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(57) Abrégé/Abstract:

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(54) Title: ENHANCED PLANT REGENERATION AND TRANSFORMATION BY USING GRF1 BOOSTER GENE

(57) Abstract: The present invention relates to the field of plant breeding and biotechnology and in particular to the generation of plants from cells and other tissues. More particularly, the invention provides methods and means for improving plant regeneration, especially from transformed or genetically modified plant cells using GRF1 booster gene.



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Enhanced Plant Regeneration and Transformation by Using GRF1 Booster Gene

5 The present invention relates to the field of plant breeding and biotechnology and in particular to the generation of plants from cells and other tissues. More particularly, the invention provides methods and means for improving plant regeneration, especially from transformed or genetically modified plant cells.

10 In plant breeding, the process of manipulation of plant species has been practiced since near the beginning of human civilization in order to create desired genotypes and phenotypes for specific purposes. With the development of genetic engineering, this field of agriculture has significantly changed during the last decades. A variety of methods for plant genetic engineering has been developed. The choice of

15 transformation method depends on a number of variables, primarily the plant species to be transformed, the purpose of the experiment and the availability of the necessary equipment. The vast majority of plant transformation techniques requires the use of explants with high regeneration capacities as starting material. In addition, gene editing constitutes a new molecular biological method by means of

20 which specific modifications such as insertions, deletions or point mutations or combinations thereof can be introduced into the genome of a plant. To this end, specific molecular instruments are required which firstly have nuclease activity, but above all can be guided to the target sequence to be modified with sufficient specificity to program and carry out a specific and site-directed mutagenesis. In the

25 past few years in plant biotechnology, specific genome editing has developed into an alternative to conventional breeding and to transgenic strategies. However, tools which are currently available, such as meganucleases, zinc finger nucleases (ZFNs), "transcription activator-like effector nucleases" (TALENs) or CRISPR systems are only used in plant biotechnology to a limited extent because of limited

30 regeneration capacities of edited starting material of plants.

A wide variety of cells have the potential to develop into embryos, including haploid gametophytic cells, such as the cells of pollen and embryo sacs (see Forster, B.P., et al. (2007) Trends Plant Sci. 12: 368-375 and Seguí-Simarro, J.M. (2010) Bot.

35 Rev. 76: 377-404), as well as somatic cells derived from all three fundamental tissue layers of the plant (Gaj, M.D. (2004) Plant Growth Regul. 43: 27-47 or Rose, R., et

al. (2010) "*Developmental biology of somatic embryogenesis*" in: Plant Developmental Biology-Biotechnological Perspectives, Pua E-C and Davey MR, Eds. (Berlin Heidelberg: Springer), pp. 3-26).

5 The ability to regenerate into plants is often limited to particular genotypes in a certain plant species and weakened in transformed and genetically modified plant cells and other precursor tissues. Even if the step of transformation and genetic modification of a plant cell is successful, this does not necessarily mean that the desired plants can actually be obtained from the modified cells. It is assumed that
10 the treatment, which the plant cells and other precursor tissues are subjected to in order to achieve a genetic modification, affects plant development and regeneration. Thus, it is an object of the present invention to improve efficacy of previously known methods for generating transgenic and genetically modified plants and to support the regeneration of plants from modified plant cells and other plant precursors.

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Lowe K, et. al (2016, "Morphogenic regulators Baby boom and Wuschel improve monocot transformation" *Plant Cell* 28:1998-2015.), report a transformation approach that takes advantage of morphogenic genes to create a biological context conducive to transformation efficiency. Their transformation constructs included
20 maize (*Zea mays*) Baby boom (BBM) and Wuschel2 (WUS2). The authors used these constructs for Agrobacterium-mediated transformation of immature embryos from four maize inbred lines. These lines showed markedly higher transformation rates, going from 0 to 2% in the controls to 25 to 51% in the presence of WUS2 and BBM. However, this approach has disadvantages and is not generally applicable as
25 plants that constitutively express ZmWUS2 and ZmBBM are often sterile or even lethal. It would therefore be desirable to have a method for increasing transformation and regeneration efficiency that leads to plants that show no recognizable phenotype.

30 In van der Knaap et al. (2000; "A novel gibberellin-induced gene from rice and its potential regulatory role in stem growth", *Plant physiology*, 122(3), 695-704.) the authors have identified and characterized the first member of the GRF gene family in rice (*OsGRF1*). This was a gibberellic acid-induced gene in intercalary meristems. Overexpression in Arabidopsis caused impaired stem growth, female sterility and
35 reduced male fertility. Application of gibberellic acid could not recover the stem elongation defect of transformed plants, suggesting that *OsGRF1* could participate in the GA-induced stem elongation. In 2003 Kim et al. ("The AtGRF family of

putative transcription factors is involved in leaf and cotyledon growth in *Arabidopsis*”, *The Plant Journal*, 36(1), 94-104.) have characterized the *Arabidopsis* GRF family and determined that the genes are mainly expressed in actively growing tissues. Analysis of null mutants and transgenic plants overexpressing *At GRF1* and *AtGRF2* indicated that some members of the GRF family are involved in the regulation of cell expansion during leaf and cotyledon growth. In addition, overexpressor plants showed delayed bolting time, revealing a putative role in flowering. In a study to determine the molecular mechanisms that coordinate cell proliferation in developing leaves, Rodriguez et al. discovered that the miR396 antagonizes the expression pattern of its targets, the GRF transcription factors, in *Arabidopsis* ((2010), “Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396”, *Development*, 137(1), 103-112.). Thus, the balance between miR396 and the GRFs controls the final number of cells in leaves. Furthermore, the authors showed that miR396-targeted GRFs can regulate the size of the shoot apical meristem.

