% identity, provided that both proteins share the same or a similar biological activity (in particular resulting in the same phenotype of interest).

Vector

[0081] The present invention particularly relates to a vector suitable for a targeted integration of at least one gene of interest in a plant in 5' or in 3' of a polyubiquitin gene.

[0082] The vector may be a plasmid.

[0083] The vector as defined above comprises a repair DNA, wherein said repair DNA comprises from 5' to 3':

[0084] a first gRNA target,

[0085] a left ubiquitin-like region,

[0086] at least one gene of interest,

[0087] a right ubiquitin-like region, and

[0088] a second gRNA target.

[0089] The gene of interest is particularly as defined above in the section “gene of interest”.

[0090] The polyubiquitin gene is an endogenous polyubiquitin gene of the plant.

[0091] The plant may comprise several polyubiquitin genes.

[0092] The polyubiquitin gene is preferably under the control of a strong promoter.

[0093] The polyubiquitin gene is for example the wheat Ubi7AL gene of sequence SEQ ID NO: 5, the wheat Ubi7BL gene of sequence SEQ ID NO: 4, wheat Ubi7DL gene of sequence SEQ ID NO: 3, the maize gene of sequence SEQ ID NO: 43, the maize gene of sequence SEQ ID NO: 44, the maize gene of sequence SEQ ID NO: 45, the maize gene of sequence SEQ ID NO: 46, the *B. napus* gene of sequence SEQ ID NO: 57, the *B. napus* gene of sequence SEQ ID NO: 58 or the *B. napus* gene of sequence SEQ ID NO: 59.

[0094] The polyubiquitin gene comprises tandem repeats, hereafter referred to as “repeats” or “Ubi repeats”, each repeat encoding an ubiquitin protein.

[0095] By the expression “targeted integration in 3' of a polyubiquitin gene”, it is herein meant that integration of the at least one gene of interest occurs upstream of the stop codon of the polyubiquitin gene, or at the stop codon of the polyubiquitin gene, such that the polyubiquitin stop codon is replaced by the first codon of the gene of interest.

[0096] By the expression “targeted integration in 5' of a polyubiquitin gene”, it is herein meant that integration of the at least one gene of interest occurs at the start codon or downstream the start codon, preferably directly following the start codon of the polyubiquitin gene and no further than 60 nucleotides from the start codon. When the integration occurs at the start codon, the start codon of the gene of interest replaces the start codon of the polyubiquitin gene. In this embodiment, the repair DNA for example comprises from 5' to 3':

- [0097] a first gRNA target,
- [0098] a left ubiquitin-like region,
- [0099] at least one gene of interest,
- [0100] a site cleavable by a Ubi protease,
- [0101] the start codon of the first Ubi repeat of the polyubiquitin gene,
- [0102] a right ubiquitin-like region, and
- [0103] a second gRNA target.

[0104] The exact location of the integration is defined by the left ubiquitin-like region and the right ubiquitin-like region of the repair DNA.

[0105] The site of integration of the at least one gene of interest is chosen so that the Ubi gene and the gene(s) of interest are in frame, in order to obtain a single RNA transcript and then a fusion protein.

[0106] The site of integration of the at least one gene of interest is chosen so that the inserted gene of interest is flanked by one or two Ubi protease domains, to obtain a cleavage of the fusion protein and release the encoded protein(s) of interest.

[0107] An Ubi protease domain is a site cleavable by an Ubi protease.

[0108] The vector may also comprise at least one site cleavable by an Ubi protease, to obtain a cleavage of the fusion protein and the release of the encoded protein(s) of interest.

[0109] The vector as defined above is preferably suitable for a targeted integration of at least one gene of interest in a plant in 5' or in 3' of a polyubiquitin gene, wherein the integrated gene is flanked by one or two sequences encoding a sequence cleavable by an Ubi protease, in particular to allow releasing of the encoded protein(s) of interest from the Ubi protein(s).

[0110] The vector as defined above preferably comprises a repair DNA, wherein said repair DNA comprises from 5' to 3':

- [0111] a first gRNA target,
- [0112] a left ubiquitin-like region,
- [0113] optionally, a site cleavable by a Ubi protease, in particular for an integration in 3' of the polyubiquitin gene,
- [0114] at least one gene of interest,
- [0115] optionally, a site cleavable by a Ubi protease, in particular for an integration in 5' of the polyubiquitin gene,
- [0116] a right ubiquitin-like region, and
- [0117] a second gRNA target.

[0118] The repair DNA in the vector as defined above preferably comprises a site cleavable by an Ubi protease, wherein said site is in 5' of the gene of interest for an integration in 3' of the polyubiquitin gene or is in 3' of the gene of interest for an integration in 3' of the polyubiquitin gene.

[0119] The site cleavable by an Ubi protease may be provided in the form of a region of the polyubiquitin gene comprising a site recognized by an Ubi protease (for example a region encoding for at least the last 6, 8, 10, 12 or 14 amino acids of the C-terminus of a protein encoded by any repeat of the polyubiquitin gene except the last repeat).

[0120] As defined herein, an ubiquitin-like region preferably comprises:

- [0121] a sequence homologous to a sequence comprising the end of the coding region of the polyubiquitin

gene and at least a portion of the 3'UTR region of the polyubiquitin gene, for a targeted insertion in 3' of the polyubiquitin gene, or

[0122] a sequence homologous to a sequence comprising at least a portion of the 5'UTR region of the polyubiquitin and the start of the coding region of the polyubiquitin gene, for a targeted insertion in 5' of the polyubiquitin gene.

[0123] The ubiquitin-like region as defined above comprises a left ubiquitin-like region in 5' and a right ubiquitin-like region in 3'. Such an ubiquitin-like region may thus comprise, between the left ubiquitin-like region and the right ubiquitin-like region, a sequence homologous to a sequence of the polyubiquitin gene, which is lost upon the targeted integration of the at least one gene of interest. Alternatively, the ubiquitin-like region may consist of a left ubiquitin-like region in 5' and a right ubiquitin-like region in 3'.

[0124] By the expression "a sequence homologous to sequence X", it is particularly meant that said sequence has at least 85% identity with sequence X, preferably at least 90%, more preferably at least 95%, more preferably at least 96%, at least 97%, at least 98% or at least 99% identity with sequence X.

[0125] In a preferred embodiment, a sequence homologous to X is identical to sequence X.

[0126] The expression "a region Y is homologous to a region X" herein means that the sequence of region Y is homologous to the sequence of region X.

[0127] The present invention preferably relates to a vector as defined above,

[0128] wherein said left ubiquitin-like region and said right ubiquitin-like region are homologous to a region in 5' and in 3', respectively, of a sequence comprising the end of the coding region of the polyubiquitin gene and at least a portion of the 3'UTR region of the polyubiquitin gene, for a targeted insertion in 3' of the polyubiquitin gene, and

[0129] wherein said left ubiquitin-like region and said right ubiquitin-like region are homologous to a region in 5' and in 3', respectively, of a sequence comprising at least a portion of the 5'UTR region of the polyubiquitin and the start of the coding region of the polyubiquitin gene for a targeted insertion in 5' of the polyubiquitin gene.

[0130] When the plant comprises more than one polyubiquitin gene, the ubiquitin-like domain preferably comprises a sequence homologous to a sequence present in only one of these polyubiquitin genes, preferably a sequence homologous to 5' UTR or 3' UTR. The 5'UTR and 3'UTR indeed comprise variations between the different polyubiquitin genes in a genome that allow the targeting of a specific polyubiquitin gene.

[0131] The ubiquitin-like region preferably comprises at least 50 nucleotides, preferably at least 100 nucleotides, preferably at least 400 nucleotides, preferably at least 700 nucleotides, preferably at least 900 nucleotides, preferably at least 1200 nucleotides, more preferably at least 1400 nucleotides and/or at most 1900 nucleotides, preferably at most 1700 nucleotides, more preferably at most 1900 nucleotides.

[0132] The left and/or right ubiquitin-like region preferably comprises at least 50 nucleotides, preferably at least 100 nucleotides, preferably at least 200 nucleotides, more preferably at least 300 nucleotides, more preferably at least 400 nucleotides, more preferably at least 500 nucleotides,

more preferably at least 600 nucleotides and/or at most 1900 nucleotides, preferably at most 1700 nucleotides, more preferably at most 1500 nucleotides, more preferably at most 1300 nucleotides, more preferably at most 1100 nucleotides, more preferably at most 900 nucleotides.

[0133] For an insertion in 3' of the polyubiquitin gene, the left ubiquitin-like region may comprise the last repeat of the polyubiquitin gene, preferably the two last repeats of the polyubiquitin gene, more preferably the three last repeats of the polyubiquitin gene. For example, the left ubiquitin-like region may comprise repeats 3 to 5 of the polyubiquitin gene for an insertion in 3' of the polyubiquitin gene.

[0134] For an insertion in 5' of the polyubiquitin gene, the left ubiquitin-like region may comprise a portion of the 5' UTR or the 5' UTR of the polyubiquitin gene.

[0135] For an insertion in 3' of the polyubiquitin gene, the right ubiquitin-like region may comprise a portion of the 3'UTR or the 3'UTR of the polyubiquitin gene. For example, the right ubiquitin-like region may comprise the terminator of the polyubiquitin gene plus adjacent intergenic regions for an insertion in 3' of the polyubiquitin gene.

[0136] For an insertion in 5' of the polyubiquitin gene, the right ubiquitin-like region may for example comprise repeats 1 to 3 of the polyubiquitin gene.

[0137] A left ubiquitin-like region, in particular suitable for a 3' insertion, may for example comprise or consist of sequence SEQ ID NO: 66, which is homologous to repeats 3 to 5 of the polyubiquitin gene Ubi7DL.

[0138] A right ubiquitin-like region, in particular suitable for a 5' insertion, may for example comprise or consist of sequence SEQ ID NO: 67, which is homologous to repeats 1 to 3 of the polyubiquitin gene Ubi7DL.

[0139] A right ubiquitin-like region, in particular suitable for a 3' insertion, may for example comprise or consist of sequence SEQ ID NO: 68, which is homologous to a region of the 3'UTR region of the polyubiquitin gene Ubi7DL.

[0140] A left ubiquitin-like region, in particular suitable for a 5' insertion, may for example comprise or consist of sequence SEQ ID NO: 69, which is homologous to a region of the 5'UTR region of the polyubiquitin gene Ubi7DL.

[0141] The first gRNA target and the second gRNA target comprise a sequence complementary to a gRNA.

[0142] In one embodiment, the first gRNA target and the second gRNA target may comprise a sequence complementary to the same gRNA.

[0143] The gRNA target preferably comprises at least 15 nucleotides, preferably at least 17 nucleotides, more preferably at least 18 nucleotides and/or at most 25 nucleotides, preferably at most 22 nucleotides, more preferably at most 20 nucleotides.

[0144] The gRNA target for example consists of 17, 18, 19 or 20 nucleotides.

[0145] The first and second gRNA target sequences are preferably identical.

[0146] The first and/or second gRNA target may for example comprise or consist of sequence SEQ ID NO: 8.

[0147] The repair DNA as defined above preferably does not encode a polypeptide comprising a cleavable sequence other than sequence(s) cleavable by an Ubi protease.

[0148] The expressions "sequence cleavable by an Ubi protease" or "site recognized by a Ubi protease" are herein synonymous.

[0149] If needed, in particular depending of the site of integration, the repair DNA as defined above may comprise

a sequence cleavable by an Ubi protease, so as to be able to separate the protein of interest from the Ubi protein.

[0150] When the repair DNA as defined above comprises at least two genes of interest, the repair DNA further comprises at least one sequence encoding one cleavable sequence between each two genes of interest, said cleavable sequence(s) being a sequence cleavable by an Ubi protease.

[0151] The proper release of the protein of interest from the polyubiquitin fusion protein is also important for proteins addressed to one or more different cellular compartments in particular to the mitochondria or chloroplast. The person skilled in the art knows that some proteins have to be addressed to one of these compartment, for example: fertility restorers are supposed to be required in mitochondria, or some herbicides as ALS are known to be active in the chloroplast. It is also one of the scope of the invention to provide a method to obtain expression of a protein active in those cellular compartments.

[0152] The repair DNA as defined above preferably does not comprise any T2A or 2A sequence, nor any IRES sequence.

[0153] The repair DNA as defined above preferably does not comprise any T2A sequence, any 2A sequence or any IRES sequence.

[0154] The repair DNA as defined above preferably does not comprise any T2A sequence, any 2A sequence and any IRES sequence.

[0155] By "T2A sequence" or "2A sequence", it is herein meant self-cleaving peptide motifs derived from certain viruses such as the Foot and Mouth Virus.

[0156] The vector as defined above preferably does not comprise any T2A sequence.

[0157] The vector as defined above preferably does not comprise any 2A sequence.

[0158] The vector as defined above preferably does not comprise any IRES sequence.

[0159] The vector as defined above preferably does not comprise any T2A, any 2A sequence and any IRES sequence.

[0160] In one preferred embodiment, the repair DNA as defined above only comprises gRNA targets at its 5' and 3' ends, to avoid cleavage within the repair DNA.

[0161] In another embodiment, the repair DNA as defined above does not comprise any gRNA target other than the first and second gRNA targets, in particular to avoid any cleavage once the repair DNA is inserted. For that purpose, mutation(s) may for example be introduced in the sequence of the gene(s) of interest, in the left ubiquitin-like region and/or in the right ubiquitin-like region, as compared to the corresponding wild-type sequences. Preferably, said mutation(s) do not result in a change in the sequence of the protein of interest encoded by the gene of interest in the repair DNA, compared to the sequence of the wild-type protein.

[0162] The vector comprising the repair DNA as defined above may further comprise:

[0163] at least one CRISPR (Clustered Regularly Inter-spaced Short Palindromic Repeats)-Cas endonuclease expression cassette and/or

[0164] at least one gRNA expression cassette.

[0165] Alternatively, (i) the at least one CRISPR-Cas endonuclease expression cassette and/or (ii) the at least one gRNA expression cassette may be provided in the form of one or several separate vector(s) comprising said cassettes.

[0166] The CRISPR-Cas endonuclease expression cassette comprises a nucleic acid encoding a Cas endonuclease under the control of a promoter.

[0167] The Cas endonuclease is an enzyme which uses a gRNA as a guide to recognize and performs a double-stranded break at a specific position in a DNA sequence. The Cas endonuclease generally requires the presence of a Protospacer Adjacent Motif (PAM) sequence in the vicinity of the specific targeted position. The PAM sequence can differ depending on the Cas endonuclease.

[0168] When the used Cas endonuclease requires a PAM sequence, the first gRNA target and/or the second gRNA target further comprises a PAM sequence.

[0169] The Cas endonuclease may be selected from the group consisting of Cas9, Cas12a, Cas12b, C2c1 and C2c2.

[0170] The Cas endonuclease is preferably Cas9 (CRISPR-associated protein 9) endonuclease. Cas9 endonuclease may for example comprise or consist of sequence SEQ ID NO: 13.

[0171] The promoter of the CRISPR-Cas endonuclease expression cassette may be a constitutive promoter selected from the group consisting of an ZmUbi promoter, the 35S promoter or the 19S promoter (Kay et al., 1987), the rice actin promoter (McElroy et al., 1990), the pCRV promoter (Depigny-This et al., 1992), the CsVMV promoter (Verdaguer et al., 1998) and the ubiquitin promoter from rice or sugarcane. The promoter of the CRISPR-Cas endonuclease expression cassette is preferably an ZmUbi promoter.

[0172] The CRISPR-Cas endonuclease expression cassette preferably comprises a terminator, for example SbHSP.

[0173] The gRNA expression cassette comprises a nucleic acid encoding a gRNA under the control of a promoter.

[0174] The gRNA comprises:

[0175] a region complementary to the first and/or second gRNA target sequence and/or to a region in 5' or 3' of the polyubiquitin gene, and

[0176] a scaffold region that allows binding to the CRISPR-Cas endonuclease encoded by the CRISPR-Cas endonuclease expression cassette.

[0177] The promoter of the gRNA expression cassette may for example be selected from the group consisting of an RNA polymerase III promoter (for example TaU6 promoter, ZmU6 promoter or ZmU3), or an RNA polymerase II promoter such as a constitutive promoter (for example ZmUbi or TaUbi). The promoter of the gRNA expression cassette is preferably TaU6 promoter.

[0178] The gRNA produced by the gRNA expression cassette is able to:

[0179] recognize the first and/or second gRNA target of the repair DNA, so as to liberate the repair DNA from the vector through the action of the corresponding CRISPR-CAS endonuclease, and/or

[0180] recognize a region in 3' or 5' of the polyubiquitin gene, so as to introduce a double stranded break in 3' or 5', respectively, of the polyubiquitin gene through the action of the corresponding CRISPR-CAS endonuclease.

[0181] In one embodiment, the vector comprising a repair DNA as defined above comprises a single gRNA expression cassette.

[0182] When a single gRNA expression cassette is used, the gRNA is able to introduce a double stranded break both in 3' or 5' of the polyubiquitin gene and to liberate the repair DNA. The sequence complementary to the gRNA is in this

case identical in the first and second gRNA targets of the repair DNA and in the 3' or 5' of the polyubiquitin gene.

[0183] When two gRNA expression cassettes are used, the first gRNA transcribed from the first gRNA expression cassette is for example able to introduce a double stranded break in 3' or 5' of the polyubiquitin gene and the second gRNA transcribed from the second gRNA expression cassette is for example able to liberate the repair DNA. The sequence complementary to the second gRNA is in this case identical in the first and second gRNA targets of the repair DNA.

[0184] When three gRNA expression cassettes are used, the first gRNA transcribed from the first gRNA expression cassette is for example able to introduce a double stranded break in 3' or 5' of the polyubiquitin gene, the second gRNA transcribed from the second gRNA expression cassette is for example able to liberate the 5' end of the repair DNA and the third gRNA transcribed from the third gRNA expression cassette is for example able to liberate the 3' end of the repair DNA.

[0185] When four gRNA expression cassettes are used,

[0186] the first gRNA transcribed from the first gRNA expression cassette is for example able to introduce a double stranded break in 3' or 5' of the polyubiquitin gene,

[0187] the second gRNA transcribed from the second gRNA expression cassette is for example able to introduce a double stranded break in 3' or 5' of the polyubiquitin gene, downstream of the double stranded break introduced by the first gRNA,

[0188] the third gRNA transcribed from the third gRNA expression cassette is for example able to liberate the 5' end of the repair DNA and

[0189] the fourth gRNA transcribed from the fourth gRNA expression cassette is for example able to liberate the 3' end of the repair DNA.

[0190] The vector comprising the repair DNA as defined above may further comprise a selection marker.

[0191] Any suitable selection marker well-known by the skilled person may be used. The selection marker may for example be NptII gene or bar gene.

[0192] NptII (neomycin phosphotransferase) inactivates aminoglycoside antibiotics, including kanamycin and neomycin.

[0193] The selection marker is preferably provided in the form of a selection marker expression cassette comprising said selection marker.

[0194] When at least two genes of interest are to be integrated in a plant genome, in particular in 5' or 3' of the polyubiquitin gene, they may be provided in the same repair DNA or in different repair DNAs.

[0195] When at least two genes of interest are to be integrated in a plant genome, in particular in 5' or 3' of the polyubiquitin gene, they may be provided in different repair DNAs either in the same vector or in different vectors.

[0196] For example, a first vector as defined above comprises a repair DNA comprising the first gene of interest and a second vector as defined above comprises a repair DNA comprising the second gene of interest

[0197] In a preferred embodiment, the at least two genes of interest are provided in the same vector, more preferably in the same repair DNA. In such case, it is preferred that the repair DNA comprises a site recognized by a Ubi protease between each two genes of interest. This allows processing

between the first and the second genes of interest, between the second and third genes of interest (if a third gene of interest is present), etc.

[0198] The site recognized by an Ubi protease is for example a 3' ubiquitin tail coding for at least the last 6 amino acids, preferably at least the last 8 amino acids, preferably at least the last 10 amino acids, preferably at least the last 12 amino acids, or still preferably at least the last 14 amino acids of the C-term of a protein encoded by any of the Ubi repeats, except the last Ubi repeat. The site recognized by an Ubi protease may for example comprise or consist of the last 14 amino acids of the C-term of a protein encoded by one repeat of Ubi7DL (except the last Ubi repeat). A site recognized by an Ubi protease may for example comprise or consist of sequence SEQ ID NO: 70.

[0199] The vector as defined above suitable for targeted integration of at least two genes of interest may for example comprise a repair DNA, wherein said repair DNA comprises from 5' to 3':

[0200] a first gRNA target, in particular as defined above,

[0201] a left ubiquitin-like domain, in particular as defined above,

[0202] optionally, a region of the polyubiquitin gene comprising a site recognized by an Ubi protease (for example a region encoding for at least the last 6, 8, 10, 12 or 14 amino acids of the C-terminus of a protein encoded by any repeat of the polyubiquitin gene except the last repeat), in particular for an integration in 3' of the polyubiquitin gene,

[0203] the first gene of interest,

[0204] a region of the polyubiquitin gene comprising a site recognized by an Ubi protease (for example a region encoding for at least the last 6, 8, 10, 12 or 14 amino acids of the C-terminus of a protein encoded by any repeat of the polyubiquitin gene except the last repeat),

[0205] the second gene of interest,

[0206] optionally, a region of the polyubiquitin gene comprising a site recognized by an Ubi protease (for example a region encoding for at least the last 6, 8, 10, 12 or 14 amino acids of the C-terminus of a protein encoded by any repeat of the polyubiquitin gene except the last repeat), in particular for an integration in 3' of the polyubiquitin gene,

[0207] the right ubiquitin-like domain, in particular as defined above, and

[0208] a second gRNA target, in particular as defined above.

[0209] If more than two genes of interest are integrated, a region of the polyubiquitin gene comprising a site recognized by a Ubi protease is introduced between each two genes of interest.

[0210] The Ubi protease is preferably the endogenous Ubi protease expressed by the plant cell.

[0211] When the fusion protein comprising the at least one gene of interest is expressed in a plant cell, the Ubi protease expressed in said cell can thus cut said fusion protein, thereby releasing the or each protein encoded by the gene(s) of interest.

[0212] The targeted insertion of at least one gene of interest in 5' or in 3' of the polyubiquitin gene is thus achieved by using at least one repair DNA, at least one CRISPR-Cas endonuclease expression cassette and at least

one gRNA expression cassette, which elements are provided either in the same vector or in separate vectors. Beside said gRNA expression cassette(s) and said CRISPR-Cas endonuclease expression cassette(s) need to provide gRNA(s) and Cas-endonuclease(s) able to:

[0213] liberate the repair DNA from the vector, and

[0214] introduce a double stranded break in 5' or in 3' of the polyubiquitin gene.

[0215] The invention also relates to an inserted fragment in the wheat Ubi sequence under the control of an Ubi promoter that allows strong expression of a GOI. A vector comprising an Ubi promoter, such as the Ubi7DL promoter, or either the Ubi7AL or Ubi7BL promoter, with ubiquitin-like regions as described above can drive strong expression of a GOI. Vectors such as the one described in example 4bis make also part of the invention. In particular said vector comprises from 5' to 3' a wheat Ubi promoter, a wheat Ubi CDS, at least one gene of interest, and a wheat Ubi terminator. Preferably, said Ubi promoter, Ubi CDS and Ubi terminator are from a same wheat Ubi gene, such as wheat Ubi7AL gene, wheat Ubi7DL gene, or wheat Ubi7BL gene. According to some embodiments, the at least one gene of interest encodes a mutated wheat ALS1 gene that confers herbicide resistance.

[0216] The present invention also relates to a plant cell or plant tissue, which is transformed with (i) at least said vector and (ii) optionally, at least one CRISPR-Cas endonuclease expression cassette as defined and/or at least one gRNA expression cassette as defined above. The two or three expression cassette may be on the same vector or different vectors.

[0217] One of GOI tested by the inventor is a mutated ALS1 gene. They showed that selection of 2 mutations in ALS1 gene, that result in either mutations at amino-acid D376 and W574 (SEQ ID NO: 79, SEQ ID NO: 81 or SEQ ID NO: 83) or W574 and S653 (SEQ ID NO: 80 or SEQ ID NO: 82 or SEQ ID NO: 84) of the encoded polypeptide (as defined by reference to *Arabidopsis* protein positions) can lead to strong herbicide resistance.

[0218] Accordingly, the invention further relates to an isolated nucleic acid that encodes a wheat ALS1 mutated polypeptide sequence at amino-acids D376 and W574, or at amino-acids W574 and S653, by reference to *Arabidopsis* protein positions, or at amino-acids D350 and W548, or W548 and S627 by reference to wheat chromosome 6 genome A, B or D. Preferably, the isolated nucleic acid encodes a mutated wheat ALS1 polypeptide comprising the amino acid substitutions D350E and W548L, or amino acid substitutions 548L and S627N in either of SEQ ID NO: 36, SEQ ID NO: 38, or SEQ ID NO: 40. Preferably, the isolated nucleic acid encodes a mutated wheat ALS1 polypeptide that does not comprise additional mutations. According to some embodiments, the isolated nucleic acid encodes a mutated wheat ALS1 polypeptide comprising or consisting of SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, or SEQ ID NO: 84.

[0219] The invention further relates to a vector comprising a nucleic acid encoding a mutated wheat ALS1 polypeptide, as described above.

[0220] Plants transformed with nucleic acid encoding a mutated wheat ALS1 polypeptide, or with a vector comprising said nucleic acid encoding a mutated wheat ALS1 polypeptide also make part of the invention.

Plant Cell, Plant Tissue or Plant Expressing at Least One Gene of Interest

[0221] The present invention also relates to a plant cell or plant tissue, which is transformed with (i) at least one vector as defined above in the section "Vector" and (ii) optionally, at least one CRISPR-Cas endonuclease expression cassette as defined above in the section "Vector" and/or at least one gRNA expression cassette as defined above in the section "Vector".

[0222] The present invention also relates to a plant cell or plant tissue comprising at least one gene of interest integrated in 5' or in 3' of the polyubiquitin gene, in particular obtained by transformation with (i) at least one vector as defined above in the section "Vector" and (ii) optionally, at least one CRISPR-Cas endonuclease expression cassette as defined above in the section "Vector" and/or at least one gRNA expression cassette as defined above in the section "Vector".

[0223] The plant cell or plant tissue as defined above may be obtained by the method for targeted insertion of a gene of interest in 5' or in 3' of a polyubiquitin gene in a plant genome as defined below.

[0224] The plant is particularly as defined above in the section "Plant".

[0225] The plant cell may be a protoplast.

[0226] The plant tissue may be an apical meristem, cotyledon, embryo, pollen and/or microspores.

[0227] The present invention also relates to a plant comprising at least one gene of interest, in particular integrated in 5' or 3' of the polyubiquitin gene, in particular obtained by transformation with the vector as defined above in the section "Vector", preferably a plant obtained from a plant cell or plant tissue as defined above.

[0228] The present invention also relates to a progeny plant of as defined above, wherein said progeny plant comprises at least one gene of interest inserted in 5' or in 3' of the polyubiquitin gene.

[0229] The plant, progeny plant, plant cell and plant tissue as defined above express at least one protein of interest encoded by at least one gene of interest, in the form of a fusion protein comprising ubiquitin protein(s) and the protein(s) of interest, said fusion protein being then cleaved by endogenous ubi protease, thereby releasing said protein(s) of interest.

Method for Targeted Insertion of a Gene of Interest

[0230] The present invention particularly relates to a method for targeted insertion of a gene of interest in 5' or in 3' of a polyubiquitin gene in a plant genome.

[0231] The method as defined above particularly comprises:

[0232] a) the transformation of a plant cell or plant tissue, in particular as defined above in the section "Plant cell, plant tissue or plant expressing at least one gene of interest", with at least one vector comprising a repair DNA, as defined above in the section "Vector", to obtain a transformed plant cell or a transformed plant tissue and

[0233] b) the regeneration of a plant from the transformed plant cell or transformed plant tissue.

[0234] Step a) comprises the transformation of a plant cell or plant tissue, in particular as defined above in the section "Plant cell, plant tissue or plant expressing a gene of

interest" with at least one vector comprising a repair DNA, optionally at least one CRISPR-Cas endonuclease expression cassette and optionally at least one gRNA expression cassette.

[0235] If the vector comprising the repair DNA does not comprise at least one CRISPR-Cas endonuclease expression cassette and/or does not comprise at least one gRNA expression cassette, the plant cell or plant tissue is transformed with separate vector(s) comprising the missing expression cassette(s), i.e. at least one CRISPR-Cas endonuclease expression cassette and/or at least one gRNA expression cassette.

[0236] If the vector comprising the repair DNA does not comprise at least one CRISPR-Cas endonuclease expression cassette, nor at least one gRNA expression cassette, the plant cell or plant tissue is preferably transformed with a second vector comprising at least one CRISPR-Cas endonuclease expression cassette and at least one gRNA expression cassette. Alternatively, the CRISPR-Cas endonuclease expression cassette and the gRNA expression cassette may be provided in separate vectors.

[0237] When more than one vector is used in step a), the plant cell or plant tissue is preferably transformed with the different vectors at the same time.

[0238] Any technique suitable for plant cell or plant tissue transformation may be used, such as biolistic particle delivery, PEG transformation, electroporation or *Agrobacterium* transgene delivery.

[0239] For *Agrobacterium* transgene delivery, the vector(s) is/are first transferred into *Agrobacterium*, to obtain a transformed *Agrobacterium* and the plant cell or plant tissue is then transformed with said transformed *Agrobacterium*. The *Agrobacterium* is preferably *Agrobacterium tumefaciens*.

[0240] Step a) particularly results in:

[0241] (a1) the expression of the Cas endonuclease(s) from the CRISPR-Cas endonuclease expression cassette(s) and the production of gRNA(s) from the gRNA expression cassette(s), thereby producing a double strand break in 3' or 5' of the polyubiquitin gene in the plant genome and two double strand breaks in the vector, thereby liberating the repair DNA, and

[0242] (a2) the homologous recombination between the repair DNA and the plant genome in 3' or 5' of the polyubiquitin gene.

[0243] The (a2) step can also occur during or after the regeneration of the plant (step (b)).

[0244] Step b) comprises regeneration of a plant.

[0245] Regeneration of a plant from a plant cell or plant tissue is well known by the skilled person.

[0246] In particular, the plant cell or plant tissue may be placed in a culture medium suitable for plant growth.

[0247] The regeneration of a plant from a plant cell or a plant tissue may comprise:

[0248] growing said plant cell or plant tissue to obtain a callus, and

[0249] regeneration of shoots from the callus.

[0250] The growth of the plant cell into a callus and the regeneration of shoots are carried out in any suitable culture medium comprising plant growth regulators.

[0251] The regeneration of a plant from a plant tissue may comprise regeneration of shoots.

[0252] The regeneration of shoots from the plant tissue is carried out in any suitable culture medium comprising plant growth regulators.

Method for Expressing a Protein of Interest in a Plant

[0253] The present invention particularly relates to a method for expressing at least one protein of interest in a plant, wherein said method comprises the steps of the method for targeted insertion of at least one gene of interest in 5' or 3' of a polyubiquitin gene in a plant genome as defined above, wherein said gene of interest codes for said protein of interest.

[0254] The method as defined above may further comprise a subsequent step of detecting the protein of interest.

[0255] Detection of a protein of interest may be performed according to any method well-known by the skilled person, such as western-blot or an immunoassay.

Use of a Vector for Expressing a Gene of Interest in a Plant

[0256] The present invention also relates to the use of a vector comprising a repair DNA as defined above in the section "Vector", for expressing at least one gene of interest in a plant, plant cell or plant tissue.

[0257] The plant, plant cell and plant tissue are particularly as defined above.

[0258] The use as defined above particularly allows expressing said at least one gene of interest under the endogenous promoter of the polyubiquitin gene.

[0259] The present invention also relates to the use of a vector comprising a repair DNA as defined above in the section "Vector" for the targeted integration of at least one gene of interest, in particular in 5' or 3' of a polyubiquitin gene.

Method of Identifying a Plant Having a Gene of Interest Inserted in 5' or 3' of a Polyubiquitin Gene

[0260] The present invention also relates to a method of identifying a plant comprising at least one gene of interest inserted in 5' or 3' of a polyubiquitin gene, wherein said method comprises:

[0261] extracting the DNA, RNA or proteins of a plant,

[0262] detecting the presence of a DNA comprising at least one gene of interest inserted in 5' or 3' of a polyubiquitin gene and/or the presence of an RNA transcript from said DNA, and

[0263] optionally, detecting the presence of a protein encoded by said at least one gene of interest.

[0264] Detecting the presence of a DNA comprising at least one gene of interest inserted in 5' or 3' of a polyubiq-

uitin gene may for example comprise detecting the presence of the repair DNA as defined above in the section "Vector" or of a fragment thereof.

[0265] Said fragment thereof may for example comprise:

[0266] the left ubiquitin-like domain and at least the 5' part of the gene of interest (or of the first gene of interest), or

[0267] at least the 3' part of the gene of interest (or of the last gene of interest) and the right ubiquitin-like domain.

[0268] In one embodiment, detecting the presence of a DNA comprising at least one gene of interest inserted in 5' or 3' of a polyubiquitin gene is performed using at least one pair of PCR primers, in particular able to amplify a region comprising a portion of the endogenous plant genome and at least a portion of the gene of interest, for example,

[0269] a pair of primers comprising one primer that recognizes a region upstream of the left ubiquitin-like region in the genome and which is not present in the repair DNA and another primer that recognizes at least one portion of the gene of interest, and/or

[0270] a pair of primers comprising a primer that recognizes at least one portion of the gene of interest and a primer that recognizes a region downstream of the right ubiquitin-like region in the genome and which is not present in the repair DNA.

[0271] Detecting the presence of a protein encoded by said at least one gene of interest allows confirming that the protein is expressed and/or assessing its level of expression.

[0272] Detecting the presence of a protein encoded by said at least one gene of interest may be performed as follows:

[0273] if the gene of interest encodes a protein that is already present in the wild-type plant, the quantity of protein produced is for example compared to the quantity of protein produced by a control wild-type plant. If the quantity produced by the transformed plant is significantly higher than the quantity produced in a control wild-type plant, the plant is identified as a plant expressing the protein encoded by the gene of interest,

[0274] if the gene of interest encodes a protein absent from the wild-type plant, the detection of the presence of the protein in the transformed plant allows the identification of a plant expressing the protein encoded by the gene of interest.

[0275] If the gene of interest in the repair DNA is also a selective marker, for example an herbicide gene like the Bar gene, the expression of the protein can be detected in a selective medium.

[0276] The invention will be further illustrated in the following figures and examples.

BRIEF DESCRIPTION OF THE SEQUENCES

Sequence number	Type	Origin (organisme or artificial)	Name of the sequence
SEQ ID NO: 1	Nucleic	<i>Streptomyces hygroscopicus</i> strain A10	phosphinothricin acetyl transferase gene (bar gene)
SEQ ID NO: 2	Protein	<i>Streptomyces hygroscopicus</i> strain A10	phosphinothricin acetyl transferase (bar protein)
SEQ ID NO: 3	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	TaUbi7DL genomic
SEQ ID NO: 4	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	TaUbi7BL genomic

-continued

Sequence number	Type	Origin (organism or artificial)	Name of the sequence
SEQ ID NO: 5	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	TaUbi7AL genomic
SEQ ID NO: 6	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	G1 gRNA spacer
SEQ ID NO: 7	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	G2 gRNA spacer
SEQ ID NO: 8	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	G3 gRNA spacer
SEQ ID NO: 9	Nucleic	<i>Triticum aestivum</i>	TaU6 promoter
SEQ ID NO: 10	Nucleic	Artificial	TaU6_G1, G2, G3 multiplex gRNA
SEQ ID NO: 11	Nucleic	<i>Zea mays</i>	ZmUbiquitin promoter + 5'UTR
SEQ ID NO: 12	Nucleic	<i>Agrobacterium tumefaciens</i>	Nopaline synthase polyadenylation sequence
SEQ ID NO: 13	Nucleic	Artificial	SV40 NLS-SpCas9-Nucleoplasmin NLS, maize optimised
SEQ ID NO: 14	Nucleic	Artificial	Ubi7DL-BAR -Ub7DL donor repair fragment flanked by G3 gRNA target sites
SEQ ID NO: 15	Nucleic	<i>Triticum aestivum</i> (var. Chinese Spring)	TraesCS1B01G038500 (RFL29b) CDS
SEQ ID NO: 16	Protein	<i>Triticum aestivum</i> (var. Chinese Spring)	TraesCS1B01G038500 (RFL29b)
SEQ ID NO: 17	Nucleic	<i>Triticum aestivum</i> (var. Spelt)	RFL29a CDS
SEQ ID NO: 18	Protein	<i>Triticum aestivum</i> (var. Spelt)	RFL29a
SEQ ID NO: 19	Nucleic	<i>Triticum timopheevii</i>	RFL79 CDS
SEQ ID NO: 20	Protein	<i>Triticum timopheevii</i>	RFL79
SEQ ID NO: 21	Nucleic	Artificial	RH-Ubi7DL-RFL29a CDS
SEQ ID NO: 22	Nucleic	Artificial	RFL29a_RH-Ubi7DL CDS
SEQ ID NO: 23	Nucleic	Artificial	RH-Ubi7DL-RFL79 CDS
SEQ ID NO: 24	Nucleic	Artificial	RFL79_RH-Ubi7DL CDS
SEQ ID NO: 25	Nucleic	Artificial	pTaU6_G3 gRNA
SEQ ID NO: 26	Nucleic	Artificial	Ubi7DL-RFL29a - Ub7DL donor repair fragment flanked by G3 gRNA target sites
SEQ ID NO: 27	Nucleic	Artificial	Ubi7DL-RFL79 - Ub7DL donor repair fragment flanked by G3 gRNA target sites
SEQ ID NO: 28	Nucleic	Artificial	Ubi7DL-RFL29a-Ubi-RFL79 -Ub7DL donor repair fragment flanked by G3 gRNA target sites
SEQ ID NO: 29	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	ACCase ChrA CDS
SEQ ID NO: 30	Protein	<i>Triticum aestivum</i> (var. Fielder)	ACCase ChrA
SEQ ID NO: 31	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	ACCase ChrB CDS
SEQ ID NO: 32	Protein	<i>Triticum aestivum</i> (var. Fielder)	ACCase ChrB
SEQ ID NO: 33	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	ACCase ChrD CDS
SEQ ID NO: 34	Protein	<i>Triticum aestivum</i> (var. Fielder)	ACCase ChrD
SEQ ID NO: 35	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	ALS ChrA CDS
SEQ ID NO: 36	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS ChrA
SEQ ID NO: 37	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	ALS ChrB CDS

-continued

Sequence number	Type	Origin (organisme or artificial)	Name of the sequence
SEQ ID NO: 38	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS ChrB
SEQ ID NO: 39	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	ALS ChrD CDS
SEQ ID NO: 40	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS ChrD
SEQ ID NO: 41	Nucleic	<i>Triticum aestivum</i> (var. Chinese Spring)	P450_Chr5A, TraesCS5A02G398000 CDS
SEQ ID NO: 42	Protein	<i>Triticum aestivum</i> (var. Chinese Spring)	P450_Chr5A, TraesCS5A02G398000
SEQ ID NO: 43	Nucleic	<i>Zea mays</i> . (var B73)	ZmUbiChr4, Zm00001d053838 genomic
SEQ ID NO: 44	Nucleic	<i>Zea mays</i> . (var B73)	ZmUbiChr5, Zm0001d015327 genomic
SEQ ID NO: 45	Nucleic	<i>Zea mays</i> . (var A188)	ZmUbiChr4 genomic
SEQ ID NO: 46	Nucleic	<i>Zea mays</i> . (var A188)	ZmUbiChr5, genomic
SEQ ID NO: 47	Nucleic	<i>Zea mays</i> . (var A188)	gRNA31 spacer
SEQ ID NO: 48	Nucleic	<i>Zea mays</i> . (var A188)	gRNA20 spacer
SEQ ID NO: 49	Nucleic	<i>Zea mays</i>	ZmU6 promoter
SEQ ID NO: 50	Nucleic	Artificial	Cry1Ac codon optimised
SEQ ID NO: 51	Protein	<i>Bacillus thuringiensis</i>	Cry1Ac
SEQ ID NO: 52	Nucleic	Artificial	RCA-Cry1Ac codon optimised
SEQ ID NO: 53	Nucleic	Artificial	ZmUbiChr5-Cry1Ac - ZmUbiChr5 donor repair fragment flanked by gRNA20 target sites
SEQ ID NO: 54	Nucleic	Artificial	ZmUbiChr5-RCA- Cry1Ac -ZmUbiChr5 donor repair fragment flanked by gRNA20 target sites
SEQ ID NO: 55	Nucleic	<i>R. sativus</i>	Rfo genomic
SEQ ID NO: 56	Protein	<i>R. sativus</i>	Rfc
SEQ ID NO: 57	Nucleic	<i>B. napus</i>	BnaA09g19810D genomic
SEQ ID NO: 58	Nucleic	<i>B. napus</i>	BnaC09g21810D genomic
SEQ ID NO: 59	Nucleic	<i>B. napus</i>	BnaA08g30590D genomic
SEQ ID NO: 60	Nucleic	<i>B. napus</i> var. Westar	BnUbiA09 genomic
SEQ ID NO: 61	Nucleic	<i>B. napus</i> var. Westar	gRNA16 spacer
SEQ ID NO: 62	Nucleic	<i>Arabidopsis thaliana</i>	AtU6 promoter
SEQ ID NO: 63	Nucleic	Artificial	BnUbiA09-Rfo - BnUbiA09 donor repair fragment flanked by gRNA16 target sites
SEQ ID NO: 64	Nucleic	Cauliflower Mosaic Virus	35S CaMV promoter
SEQ ID NO: 65	Nucleic	Artificial	SV40 NLS-Cas9- Nucleoplasmin NLS
SEQ ID NO: 66	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	left ubiquitin-like region homologous to repeats 3 to 5 of the polyubiquitin gene Ubi7DL
SEQ ID NO: 67	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	right ubiquitin-like region homologous to repeats 1 to 3 of the polyubiquitin gene Ubi7DL
SEQ ID NO: 68	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	right ubiquitin-like region homologous to a region of the 3'UTR region of the polyubiquitin gene Ubi7DL

-continued

Sequence number	Type	Origin (organism or artificial)	Name of the sequence
SEQ ID NO: 69	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	left ubiquitin-like region homologous to a region of the 5'UTR region of the polyubiquitin gene Ubi7DL
SEQ ID NO: 70	Protein	<i>Triticum aestivum</i> (var. Fielder)	Cleavable sequence by Ubi protease from one repeat of Ubi7DL
SEQ ID NO 71	Nucleic	<i>Lolium rigidum</i>	P450 CYP81A10v7
SEQ ID NO 72	Protein	<i>Lolium rigidum</i>	P450 CYP81A10v7
SEQ ID NO 73	Nucleic	<i>Zea mays</i>	P450 CYP81A2
SEQ ID NO 74	Protein	<i>Zea mays</i>	P450 CYP81A2
SEQ ID NO 75	Nucleic	<i>Zea mays</i>	P450 CYP81A9
SEQ ID NO 77	Protein	<i>Zea mays</i>	P450 CYP81A9
SEQ ID NO77	Nucleic	<i>Triticum aestivum</i> (var. Fielder)	Su1, P450 CYP71
SEQ ID NO 78	Protein	<i>Triticum aestivum</i> (var. Fielder)	Su1, P450 CYP71
SEQ ID NO 79	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS1_chr6D mutant D350E_W548L
SEQ ID NO 80	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS1_chr6D mutant W548L_S627N
SEQ ID NO 81	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS1_chr6A mutant D353E_W551L
SEQ ID NO 82	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS1_chr6A mutant W551L_S630N
SEQ ID NO 83	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS1_chr6B mutant D350E_W548L
SEQ ID NO 84	Protein	<i>Triticum aestivum</i> (var. Fielder)	ALS1_chr6B mutant W548L_S627N
SEQ ID NO 85	Nucleic	Artificial	TaUbi7DL_promoter::TaUbi7DL_cds::TaAL S1_chr6D_cds::TaUbi7DL_terminator

DESCRIPTION OF THE FIGURES

[0277] FIG. 1: Strategy of Gene Targeting (GT) at the Ubi7DL locus. A: landing pad: target locus in wheat; B: T-DNA from *Agrobacterium* strain (T11561), from pBIOS12163.

[0278] FIG. 2: PCR Analysis of BASTA resistant T1 Progeny of T11561 plants. Schematic showing the position of primers used to amplify left (1694 bp) and right (988 bp or 1171 bp) homologous recombination junctions. One primer is within Bar to other is in TaUbi7DL outside of the regions of homology between T11561 and TaUbi7DL (RH Left and RH Right).

[0279] FIG. 3: PCR Analysis of BASTA resistant T1 Progeny of T11561 plants. Examples of PCR products from the Left junction amplification (1694 bp), Ubi-bar on the figure. T11561_028 plants have a product of the expected size.

[0280] FIG. 4: Constructs designed to insert RFL29a (A) from pBIOS12979 or RFL79 (B) from pBIOS12980 into the TaUbi7DL Landing Pad.

[0281] FIG. 5: Construct designed to insert CoAXium mutated (T6123C) ACCase into the TaUbi7DL Landing Pad.

[0282] FIG. 6: Construct designed to insert a mutated version of the ALS gene into the TaUbi7DL Landing Pad.

[0283] FIG. 7: Construct designed to insert the wheat homologue of the *Lolium rigidum* P450 CYP81A10v7 gene into the TaUbi7DL Landing Pad.

[0284] FIG. 8: Constructs designed to over express ALS native (from pBIOS13536) and mutated gene (from pBIOS13535 and pBIOS13534) in a TaUbi context.

[0285] FIG. 9: Herbicide treatment on plants over expressing ALS native or mutated in a TaUbi context.

EXAMPLES

Example 1: Expression of an Herbicide Resistance from Wheat PolyUbiquitin: Bar Gene Fusion

[0286] As a proof of concept, the BAR gene for herbicide resistance (of sequence SEQ ID NO: 1 encoding SEQ ID NO: 2) was fused to the 3' of a wheat polyubiquitin gene using homologous recombination (Gene Targeting or GT). The use of Bar allows a positive selection for desired insertion events at the Landing Pad.

[0287] A polyubiquitin gene on Chr7DL of Chinese Spring (*TraesCS7D01G443100*) was found by BLAST analysis to be closest to the gene of the strong Ubiquitin Maize promoter on Chromosome 5 widely used in monocot transgenesis. This gene has homologs on Chr7BL (*TraesCS7B01G354200*) and Chr7AL (*TraesCS7A01G453500*). RNAseq data confirmed that all 3 genes are strongly expressed and could thus be used as Landing Pads. The Ubi7DL was chosen as the target for GT and sequenced along with Ubi7AL and Ubi7BL in the wheat variety Fielder to be used for transformation. The 3 Ubi genes (Ubi7DL SEQ ID NO: 3, Ubi7BL SEQ ID NO: 4, Ubi7AL SEQ ID NO: 5) have a good level of homology in

the CDS but are divergent in the 3' UTR region suggesting that a GT repair fragment including the Ubi7DL 3' UTR as one of the arms of homology used for homologous recombination should allow specific targeting to Ubi7DL.

[0288] The strategy for GT at Ubi7DL is outlined in FIG. 1 and is based on the method of in planta GT (Fauser et al. 2012). A Cas9 gRNA (G3 SEQ ID NO: 8) that efficiently makes a DNA double strand break (DSB) around the STOP codon of the Ubi7DL gene was first identified by *Agrobacterium*-mediated transformation into wheat var. Fielder with a binary plasmid containing a TaU6-tRNA multiplex guide (SEQ ID NO: 10) expressing 3 guides G1 (SEQ ID NO: 6), G2 (SEQ ID NO: 7) and G3 (SEQ ID NO: 8) under control of promoter TaU6 (SEQ ID NO: 9), together with ZmUbi (SEQ ID NO: 11)-Cas9 (SEQ ID NO: 13)-nos terminator (SEQ ID NO: 12) and pActin-Bar-nos cassettes. These 3 target sites are around the STOP codon of Ubi7DL. Fielder wheat cultivar was transformed with these *Agrobacterium* strains essentially as described by WO2000/063398. High-throughput (NGS) sequencing of transformed plantlets showed 59 of 97 (61%) independent transformation events had mutations at the target site and all were generated with the G3 gRNA.

[0289] WT Fielder was then transformed with an *Agrobacterium* strain (T11561) with a binary plasmid pBIOS12163 with a T-DNA that contains the repair DNA (SEQ ID NO: 14 BAR gene flanked by Left (680 bp) and Right homology regions (740 bp) to the Ubi7DL target) flanked by G3 sites. The G3 sites contain 6 bp upstream and 6 bp downstream of Ta7DL sequences flanking the G3 target to help maintain the context of the G3 target. This T-DNA expresses Cas9 (SEQ ID NO: 13) from the constitutive ZmUbi promoter (SEQ ID NO: 11), produces the G3 gRNA from the ZmU6 promoter (SEQ ID NO: 49) and also has NptII under the control of a VirSc4 promoter allowing transient or stable selection of transformants. Expression of Cas9 and the G3 gRNA both produce a DSB at the Ubi7DL target and also liberate the repair DNA from the T-DNA making the repair DNA available for GT at the Ubi7DL target.

[0290] Wheat plants stability transformed with T11561 were generated via selection on Kanamycin. In these transformants GT may occur throughout plant growth (provided that the G3 gRNA target sites are not mutated and that the repair DNA is still present). Direct selection on BASTA was also performed on T0 plants resistant plants were obtained but molecular analysis shows no GT (table 1).

[0291] 92 independent transformation events on Kanamycin selection (365 plants with sister plants) were obtained and T1 seed harvested. T1 progeny were sown and sprayed 2x with BASTA to look for resistant plants (data not shown). Several T1 families exhibited BASTA resistance (Table 1). 2 T1 plants of 55 T1T11561_028 events exhibited full resistance (data not shown). Molecular analysis of these plants by PCR (FIGS. 2 and 3) and DNA sequencing showed that these plants had the predicted insertion of Bar into the Ubi7D gene. Both left and right junction sequences were as predicted indicating insertion of Bar by a double homologous recombination event.

TABLE 1

Line	T0 Selection	Sister plant	T1 seed sown	Resistant plants	% Resistance	Level of BASTA R on T1	
						BASTA	R on T1
T11561_002	Kan	N	76	1	1%	med	
T11561_006	Kan	N	86	4	5%	low	
T11561_013	Kan	N	33	1	3%	med	
T11561_014	Kan	N	28	14	50%	med	
T11561_017	Kan	N	59	1	2%	low	
T11561_028	Kan	N	55	2	4%	good	
T11561_030	Kan	N	43	1	2%	low	
T11561_056	Kan	N	88	1	1%	med	
T11561_059	Kan	N	50	1	2%	low	
T11561_063	Kan	N	23	1	4%	low	
T11561_065	Kan	N	27	7	26%	low	
T11561_067	Kan	N	72	36	50%	good	
T11561_069	Kan	N	59	2	3%	low	
T11561_073	Kan	Y	30	11	37%	good	
T11561_074	Kan	Y	28	11	39%	good	
T11561_075	Kan	Y	28	9	32%	good	
T11561_078	Kan	N	20	2	10%	low	
T11561_091	Kan	N	105	57	54%	good	
T11561_093	Basta Evnt1	Y	70	6	9%	low	
T11561_094	Basta Evnt1	Y	64	39	61%	low	
T11561_095	Basta Evnt2	Y	59	38	64%	good	
T11561_096	Basta Evnt2	Y	67	35	52%	good	
T11561_097	Basta Evnt2	Y	44	26	59%	good	
T11561_098	Basta Evnt2	Y	40	19	48%	good	

Low: Plant with significant BASTA herbicide damages;

good: plant with no damage;

med: plant with intermediate damages

Example 2: Use of the TaUbi Landing Pad to Generate Cytoplasmic Male Sterility (CMS) Restorer Plants

[0292] It is a goal of wheat seed companies to move towards the sale of hybrid wheat, since hybrid varieties usually outperform inbreds. Since wheat is dioecious and largely autogamous the production of hybrid seed requires systems to facilitate crossing and reduce the cost of hybrid seed production. Such a system is the use of male-sterile ‘female’ plant line crossed to a male fertile line such that all the seed harvested from the female, male-sterile plants will be F1 hybrid seed. Male-sterile plants can be produced using cytoplasmic male sterility (CMS) where the female plant carries ‘defective’ mitochondria that often express novel ORFs leading to the production of no or defective pollen. Use of CMS systems for hybrid seed production requires that the male line used in the hybrid seed production cross carries a nuclear gene or genes that repair the defective mitochondria in the F1. This leads to full male-fertility of the F1 plants that are grown by the farmer. These nuclear genes in the male line are referred to as CMS restorer genes. One potential CMS system for hybrid wheat production is that using *T. timopheevii* CMS (WO 2019/086510 A1 or PCT/EP2022/064472). A drawback of this system is that a combination of several restorer genes (Rf1, Rf3, Rf4 and Rf7) is required to give full male fertility to the F1. For the breeder this makes the system more complex to use since each male line has to be converted to contain 3 or 4 independently segregating restorer genes. It is thus desirable to identify or create a single effective restorer locus.

[0293] The *T. timopheevii* CMS restorer gene Rf3 has been identified as a PPR protein on Chr1B referred to as RFL29

(TraesCS1B01G038500) (WO 2019/086510 A1). This gene is present in most wheat lines such as Chinese Spring though its level of expression is very low as measured by RNAseq data. There are at least 3 RFL29 variants in wheat. RFL29b (SEQ ID NO: 15 encoding SEQ ID NO: 16), present in Chinese Spring is a less effective restorer than the RFL29a allele (SEQ ID NO: 17 encoding SEQ ID NO: 18) found in lines such as Spelt. Some lines such as Fielder contain an inactive RFL29 variant, RFL29c, with a frameshift in the coding region. To determine if RFL29-mediated fertility restoration can be improved, RFL29a and RFL29b were placed under the control of the strong ZmUbiquitin (ZmUbi) promoter and transformed into a wheat line containing *T. timopheevii* CMS. Full male fertility was observed in single copy T-DNA transformants.

[0294] Similarly, Rf1 has been found to be a PPR gene (RFL79) (WO 2019/086510 A1) on Chr1A (SEQ ID NO: 19 encoding SEQ ID NO: 20). As for RFL29, overexpression of RFL79 under the strong ZmUbi promoter restores full male fertility in a wheat line containing *T. timopheevii* CMS.

[0295] Wheat 7DL Polyubiquitin::RFL29 and polyubiquitin::RFL79 fusions also restore male fertility to a wheat line containing *T. timopheevii* CMS when expressed as a transgene from the maize Ubi promoter or the wheat Ubi promoter. This is the case when the RFL genes are expressed as 5' or 3' fusions to polyubiquitin (table 2). In the case of 5' fusions, the RFL29a or RFL79 sequence has an added 3' ubiquitin tail of 14 amino acids (Walker and Vierstra (2007) which are the C-terminal amino acids of the first Ubi repeat in Ubi7DL (SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24).

integration of RFL79 at the wheat Ubi7DL Landing Pad was constructed (SEQ ID NO: 27).

[0297] Progeny of the TO transformed lines are screened by PCR to identify GT events where the RFL29a or RFL79 gene has been integrated into the TaUbi7DL Landing Pad. These plants are fertile. The full restoration of fertility by plants expression a single copy of these both gene in the landing pad context confirms the potential of the method to give a high level of expression of a sequence introduced in this context.

Example 3: Co-Expression of CMS Restorer Genes from a Wheat Polyubiquitin Landing Pad in Order to Restore Male Fertility

[0298] More than one gene can be integrated into a polyubiquitin Landing Pad. To improve the *T. timopheevii* CMS system, it would be advantageous to express the two restorer genes RFL29a and RFL79 (see example 2) from the Polyubiquitin Landing Pad. This would assure sufficient expression of each restorer and in addition create a single locus that can be introgressed by the breeder to convert wheat lines into *T. timopheevii* CMS restorer lines. In example 2, it was shown that an N-terminal fusion of RFL29a to polyubiquitin when expressed in CMS wheat can restore male fertility (Table 2, strain T11634). Thus, a donor fragment for homologous recombination (HR) at the wheat Ubi7DL Landing Pad comprises the Ubi7DL right RH region (repeats 3, 4 and 5): RFL29a: 14aa of the C-terminus of Ubi7DL repeat1: Ubi7DL repeat1: RFL79: Ubi7DL terminator (right HR region) (SEQ ID NO: 28). This HR region is flanked by G3 gRNA sites.

TABLE 2

Restoration of fertility in a Wheat <i>T. timopheevii</i> CMS line transformed with TaUbi7DL::RFL fusion genes					
Strain	Promoter	GOI	Terminator	Restoration efficiency (%)	Fertile plants
T11635	ZmUbi	RH_TaUbi7D_TaRFL29a (SEQ ID NO: 21)	SbHSP	77	14/18
T11634	ZmUbi	TaRFL29a_RH_TaUbi7D (SEQ ID NO: 22)	SbHSP	82	18/22
T11549	ZmUbi	RH_TaUbi7D_TaRFL79 (SEQ ID NO: 23)	SbHSP	93	42/45
T11548	ZmUbi	TaRFL79_RH_TaUbi7D (SEQ ID NO: 24)	SbHSP	61	19/31

[0296] These results demonstrate the ability of a maize Ubi promoter to drive sufficient expression of the fertility restorer sequence to have fertile plants, but also to have a fusion protein properly processed to restore sterility. The restoration of fertility implies that the processed protein is correctly imported into the mitochondria where it will restore mitochondrial function and fertility. Thus, integration of RFL29 or RFL79 or both into the wheat polyubiquitin landing pad should create a single locus *T. timopheevii* CMS restorer gene. Transformation constructs to achieve this are shown in FIG. 4 and are transformed into a wheat line containing *T. timopheevii* CMS. The donor fragment for homologous recombination (HR) of RFL29a at the wheat Ubi7DL Landing Pad comprises of the Ubi7DL left HR region (repeats 3, 4 and 5): RFL29a: Ubi7DL terminator (right HR region) (SEQ ID NO: 26). This HR region is flanked by G3 gRNA sites. A similar donor fragment for

[0299] As in example 2, the HR donor region plus flanking G3 gRNA sites is assembled into a plant binary vector for *Agrobacterium*-mediated transformation of a wheat line containing *T. timopheevii* CMS. This binary vector contains a pZmUbi:Cas9 expression cassette, a pTaU6 G3 gRNA expression cassette (SEQ ID NO: 25) and a bar selectable marker cassette.

[0300] Progeny of the TO transformed lines are screened by PCR to identify GT events where the RFL29a and RFL79 genes have been integrated into the TaUbi7DL Landing Pad. These plants are fertile.

Example 4: Use of TaUbi Landing Pad to Generate Herbicide Tolerant Wheat Plants

[0301] The control of weeds is a major agronomical goal for the production of wheat with respect to competition for water and nutrients and to avoid pollution of seed stocks

with undesired weed seeds. The use of chemical herbicide is preferred to mechanical approaches in the sense that it avoids damages to soil structure and erosion. There exists today a large panoply of chemical herbicides which have been developed to this purpose. Ideally wheat should be tolerant to numerous types of herbicides and not just to a single type to avoid the presence of weed types tolerant to a given class of herbicides.

[0302] Working with and introgressing several types of herbicide tolerance genes is a real challenge to breeders, and it would be desirable to have those genes sufficiently expressed and stacked into a single locus. To this aim, the Ubiquitin locus is well adapted since it produces a polyprotein which is subsequently cleaved into single units by cytoplasmic proteases. Furthermore, this locus is expressed in a constitutive fashion and at levels suited to provide good herbicide tolerance.

[0303] Examples of herbicide tolerance genes which may be expressed in this way may be (but not limited to) the wheat acetyl-CoA Carboxylase (ACCase) (ACCase chrA SEQ ID NO: 29 encoding SEQ ID NO: 30, ACCase chrB SEQ ID NO: 31 encoding SEQ ID NO: 32, ACCase chrD SEQ ID NO: 33 encoding SEQ ID NO: 34) comprising the CoAXium mutation Ala2004Val (U.S. Pat. No. 9,578,880-B2) (or other mutations from EP2473022_B1) (FIG. 5), wheat Acetolactate Synthase (ALS) (FIG. 6) mutated individually in amino-acids (numbering according to the reference *Arabidopsis* ALS protein encoded by AT3G48560) A122, P197, A205, D376, W574, S653 or any of the 4 mutations P197, A205, D376 or W574 in combination between them or with A122 or S653 (ALS chr6A SEQ ID NO: 35 encoding SEQ ID NO: 36, ALS chr6B SEQ ID NO: 37 encoding SEQ ID NO: 38, ALS chr6D SEQ ID NO: 39 encoding SEQ ID NO: 40), or a gene encoding a cytochrome P450 involved in herbicide detoxification. An example for a cytochrome P450 enzyme may be the wheat orthologous gene (TraesCS5A02G398000, SEQ ID NO: 41 encoding SEQ ID NO: 42) which is orthologous to a P450 gene from *Lolium rigidum* described by Han et al. (2021) (FIG. 7), another example is the one described in EP22306134.2.

[0304] These herbicide resistance genes are cloned into a plant binary vector essentially identical to pBIOS12979 (example 2) apart from the replacement of RFL29a with the herbicide resistance gene. The T-DNA regions of these plant binary vectors is shown in FIGS. 5, 6 and 7. These plant binary vectors are transferred into *Agrobacterium* and used in *Agrobacterium*-mediated transformation of the wheat variety Fielder. Progeny of the TO transformed lines are screened by PCR to identify GT events where the herbicide-resistance genes have been integrated into the TaUbi7DL Landing Pad. These plants are screened for herbicide resistance using an appropriate herbicide.

Example 4bis: Over-Expression of a Mutated ALS Gene Fused to the Polyubiquitin Coding Region

[0305] The coding region of the wheat ALS1 gene (TraesFLD6D01G329900) was fused to the polyubiquitin gene on chromosome 6D from wheat genotype Fielder (TraesFLD7D01G490700) between the 3'end of the coding region and the terminator. The introduced ALS1 coding region was either the wild-type sequence, or a mutated sequence with amino-acids D350E and W548L (SEQ ID NO: 79) or W548L and S627N (SEQ ID NO: 80). Those amino-acids are equivalent to the *Arabidopsis* ALS amino-

acids D376, W574 or S653. The resulting fragments (Ubi7D_promoter::Ubi7D_cds::ALS1 cds::Ubi7D_terminator, SEQ ID NO: 85) were introduced via a Golden Gate reaction into the destination binary plasmid pBIOS10746 which is a derivative of the binary vector pMRT (WO200101819A3), FIG. 8. The final plasmids pBIOS13536 (fusion with wild-type ALS1), pBIOS13535 (fusion with D350E, W548L mutated ALS1) or pBIOS13534 (fusion with W548L, S627N mutated ALS1) were transformed into *Agrobacterium* EHA105.

[0306] Fielder wheat cultivar was transformed with these *Agrobacterium* strains essentially as described by WO2000/063398. Wheat transgenic events were generated for each construct described above. All wheat transgenic plants were grown in a glasshouse under standard wheat growth conditions (16 h of light period at 20° C. and 8 h of dark period at 15° C. with constant 60% humidity).

ALS1 Inhibiting Herbicide Assay

[0307] To assay for ALS1-inhibiting herbicide of the sulfonylurea family nicosulfuron, T1 plants (progeny of transformed wheat plants) were grown in the glasshouse until the growth stage BBCH13 (3 developed leaves) and sprayed with a solution of nicosulfuron (Pampa herbicide) at a concentration of 0.1 g/L and a spraying rate equivalent to what is used by farmers (600 L/ha).

[0308] Herbicide effect was evaluated between 8 and 16 days after herbicide treatment (FIG. 9). Plants transformed with the mutated ALS1 gene fused to the polyubiquitin gene were resistant to nicosulfuron treatment, whereas untransformed plants or plants transformed with the wild-type ALS1 gene fused to the polyubiquitin gene were susceptible to the herbicide.

[0309] These results demonstrate the ability of the wheat Ubi promoter in this context to drive an expression strong enough to obtain resistance to the herbicide and also that the fusion with ubi sequences allows a correct processing of the protein allowing it to be correctly targeted to the chloroplast.

[0310] ALS1 inhibiting herbicides include molecules belonging to various families like Sulfonylurea (nicosulfuron), Imidazolinone (imazamox), Triazolinones (carfentrazone-ethyl), or Triazolopyrimidine (florasulam). Weeds tolerant to those herbicides were identified in nature and tolerance shown to result from mutations in their ALS1 gene at amino-acids D376 or W574 (*Arabidopsis* protein position). The introduced ALS1 mutations in wheat correspond to those changes and the transformed plants over-expressing those mutations are tolerant to those different herbicides.

Example 5: Use of a ZmUbi Landing Pad to Generate Insect-Resistant Plants

[0311] Maize line B73 has two polyubiquitin genes that are highly and ‘constitutively’ expressed, Zm00001d053838 on Chr4 (SEQ ID NO: 43) and Zm0001d015327 (SEQ ID NO: 44) on Chr5 (genome B73 v4). Equivalent genes in A188 on Chr4 (SEQ ID NO: 45) and Chr5 (SEQ ID NO: 46) can be identified by sequence homology to B73. The promoter of the ZmUbiChr5 gene is widely used in plant transgenesis as a strong and constitutive promoter. Specific Cas9 gRNA can be identified that create a double strand break (DSB) near to the Stop codon of ZmUbiChr4 or ZmUbiChr5. Both ZmUbiChr4 and ZmUbiChr5 can be used as Landing Pads. ZmUbiChr4 is located near to the telomere

of Chr4 thus gene insertions into this Landing Pad may be easier to introgress into other maize varieties than for insertions in ZmUbiChr5 which is near to the centromere of Chr5. However, ZmUbiChr5 seems to be more highly expressed thus depending on the application, one or other of the Landing Pads may be more appropriate.

[0312] A guide targeting an analogous position to that of the wheat gRNA3 of example 1 and example 2 in ZmUbiChr4, gRNA31 (SEQ ID NO: 47) can be used to create a DSB adjacent to the Stop codon of ZmUbiChr4 in both B73 and A188. Similarly, gRNA20 ((SEQ ID NO: 48) can be used to create a DSB adjacent to the Stop codon of ZmUbiChr5 in both B73 and A188. As in examples 1 and 2 regions flanking the stop codon of ZmUbiChr4 can be used are homology regions for homologous recombination of a coding region of interest into the ZmUbiChr4 Landing Pad. Also, regions flanking the stop codon of ZmUbiChr5 can be used are homology regions for homologous recombination of a coding region of interest into the ZmUbiChr5 Landing Pad.

[0313] An example of a coding region of interest is a gene for insect resistance Bt Cry1Ac (SEQ ID NO: 50 encoding SEQ ID NO: 51) that is introduced into the ZmUbiChr5 Landing Pad. ZmUbiChr5 homology flanking regions are cloned upstream and downstream of a maize-codon-optimised Cry1Ac gene which is in turn flanked by target sequences for gRNA20 (The gRNA20 sites contain 6 bp upstream and 6 bp downstream of ZmUbiChr5 sequences flanking the gRNA20 target to help maintain the context of the gRNA20 target). The Cry1Ac gene can also contain sub-cellular targeting signals. SEQ ID NO: 52 shows a Cry1Ac gene that has an N-terminal chloroplast targeting signal from Rubisco Activase (RCA). The homologous recombination Cry1Ac and RCA-Cry1Ac repair fragments (SEQ ID NO: 53 and SEQ ID NO: 54) are then cloned into a plant binary vector containing a rice Actin promoter-BAR nos terminator selectable marker gene together with a ZmUbi promoter-Cas9-Nos terminator cassette and a Maize U6-gRNA20 cassette. The resulting binary plasmids are transferred to Agrobacteria and used in A188 maize transformation using a standard maize *Agrobacterium* protocol (Ishida et al., 1996)

[0314] Progeny of the TO transformed lines are screened by PCR to identify GT events where the Cry1 Ac or RCA-Cry1 Ac genes has been integrated into the ZmUbiChr5 Landing Pad.

Example 6: Use of a BnUbi Landing Pad to Generate Fertility Restorer Plants

[0315] The Ogura CMS system is used by seed companies to produce hybrid F1 rapeseed. This system requires a fertility restorer gene, Rfo, that originates from an introgression from radish (*Raphanus sativus*) (Qui et al., 2014). The original introgression also contained agronomically undesirable linked traits such as increased pod shatter and glucosinolate levels. Thus, considerable effort has been undertaken to reduce the size of the introgression which had proven difficult probably due to limited homology with *B. napus* or to create new introgressions (see Wang et al., 2020). Since the restorer gene Rfo (SEQ ID NO: 55 encoding SEQ ID NO: 56) has been identified and functionally characterized (see Qui et al., 2014) an alternative is to

introduce Rfo into a polyubiquitin Landing Pad. As such there will be good expression of Rfo without any effects due to linkage drag.

[0316] The *B. napus* gene expression site (*Brassica* EDB) described in Chao et al, (2020) was examined to identify polyubiquitin genes with a good constitutive expression. Of the 13 polyubiquitin genes in *Brassica* EDB three appeared to have high and relatively constitutive expression (BnaA09g19810D (SEQ ID NO: 57), BnaC09g21810D (SEQ ID NO: 58) and BnaA08g30590D (SEQ ID NO: 59). BnaA09g19810D was chosen as a Landing Pad, the other two also being suitable candidates (in addition, depending on the desired expression pattern of the Gene of Interest the other polyubiquitin genes can be used as Landing Pads). The *B. napus* var. Westar BnaA09g19810D genomic sequence (SEQ ID NO: 60) (BnUbiA09) was identified by homology with the BnaA09g19810D sequence. A guide targeting an analogous position to that of the wheat gRNA3 of example 1 and example 2 in BnUbiA09; gRNA16 (SEQ ID NO: 61) can be used to create a DSB adjacent to the Stop codon of BnUbiA09. As in examples 1 and 2 regions flanking the stop codon of BnUbiA09 can be used as homology regions for homologous recombination of a coding region of interest into the BnUbiA09 Landing Pad. Homology flanking regions are cloned upstream and downstream of the Rfo genomic coding region which is in turn flanked by target sequences for gRNA16 (The gRNA16 sites contain 6 bp upstream and 6 bp downstream of BnUbiA09 sequences flanking the gRNA16 target to help maintain the context of the gRNA16 target). The homologous recombination Rfo cassette (SEQ ID NO: 63) is then cloned into a plant binary vector containing Nos nptII nos terminator selectable marker gene together with a 35S promoter (SEQ ID NO: 64)-Cas9 (SEQ ID NO: 65)-CaMV terminator cassette and an *Arabidopsis* U6 (SEQ ID NO: 62)-gRNA16 cassette. The resulting binary plasmid is transferred to Agrobacteria and used in *B. napus* var. Westar transformation using a standard *B. napus agrobacterium* protocol (Moloney et al, 1989). Progeny of the TO transformed lines are screened by PCR to identify GT events where the Rfo gene has been integrated into the BnUbiA09 Landing Pad.

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- [0334] WO 2019/086510 A1. WHEAT COMPRISING MALE FERTILITY RESTORER ALLELES WO 2021/000870 A1 GLUTAMINE SYNTHETASE MUTANT HAVING GLUFOSINATE AMMONIUM RESISTANCE AND APPLICATION THEREOF AND CULTIVATION METHOD THEREFOR

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source 1..20	
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organism = Triticum aestivum	
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FEATURE Location/Qualifiers	
source 1..20	
mol_type = genomic DNA	
organism = Triticum aestivum	
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FEATURE Location/Qualifiers	
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FEATURE Location/Qualifiers	
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organism = Triticum aestivum	
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FEATURE Location/Qualifiers	
source 1..1992	
mol_type = genomic DNA	
organism = Zea mays	
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 mol_type = genomic DNA
 organism = Agrobacterium tumefaciens

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SEQ ID NO: 13 moltype = DNA length = 4206
 FEATURE Location/Qualifiers
 misc_feature 1..4206
 note = SV40 NLS-SpCas9-Nucleoplasmin NLS, maize optimised
 source 1..4206
 mol_type = other DNA
 organism = synthetic construct

SEQUENCE: 13
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source                 1..2043
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VFTYGVLMDC	CCRACRTDLV	LAFFGRLLKT	GLEANQVVFN	TLLKGLCHTK	RADEALDVLL	180

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RAMDKAEVVL	RQMIDDGVP	DNVTYSSLIH	GYSSSGHWKE	AVRVFKEMTS	RRVTADVHTY	300
NMFMTFLCKH	GRSKEAGIF	DTMAIKGLKP	DNVSYAIRLH	GYATEGCLVD	MINLFNSMAT	360
HCLLPNCHIF	NILINAYAKS	GKLKAMLI	NEMQKQGVSP	NAVYSTVIH	AFCKKGRLDD	420
AVIKFNQMID	TGVRPDASVY	RPLIQGFCTH	GDLVKAKEYV	TEMMKKGMPP	PDIMFFSSIM	480
QNLCTEGRVT	EARDILDIV	HIGMRPNVII	FNLILLGGYCL	VRKMADALKV	FDDMVSYGLE	540
PCNFPTYGILI	NGYCKNRRID	DGLLIFKEML	HKGLKPTTFN	YNVILDGLFL	AGQTVAAKEK	600
FDEMVESGSV	VCIDTYSIIL	GGLCRNSCSS	EAITLFRKLS	AMNVKFDITI	VNIIGALYR	660
VERNQEAKDL	FAAMPANGLV	PNAVTVTVM	TNLIKEGSVE	EADNLFLSME	KSGCTANSCL	720
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SEQ ID NO: 17	moltype = DNA	length = 2367			
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FMTFLCKHGR SKEAAGIFDT	MAIKGLKP	VSYAILLHG	AAEGCLVDMI	NLFNSMERDC	360	
ILPDCRIFNI LINAYAKSGK	LDKAMLI	MQKQGVSPNA	VTYSTVIHAF	CKKGRLDDAV	420	
IKPNQMIDTG VRPDASVYRP	LIQGFCTHGD	LVKAKEYVTE	MMKKGMPPD	IMFFSSIMQN	480	
LCTEGRVTEA RDILDLIVHI	GMRPNVIIFN	LLIGGYCLVR	KMADALKVFD	DMVSYGLEPC	540	
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SEQ ID NO: 20 moltype = AA length = 792
FEATURE Location/Qualifiers
source 1..792
mol_type = protein
organism = Triticum timopheevi

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note = RH-Ubi7DL-RFL79	CDS					
source	1..3063					
mol_type = other DNA						
organism = synthetic construct						
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source
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mol_type = other DNA
organism = synthetic construct

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SEQ ID NO: 40	moltype = AA length = 644
FEATURE	Location/Qualifiers
source	1..644
	mol_type = protein
	organism = Triticum aestivum
SEQUENCE: 40	
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EAFAAASGYAR ASGRVGVCVA TSGPATNLV SALADALLDS IPMVATGQV PRRMIGTDAF 180	
QETPIVEVT R SITKHNYLVL DVDEDIPRVIQ EAFFLASSGR PGPVLVDIPK DIQQQMAVPV 240	
WDTPMSLPGY IARLPKPPST ESLEQVLRV GESRRPILYV GGGCAASGEE LRRFVELTGI 300	
PVTTTLMGLG NFPSSDPLSL RMLGMHGTVY ANYAVDKADL LIAFGVRFDD RVTGKIEAFA 360	
SRSKIVHIDI DPAEIGKNKQ PHVSICADV LALQGLNLLD NGSKAQOGLD FGPWHKELDQ 420	
QKREFPLGFK TFGEAIPQY AIVQLDELTK GEAIATGVG QHQMWAAQQYY TYKRPRQWLS 480	
SSGLGAMGFG LPAAAGAAVA NPGVTVVDid GDGSFLMNIQ ELALIRIENL PVKVMILNNQ 540	
HLMVVQWED RFYKANRAHT YLGNPENESE IYPDFVTIAK GPNVPARV KKSEVTAIK 600	
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SEQ ID NO: 41	moltype = DNA length = 1551
FEATURE	Location/Qualifiers
source	1..1551
	mol_type = genomic DNA
	organism = Triticum aestivum
SEQUENCE: 41	
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cgccgcattcc cattccatgc ccacccatcc ctccatggaga agccgttccca cgcgcgtctg 180	
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SEQ ID NO: 42 moltype = AA length = 516
FEATURE Location/Qualifiers
source 1..516
mol_type = protein
organism = Triticum aestivum

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TSSYGPWHRN	LRRRAAVQQL	SARHVACMSG	VIEGEVRAMA RRLFRASVAS PGGAARVELK	180
RRLFELLSLV	LMETIAATQKG	TRSEADADPT	MSVNEAQEFKK DWEDEIPIHIG AANLWDLYPV	240
LRWFDVFVGVR	NKILAAVSRR	DAFMLRLIDA	ERRRLEDGGA EGDKKSMIAV LLTLQKTEPE	300
YVTDTMTAL	CANLEGAGTE	TTSTTTEWAM	SLLLNHPEAL RKAQAEIDVA VGTTSRLLTAD	360
DVPRFLAYLQC	IVSETRLRYA	AAPMPLLPHQS	SADCKVGGYN VPSGTMMLMVN AYAIHRDPAA	420
WERPLELFVPU	RFDGKAEGR	FMPIPFGMGR	RCPGETLALR TIGMVLATLV QCFDWERVGD	480
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SEQ ID NO: 43 moltype = DNA length = 3602
FEATURE Location/Qualifiers
source 1..3602
mol_type = genomic DNA
organism = Zea mays

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SEQ ID NO: 44 moltype = DNA length = 3602
 FEATURE Location/Qualifiers
 source 1..3602
 mol_type = genomic DNA
 organism = Zea mays

SEQUENCE: 44

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SEQ ID NO: 45 moltype = DNA length = 8385
 FEATURE Location/Qualifiers
 source 1..8385
 mol_type = genomic DNA
 organism = Zea mays

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 organism = Zea mays

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FEATURE          Location/Qualifiers
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SEQ ID NO: 52      moltype = DNA  length = 3696
FEATURE          Location/Qualifiers
misc_feature     1..3696
                  note = RCA-Cry1Ac codon optimised
source           1..3696
                  mol_type = other DNA
                  organism = synthetic construct

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SEQ ID NO: 53 moltype = DNA length = 5041
 FEATURE Location/Qualifiers
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 flanked by gRNA20 target sites
 source 1..5041
 mol_type = other DNA
 organism = synthetic construct

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source 1..5200
mol_type = other DNA
organism = synthetic construct
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 organism = Raphanus sativus

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SEQ ID NO: 56 moltype = AA length = 687
FEATURE Location/Qualifiers
source 1..687
mol_type = protein
organism = Raphanus sativus

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	540	600
	660	687

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FEATURE              Location/Qualifiers
source               1..1316
                     mol_type = genomic DNA
                     organism = Brassica napus
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SEQ ID NO: 59	moltype = DNA length = 335		
FEATURE	Location/Qualifiers		
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	mol_type = genomic DNA		
	organism = Brassica napus		
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SEQ ID NO: 60	moltype = DNA length = 4640
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SEQ ID NO: 61 moltype = DNA length = 20
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mol_type = genomic DNA
organism = Brassica napus

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SEQ ID NO: 62 moltype = DNA length = 361
FEATURE Location/Qualifiers
source 1..361
mol_type = genomic DNA
organism = Arabidopsis thaliana

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SEQ ID NO: 63 moltype = DNA length = 3695
FEATURE Location/Qualifiers
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note = BnUbiA09-Rfo -BnUbiA09 donor repair fragment flanked by gRNA16 target sites
source 1..3695
mol_type = other DNA
organism = synthetic construct

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SEQ ID NO: 64	moltype = DNA length = 732
FEATURE	Location/Qualifiers
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	organism = Cauliflower mosaic virus

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tctgtcactt	cattcaaaa	acatgat	aaaggatgg	cacccat	ttggcat	180
gctgat	aaaggatc	tttcaatgt	tttgcattt	tttgcattt	tttgcattt	240
ccccaccc	tttccatgt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	300
tggattgt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	360
atacatgtt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	420
acctcc	tttgcattt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	480
aagg	tttgcattt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	540
ctggcc	tttgcattt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	600
acgttcc	tttgcattt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	660
atgacg	tttgcattt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	720
tttggagg	tttgcattt	tttgcattt	tttgcattt	tttgcattt	tttgcattt	732

SEQ ID NO: 65	moltype = DNA length = 4212
FEATURE	Location/Qualifiers
misc_feature	1..4212
	note = SV40 NLS-Cas9-Nucleoplasmin NLS
source	1..4212
	mol_type = other DNA
	organism = synthetic construct

SEQUENCE: 65						
atggcccaa	agaagaagcg	gaaggtcgtt	atccacgg	tccacgg	cgacaaga	60
tacagcatcg	gcctggacat	cgccacaa	tctgtgg	ggccgt	gacccgac	120
tacaagg	tcacaa	atcaagg	ctggca	ccgaccc	cacccat	180
aagaacct	gtcgac	gtcttc	agccgg	cacccgg	gttgg	240
aagagaacc	ccagaaga	ataccac	cgaaagaa	ggatctcg	tctca	300
atcttc	cgacat	cgacat	cgacat	tccac	cgacat	360
ttctgtt	gggg	gggg	gggg	gggg	gggg	420
gagg	gggg	gggg	gggg	gggg	gggg	480
accac	gggg	gggg	gggg	gggg	gggg	540
agcaccg	gggg	gggg	gggg	gggg	gggg	600
ttcttc	gggg	gggg	gggg	gggg	gggg	660
ggcg	gggg	gggg	gggg	gggg	gggg	720
ctgt	gggg	gggg	gggg	gggg	gggg	780
agcct	gggg	gggg	gggg	gggg	gggg	840
tgac	gggg	gggg	gggg	gggg	gggg	900
cact	gggg	gggg	gggg	gggg	gggg	960
cgt	gggg	gggg	gggg	gggg	gggg	1020
atct	gggg	gggg	gggg	gggg	gggg	1080
tgac	gggg	gggg	gggg	gggg	gggg	1140
gaga	gggg	gggg	gggg	gggg	gggg	1200
ggcg	gggg	gggg	gggg	gggg	gggg	1260
ggcacc	gggg	gggg	gggg	gggg	gggg	1320
ttcgacaa	gggg	gggg	gggg	gggg	gggg	

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ccgcaggaaat	tttttaccc	attctgtaa	gacaaccggg	aaaagatcga	gaagatcctg	1380
accttcggca	tcccacta	cgtggccct	ctggccagg	gaaacacgag	atccgcctgg	1440
atgaccggaa	agagcgag	aaccatcacc	ccttggaaact	tcgaggaat	ggtgacaaag	1500
ggcgcttcg	cccgagctt	catcgagcg	atgaccaact	tcgataaaggaa	cctgccccac	1560
gagaagggtgc	tgcccaagca	cagectgtcg	tacgagtact	tcaccgtgt	taacgagctg	1620
accaaagtga	aatacgtgac	cgaggaaatg	agaaaggccc	ccttcctgag	cgcgagccag	1680
aaaaaggcca	tcgtggacct	gtgttcaag	accacccgg	aagtgcacgt	gaagcgactg	1740
aaagaggact	atctcaagaa	aatcgagtgc	ttcgactccg	tggaaatctc	cgcgctggaa	1800
gatcggttca	acgcctccct	gggcacatac	cacgatctgc	tgaaaattat	caaggacaaag	1860
gacttcttgg	acaatggag	aaacggagg	atcttggaa	atctcggt	gaccctgaca	1920
ctgtttgggg	acagagagat	gatcgaggaa	cggtgtaaaat	cctatggcca	cctgttcgac	1980
gacaaagtga	tgaagcagct	gaaggccgg	agatacacccg	gttggggccag	gttgagccgg	2040
aagctgtatca	acggccatccg	ggacaaggcg	tccggcaaga	caatcttgg	tttcttgaag	2100
tccgacggct	tccggcaacag	aaacttcatcg	cagctgtatcc	acgacgacag	cctgaccctt	2160
aaagaggaga	tccagaaaggc	ccagggtgtc	ggccaggccg	atgcctgca	cgagcacatt	2220
gccaatctgg	ccggcagccc	cgccattaa	aagggcac	tgcagacagi	gaagggtgt	2280
gacgagctcg	tgaaaagtgtat	gggcggcac	aaggccgaga	acatcgat	cgaaatggcc	2340
agagagaaacc	agaccacccca	gaaggggac	aagaacacgg	cgagagaaat	gaagcggtac	2400
gaagaggggca	tcaaaaggct	ggggccgg	atcttggaa	aacaccccg	ggaaaacaccc	2460
cagctgtcg	acggaaagct	gtacccgtac	tacccgtcga	atggccgg	tatgtacgt	2520
gaccaggaaac	ttggacatcaa	ccgggtgtcc	gactacgtat	tgaccat	cgtgcctcag	2580
agttttctga	aggacgactc	catecgacaa	aagggtgtca	ccagaacgca	caagaacccg	2640
ggoaagagcg	acaacgtgtcc	ctccggaa	gtcggtgaga	agatgagaa	ctactggcg	2700
cagctgtcg	acggcaagct	gattaccgg	agaaaatgtc	acaatctgac	caaggcccg	2760
agaggccggcc	tgagcgtact	ggataaggcc	ggtttcatca	agagacagct	gttgaaaacc	2820
cgccagatca	caaagcacgt	ggcagacatc	ctgactcc	ggatgaaac	taagtacgac	2880
gagaatgtaca	agctgtgtcc	ggaaggtgtaa	gtgtatcacc	tgaagtccaa	gttgggtgtcc	2940
gattttccgg	aggatttcca	gttttacaaa	gtcgccgaga	tcaaaacta	ccaccacgccc	3000
cacgacgcct	acctgtaccc	cgtegtgg	accgcctgt	tcaaaaatgt	ccctaagctg	3060
gaaagcgagt	tgctgtacgg	cgactacaag	gtgtacgtc	tgcggaaat	gatcgccaa	3120
agcggcggcc	aaatccggca	ggcttccgg	aagtacttct	tctacaccaa	catcatgaa	3180
tttttcaaga	ccggatattac	cctggccaa	ggcgagatc	ggaaggccg	tctgtatcg	3240
acaaacccgg	aaacccggg	gategtgtgg	gataaggccc	gggatttgc	caccgtcg	3300
aaagtgtcg	gatgtcccc	agtgtatata	gtgaaaaggaa	ccgaggatc	gacaggccg	3360
ttcagcaaaag	agtctatcc	gccccaaagg	aacagcgata	agctgtatcg	cagaagaaag	3420
gactgggacc	tttccggat	cgccggcc	gacagcccc	ccgtggcc	ttctgtgt	3480
gtgtgtggcca	aaatggggaa	ggccggatcc	aagaaactgt	agatgtgt	agatgtgt	3540
gggatccacca	tcatggaa	aagcagctt	gagaagaaatc	ccatcgactt	tctggaa	3600
aagggttacca	aaatgggtgt	aaaggacat	atcatcaac	tgccctaa	ctccctgtt	3660
gagctggaaa	acggccggaa	gagaatgtc	ggctctgg	cgcaactgt	gaaggaaac	3720
gaactggccc	tgccctccaa	atatgtgt	ttctgtacc	tggccacca	ctatgaa	3780
ctgaagggtc	cccccgagga	taatgagc	aaacagctgt	ttgtggaa	gcacaagcac	3840
tacccgtac	agatcatcg	gcacatcg	gagtttccca	agagatgt	cctggccg	3900
gctaatttgt	aaatgggtgt	gtccgttac	aacaaggccc	gggataggcc	catcagag	3960
caggccgaga	atatcttca	cctgtttacc	ctgacca	tggggccccc	tgccgc	4020
aagtacttt	acccacccat	cgaccggaa	aggtacca	gcaccaaa	ggtgtgt	4080
gccaccctgt	tccaccagag	catcaccgg	ctgtacgt	cacggatc	cctgtctc	4140
ctggggaggcc	acaaaaaggcc	ggccggccac	aaaaaggcc	gcccggaa	aaagaaaa	4200
ggcacatct	ag					4212

SEQ ID NO: 66 moltype = DNA length = 680
 FEATURE Location/Qualifiers
 source 1..680
 mol_type = genomic DNA
 organism = Triticum aestivum

SEQUENCE: 66

atgcagatct	tcgtcaagac	ccttacccgg	aaagaccatca	ccctggaggt	tgagtcc	60
gacactattt	acaatgttac	ggccaagatc	caggacaagg	agggcattcc	cccgaggcc	120
cacgcgttta	tctttggcc	caagcagat	gaggatggcc	gcacccttgc	ggattaca	180
atcccagaagg	aatccaccc	ccacccat	ctccgccttgc	ctggggat	gttggat	240
gtcaagaccc	tccacccgg	gaccatcc	ctggggat	atgccttgc	caccattgc	300
aatgtgttgc	ccaaatgtca	ggacaaggag	ggcattcccc	cgaccacg	gcccgtatc	360
tttgcggca	agcagctcg	ggatgtgtc	acccttgc	attacaaat	ccagaaggag	420
ttccaccctt	atctgggt	caggatcc	cgatatttgc	ggatgtgt	ggacacc	480
accggccaga	ccatcc	ggggatgg	tcatctgt	ccattgc	cgtaaggcc	540
aagatccagg	acaaggagg	cattccccc	gaccacg	gtctcat	tgccggta	600
cagcttgagg	atggccgcac	cttggcc	tacaatc	agaaggatc	cacccttac	660
tttgtgttcc	gtctccgt	ggccat				680

SEQ ID NO: 67 moltype = DNA length = 684
 FEATURE Location/Qualifiers
 source 1..684
 mol_type = genomic DNA
 organism = Triticum aestivum

SEQUENCE: 67

atgcagatct	tcgtcaagac	ccttacccgg	aaagaccatca	cccttgaggt	cgagtcc	60
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gacaccatcg acaacgtcaa ggc当地 120
cagcgttcca tcttttgtgg caagc当地 180
atccagaagg agtccacccct cccac当地 240
gtcaagaccc tcaactggcaa aaccatcaco ctc当地 300
aatgtgaagg ccaagatccaa ggacaaggag ggtatccc当地 360
tttgctggca agcagctgaa ggatggcccg accctt当地 420
tccactctcc acttgggtct caggctt当地 480
accggcaaga ccatacaccct ggagggttag tcc当地 540
aagatccagg acaaggaggg cattccccgg gaccagc当地 600
cagcttgagg atggccgcac cctt当地 660
cttgc当地 cctcc gcctt当地 tggc 684

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SEQ ID NO: 68      moltype = DNA length = 740
FEATURE          Location/Qualifiers
source           1..740
mol_type = genomic DNA
organism = Triticum aestivum
SEQUENCE: 68
cagtaagctc ctggccatgg agctgcttct gtctctggg当地 60
ggatatccaa aatggagtct ggtctgtc tgc当地 120
accatactgt gatc当地 tatec当地 ttctcaac ttctgetg当地 180
tttggtaac tatgaataag tgagc当地 atttggt当地 taat当地 ttgtctt当地 240
gatgttgcg tggatggatgg cggat当地 ggtgc当地 atctgctt当地 300
tttggacatg tttctgggat ggtacccct atc当地 tttgtt当地 tgc当地 360
tactaagtgc caactt当地 ttctggctg taga当地 cc当地 taat当地 tcaat当地 420
aaagatatgt ggaatcacct gtgc当地 tggatggg当地 cccat当地 tac gc当地 480
gc当地 gctt当地 tggatggatgg tggatggatgg tggatggatgg tggatggatgg 540
attttgtgtc atc当地 ctggatggatgg gtc当地 tggatggatgg tggatggatgg 600
tacaaggatgc tagctt当地 gcaat当地 gagaca tgc当地 tggatggatgg tggatggatgg 660
acaatttgc当地 caatgtgaaag agagaatagg atc当地 gtgagac cagactggc当地 gtgacccggc 720
cgatctttt当地 caaagatcag 740

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SEQ ID NO: 69      moltype = DNA length = 650
FEATURE          Location/Qualifiers
source           1..650
mol_type = genomic DNA
organism = Triticum aestivum
SEQUENCE: 69
gc当地 gggatgtt ctgtggggtc ggatggatgtt ctttctt当地 agttctgtt当地 60
gattatgtac cgctcatgatgatgtt cctgat当地 tgaaggatc acctt当地 ctggatgg当地 120
atc当地 cgtc当地 atgatgtt当地 gt当地 cctt当地 tgc当地 tgc当地 tgc当地 180
tttctgat当地 ttat当地 tgc当地 atgatccgtg gaaggatc acctt当地 ctggatgg当地 240
tagttctgtc ttat当地 tgc当地 ctgtatgtt当地 cctt当地 tagt当地 gt当地 ctgtat当地 300
atttcatgtc gcatgtc当地 ctgtatgtt当地 tgc当地 gagatc acctt当地 ctggatgg当地 360
tttctgatc aactatgtt当地 cttt当地 tgc当地 tgc当地 tgc当地 tgc当地 420
tgctaatata ttat当地 tgc当地 aaaat当地 tgc当地 tgc当地 tgc当地 tgc当地 480
tctaggatggc agtctt当地 ctggatggatgg tgc当地 tgc当地 tgc当地 tgc当地 540
caatatttgc当地 ttgtatgtt当地 ctgtatgtt当地 tgc当地 tgc当地 tgc当地 tgc当地 600
tatccctgtatc ctgtatgtt当地 tgc当地 tgc当地 tgc当地 tgc当地 tgc当地 650

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SEQ ID NO: 70      moltype = AA length = 14
FEATURE          Location/Qualifiers
source           1..14
mol_type = protein
organism = Triticum aestivum
SEQUENCE: 70
KESTLHLVLR LRGG 14

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SEQ ID NO: 71      moltype = DNA length = 1554
FEATURE          Location/Qualifiers
source           1..1554
mol_type = genomic DNA
organism = Lolium rigidum
SEQUENCE: 71
atggataagg catatattgc catcttctcc tgc当地 ctcc当地 tccactacgtt 60
ctc当地 ggcaagg tcaatggatgg caggccggc当地 aagaaggccg cccgtc当地 gagt 120
ccccccggccca tccctgtcat cggccacccctt caccctgtgg当地 agaaggccat ccacgccc当地 180
atgtgccc当地 tccggccgatc cctc当地 ggccggccg gtcttctccgc tgc当地 ctcc当地 240
gc当地 ctggatggatgg tgc当地 ctggatggatgg cggatggatgg tgc当地 ctggatggatgg 300
ttc当地 cccatgtt当地 ctggatggatgg cccgtc当地 gagt 360
gtc当地 acgtt当地 cccatgtt当地 ctggatggatgg cccgtc当地 gagt 420
cttctccgatc acccgtt当地 ctggatggatgg cccatgtt当地 ctggatggatgg cccgtc当地 gagt 480
gc当地 acgtt当地 ctggatggatgg cccatgtt当地 ctggatggatgg cccgtc当地 gagt 540
aagcggatggc tggatggatgg tgc当地 ctggatggatgg agaaggccat ccacgccc当地 600
gccaccatgtt当地 ctggatggatgg cccatgtt当地 ctggatggatgg cccgtc当地 gagt 660
ggatgtt当地 ctggatggatgg aacatgtt当地 ctggatggatgg cccatgtt当地 ctggatggatgg 720

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gtgtatgcgggt	ggttcgacgt	cttcggcgtg	aggaacaaga	tcttgcacgc	tgtgagccgg	780
agggacgcgt	tcctgcggcg	actcatcgat	gccgaacgccc	ggaggttgcgc	cgacggccggc	840
aggcgatggcc	ataagaagag	catgtatcgcc	gtgtgtgtca	ctctcgacagaa	gacggagggcca	900
aaggctctaa	cggatcacat	gatcacaaggct	ctctgcgcga	attttatgg	ggccgggacg	960
qagaccacgt	caaccacgac	agaqtggggcg	atgtcgctcc	tgtgtqaacca	ccccggcaged	1020
ctgaaagaagg	cccagggcga	gatcgacgcg	tcagtccggga	cctcccgctct	ggtgtccgtc	1080
gacgcgtgc	ccaggtctcg	ctacccgtcg	tgcatcgta	gcgagacgt	ccgcctctac	1140
ccggccggcc	cgctgtctgt	tccacacgacg	tcctccggcg	actgaaagggt	tggccggctac	1200
aacgttgcggc	cggacacat	gtgtatcgatc	aacgtttaacg	ccatccatccg	ggacccggcg	1260
gggtggggac	accggctgga	gttttagccg	gacaggatcg	aggaaeggca	ggccggagggt	1320
ctgttcatga	taccgttccg	gatgggggggg	cgagggttgc	ccggggggagac	gcttggccgt	1380
cgaggacgtac	gaatgttctt	ggcgcacgtct	gtgcgtgtct	tgcgtatggaa	accgggttggac	1440
ggcgttggaa	tggacatgac	gggggggggg	gggttcaacca	tcccaaaggc	cgtgcgttgg	1500
qagggccgtgt	qcaaggccgg	cgccgtatcg	cgcqacgttc	ttcqaaacct	ctaa	1554

SEQ ID NO: 72 moltype = AA length = 517
FEATURE Location/Qualifiers
source 1..517
mol_type = protein
organism = Lolium rigidum

SEQUENCE:	72	MDKAYIAILS	CAFLFLVHYY	LGKVSHGRRG	KKGAVQLPPS	PPAIPFIGHL	HLVEKPIHAT	60
MCRLAARLGP	VFSLRLGSRR	AVVSSSECA	RECFTEDVT	FANRPKEPSQ	LLASPGNTAL			120
VTSSYGPHWL	NLRRRVATVQL	LSAHRVACMS	VGIAAEVRP	ARRLFHAAEE	SPDGAARVQL			180
KRRLFLSLSL	VLMETIAQTQ	ATRSEADADT	DMSVEAQEFK	EVDVKLIPHIL	GAANNWDYLP			240
VMRWFDFVFGV	RNKILHAVSR	RDAFLRRLID	AERRRLADGG	SDGDKKSMA	VLLTLQKTEP			300
KVYTDTMITA	LCANLFGAGT	ETTSTTTWEA	MSLLLNPAA	LKKAQAEIDA	SVGTSLRVSV			360
DDVPSSLAYLQ	CIVSETLRYL	PAAPLLLPH	SSADCKVGGY	NVPADTMLIV	NAYAIHRDPA			420
AWEHPLEFRP	DRFEDGKAEG	LFMIPFGMGR	RRCPGTELAL	RTIGMVLATL	VQCFCDEWPVD			480
GKVMDTEGG	GFTKIPAKVPL	EAVCRPRVAM	RDVLQNL					517

SEQ ID NO: 73 moltype = DNA length = 1892
FEATURE Location/Qualifiers
source 1..1892
mol_type = genomic DNA
organism = Zea mays

SEQUENCE: 73 Organism - Zea mays

tatgtgtgtatatatatgtc	cagccatgcaggatgggcc	acgcgcacataaaacacca	60
aaggcagcgccatgcgtatca	accaaaagtcgtggggccaga	acatcttcctgtccacacgacc	120
acgaccatgttataaggccta	cgtggccgtgccttcgttcc	ccttcattttcgatgtatccac	180
taccttcgtggacgtgtgttgg	ccggcaaggaaatggcaagg	gcaaggaaacgcacgggttg	240
cctccccacgcctccgttccc	ggccacactccgttccgtca	gacgcgttccatcgatgggg	300
cacggaggcgtggccggct	cgccgcgcgcacggggccgg	tgttccatgtgcgttccat	360
tecccgccggcgctgtgtgttgg	gactgtgtgtgtgtgtgtgt	aggagtgttccatcacggggac	420
gacgtgtgttgtcgccaaaccc	ggccggagggttcccaacgg	accttcgtgttccgttccgttcc	480
ggccggctcgccggggccat	tacggggccctactggcgca	acctccggccgtgttccgttcc	540
gtgcagcttcgttccgtcgca	ccgcgtcgccgtcatgttcc	ccgtcgctcgccgcggagggt	600
cgcgcgtatggcgccgcgt	ggggccgcggccgcggccgg	ccggccggccgcgcggccgc	660
cgccatgttccatgtgtgtgt	gttccgttccatgtgtgtgt	tgctcatgttccatgtgtgtgt	720
cgcaccaaaatgtccgtcgca	cgagggggccgcggactcg	acatgtgttccgttccgttcc	780
gaattcaaggatgtgtcgca	cgagatgtgtgtccgtcg	gcacggccaaatgtgtgtgt	840
tacctggccgtgtgtgtgtgt	gtcgacgttccgtgtgtgtgt	ggaaacaaatgtgtgtgtgt	900
gtggccggaggatgtgtgtgtgt	gttccgtcgccgttccatgtgt	cgggccggccgtgtgtgtgt	960
gacggccggccgtgtgtgtgt	cgacgacagatgtgtgtgtgt	aaagacatgtatgtgtgtgt	1020
cagaagtcaagccgggggtgt	gtacacggatgtgtgtgtgt	tggcacttgcgggaaactta	1080
tttggccggccgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	ggccatgtgtgtgtgtgtgt	1140
aaccatccccggggccgtgt	ggggccatgtgtgtgtgtgt	gtcccttcgtgtgtgtgtgt	1200
cgccatctcgccgcaggatgt	gtgtgtgtgtgtgtgtgtgt	ggggccatgtgtgtgtgtgt	1260
acccctgtcgatgtgtgtgtgt	gtgtgtgtgtgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	1320
aagggtcgccgtgtgtgtgtgt	gtgtgtgtgtgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	1380
cacagggtggccgtgtgtgtgt	ggggccgtgtgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	1440
ggcaaggccgtgtgtgtgtgt	gtgtgtgtgtgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	1500
gagaccatccgtgtgtgtgtgt	gtgtgtgtgtgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	1560
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aggggccgtccgtgtgtgtgtgt	gtgtgtgtgtgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	1680
gacgtgtgtgtgtgtgtgtgtgt	gtgtgtgtgtgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	1740
gtgtgtgtgtgtgtgtgtgtgtgt	gtgtgtgtgtgtgtgtgtgt	gttccatgtgtgtgtgtgtgt	1800
qtatqtttaataqcaatqttgttcttqca	ttgtgtgtgtgtgtgtgtgtgt	ttgtgtgtgtgtgtgtgtgtgt	1860
			1892

SEQ ID NO: 74 moltype = AA length = 557
FEATURE Location/Qualifiers
source 1..557
mol_type = protein
organism = Zea mays

SEQUENCE : 74

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MCSHAGWCQR	HIQTPKQRSI	DQSKLVGQNI	SCSTTTMDK	AYVAVLFAF	LFVIIHVLGR	60
AGRKGNGKKG	GTQRLLPPSPP	AVPFLGLHL	VKTPFHEAL	GLAARHGPVF	SMRGMGSRRAL	120
VVSPECAMAE	CFTEHDVVFA	NRPFRATQDL	VSPFGAAALAA	ASYGPWYRN	RRVATVQLLS	180
AHRVACMSVA	VAEAVRAMAR	RMGRAAAAAP	GGAARVQLKR	RLFEVSLSVL	METIARTKTS	240
RAEADADSDM	SPEAHFFKQI	VDEIVPHLT	ANLNDWLYPLW	RWLDFVGFVRN	KITAAGVRD	300
AFLRRLLIDAE	RRRLDDGGGD	SDSDKSMMIA	VLLSLQKSEP	EVYTDTMIMA	LCGNLFGAGT	360
ETTSTTTEWA	MSLLLNHPEA	LKKAQAEIDA	VVGTSRLLAA	EDVPRLGYLH	RVISETLRMY	420
PAAPLLLPHE	SSADCKVGGY	DVARGTLIV	NAYAIHRDPL	VWEDPDEFRP	ERFEGDKAEG	480
RLLMPFGMGR	RKCPGETLAL	RTISLVLGTL	IQCFCWDWRVD	GLEIDMAAGG	GLTPLPRAVPL	540
EATCKPRAAV	RHLLEL					557

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SEQ ID NO: 75 moltype = DNA length = 1851
FEATURE Location/Qualifiers
source 1..1851
mol_type = genomic DNA
organism = Zea mays

SEQUENCE: 75
aaaccacccaa ctcgcgatcc atcgatttc agaacaccac ccaacagcca aaccatca 60
cgatcggttag acatggataa ggcctacatc gcccgcctct ccgcgcgcgc ccttttcttgc 120
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FEATURE Location/Qualifiers
source 1..521
mol_type = protein
organism = Zea mays

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SEQ ID NO: 78 moltype = AA length = 516
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 mol_type = protein
 organism = Triticella sp.

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 TSSYGPWHWRN LRRVAAVQOLL SAHRVACMSG VIEGEVRAMA RRLFRASVAS PGGAARVELK 180
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 DVPRLAYLQC IVSETLRLYP AAPMLLPHQS SADCKVGGYN VPSGTMMLMVN AYAIHRDPAA 420
 WERPLEFVPE RFEDGKAEGR FMIPFGMGR RCPGETLALAR TIGMVLATLV QCFDWERVDG 480
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SEQ ID NO: 79 moltype = AA length = 644
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 mol_type = protein
 organism = Triticum aestivum

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SEQ ID NO: 81 moltype = AA length = 647
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SEQ ID NO: 82	moltype = AA length = 647	
FEATURE	Location/Qualifiers	
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	mol_type = protein	
	organism = Triticum aestivum	
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SEQ ID NO: 83	moltype = AA length = 644	
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	organism = Triticum aestivum	
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SEQ ID NO: 84	moltype = AA length = 644	
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	organism = Triticum aestivum	
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	mol_type = other DNA	
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misc_feature	1..5442	

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caccatgttgcgtat cttttgcgtat cttttgcgtat cttttgcgtat cttttgcgtat 4200
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ccaggatgttgcgtat cttttgcgtat cttttgcgtat cttttgcgtat cttttgcgtat 4320

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ttggcgtga tccgcattga gaacctccca gtgaaggta tgatattgaa caaccagcat	4380
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cttggcaacc cagaaaatga gagtggata tatccagatt ttgtacgat tgctaaagga	4500
tccaacgttc cagcgttcg agtgacgaag aagagtgaag tcactcgac aatcaagaag	4560
atgcttggaa ccccagggcc atacttggg gatatcatag tccccatca ggagcacgtg	4620
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ttgtcgtgac cggccgatc ttttcaag atcagccggc cg	5442

1. A vector suitable for a targeted integration of at least one gene of interest in 5' or 3' of a polyubiquitin gene in a plant, wherein said vector comprises a repair DNA comprising from 5' to 3':

a first gRNA target,
a left ubiquitin-like region,
at least one gene of interest,
a right ubiquitin-like region, and
a second gRNA target.

2. The vector according to claim 1, further comprising:
at least one CRISPR-Cas endonuclease expression cassette and/or
at least one gRNA expression cassette encoding a gRNA able to recognize a region in 3' or 5' of the polyubiquitin gene.

3. The vector according to claim 2, wherein the vector comprises a single gRNA expression cassette.

4. The vector according to claim 1, wherein the gene of interest is selected from the group consisting of a herbicide tolerance gene, an insect resistance gene, a fungal resistance gene, a bacterial resistance gene, a stress resistance gene, a gene involved in reproductive capability, a gene involved in performance in the fields, a gene involved in performance in an industrial process and a gene involved in nutritional value of a plant.

5. The vector according to claim 1, wherein the gene of interest is selected from the group consisting of BAR gene, ALS gene, GS, cyt P450 gene, RFL29a gene, RFL79 gene, Rfo gene, Cry1Ac gene and RCA-Cry1Ac gene.

6. A plant cell or plant tissue comprising at least one gene of interest inserted in 5' or in 3' of the polyubiquitin gene, obtained by transformation with a vector, wherein said vector comprises a repair DNA comprising from 5' to 3':
a first qRNA target,
a left ubiquitin-like region,
at least one gene of interest,
a right ubiquitin-like region, and
a second qRNA target.

7. The plant cell or plant tissue according to claim 6, which is a protoplast, apical meristem, cotyledon, embryo, pollen or microspores.

8. A plant comprising at least one gene of interest inserted in 5' or in 3' of the polyubiquitin gene, obtained by transformation with a vector, wherein said vector comprises a repair DNA comprising from 5' to 3':

a first qRNA target,
a left ubiquitin-like region,
at least one gene of interest,
a right ubiquitin-like region, and
a second qRNA target.

9. The plant cell or plant tissue according to claim 6, wherein said plant cell or plant tissue comprises at least one polyubiquitin gene.

10. A progeny plant of a plant according to claim 8, wherein said progeny plant comprises at least one gene of interest inserted in 5' or in 3' of the polyubiquitin gene.

11. A method for targeted insertion of at least one gene of interest in 5' or 3' of a polyubiquitin gene in a plant genome, comprising:

a. transformation of a plant cell or plant tissue with at least one vector, wherein said vector comprises a repair DNA comprising from 5' to 3':
a first gRNA target,
a left ubiquitin-like region,
at least one gene of interest,
a right ubiquitin-like region, and
a second gRNA target,
to obtain a transformed plant cell or plant tissue, and
b. the regeneration of the plant from the transformed plant cell or plant tissue.

12. The method of claim 11, wherein at least one CRISPR-Cas endonuclease expression cassette is provided by said vector or in a separate vector and wherein at least one gRNA expression cassette is provided by said vector or in a separate vector.

13. A method for expressing at least one protein of interest in a plant, comprising the steps of the method according to claim 11, wherein said gene of interest codes for said protein of interest.

14. A method for expressing at least one gene of interest in a plant, in a plant cell or in a plant tissue, comprising transforming a plant cell or a plant tissue with a vector, wherein said vector comprises a repair DNA comprising from 5' to 3':

a first qRNA target,
a left ubiquitin-like region,
at least one gene of interest,
a right ubiquitin-like region, and
a second gRNA target.

15. A method of identifying a plant comprising at least one gene of interest inserted in 5' or 3' of a polyubiquitin gene, wherein said method comprises:

extracting the DNA, RNA or proteins of a plant,
detecting the presence of a DNA comprising said at least one gene of interest inserted in 5' or 3' of a polyubiquitin gene and/or the presence of a RNA transcript from said DNA, and
optionally, detecting the presence of a protein encoded by said at least one gene of interest.

16. The plant according to claim **8**, wherein said plant comprises at least one polyubiquitin gene.

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