Kuijt et al. (2014, “Interaction between the *GROWTH-REGULATING FACTOR* and *KNOTTED1-LIKE HOMEBOX* Families of Transcription Factors”, *Plant physiology*, 164(4), 1952-1966.) showed that members of the GRF family act as players in the network controlling the expression of *KNOTTED1-LIKE HOMEBOX* (*KNOX*) genes which are involved in restriction of cell differentiation in the shoot apical meristem. *AtGRF4*, *AtGRF1* and *AtGRF6* are able to bind to the promoter of a *KNOX* gene, repressing its expression. *Arabidopsis* seedlings overexpressing *AtGRF4*, *AtGRF1*, or *AtGRF6* show developmental aberrations in the shoot apical meristem.

Nelissen H, et al (2015, “Dynamic Changes in *ANGUSTIFOLIA3* Complex Composition Reveal a Growth Regulatory Mechanism in the Maize Leaf.” *Plant Cell* 287:1605-1619.) describe the function of *ZmGRF1* during maize leaf development.

In summary, it is well-known that several GRF genes play a role in leaf morphogenesis and stem development. In addition, GRF genes were reported to function in flowering, seed and root development, to control the plant growth under stress conditions and to regulate the plant longevity.

However, during their studies, the inventors of the present invention surprisingly found another and novel function of GRF1 (*GRF1*). *GRF1* is capable of providing a positive effect on boosting plant regeneration and thus allowing a more efficient recovery of transgenic plants. The present invention allows to improve the

regeneration from diverse tissues or cells (e.g. microspores), may overcome recalcitrance to plant regeneration, in particular genotype dependency, improve the recovery of transgenic plants by e.g. co-expression of gene of interest and *GRF1*, and of genome-engineered plants by e.g. transient co-expression of genome-editing components and *GRF1*, as well as shorten the time for the production of transgenic lines and the recovery. Further, it was found that plants carrying *GRF1* are healthy and do not show any obvious phenotype. Thus, a first aspect of the present invention is the use of *GRF1* polypeptide for improving the regenerative ability of a plant.

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Any reference hereinafter to a polypeptide or protein useful in the methods of the present invention is taken to mean a *GRF1* polypeptide or *GRF1* protein as defined herein. Any reference hereinafter to a nucleic acid or polynucleotide useful in the methods of the invention (except for the nucleotide sequence of interest being transformed or the nucleic acid molecule optionally used as repair template for modifying the genome of a plant) is taken to mean a nucleic acid or polynucleotide capable of encoding such a *GRF1* polypeptide or *GRF1* protein. In one embodiment, any reference to a polypeptide/protein or nucleic acid/polynucleotide useful in the methods of the invention is to be understood to mean proteins or nucleic acids useful in the methods, constructs, expression cassettes, plant cells, plants, seeds, harvestable parts and products of the invention. The nucleic acid/polynucleotide including the mRNA(s) to be introduced into a plant cell or plant (and therefore useful in performing the methods of the invention) is any nucleic acid/polynucleotide encoding the type of polypeptide/protein which will now be described, hereafter also named "GRF1 nucleic acid", "GRF1 polynucleotide", "GRF1 gene" or "GRF1 mRNA" or the like.

A "*GRF1 polypeptide*" or "*GRF1 protein*" as defined herein refers to a transcription factor preferably a 14-3-3-like protein GF14-6, more preferably comprising a PFAM domain PF08880 (also known as QLQ domain) and a PFAM domain PF08879 (also known as WRC domain) when analyzed with the Interproscan software (www.ebi.ac.uk/interpro), and even more preferably comprises a PFAM domain PF08880 that finds a match of at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% coverage at or near the N-terminus of the *GRF1* polypeptide and the PFAM domain PF08879 that finds a match of at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least

98%, at least 99% or 100% coverage C-terminally located to the PFAM domain PF08880 of the GRF1 polypeptide, i.e. the amino acid stretch matching PF08879 is located in direction of translation behind the amino acid stretch matching PF08880. Preferably, at least one of the matches has a coverage of at least 95%, at least 5 96%, at least 97%, at least 98%, at least 99% or 100%.

In one embodiment, both matching amino acid stretches are located in the N-terminal half of the GRF1 polypeptide, preferably the amino acid stretch matching PFAM domain PF08880 is located in the N-terminal quarter of the *GRF1* polypeptide. Preferably, the PFAM domain PF08880 matches the amino acid residues of the *GRF1* polypeptide starting from residue 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 of the *GRF1* polypeptide, preferably from residue 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21, and the PFAM domain PF08879 matches the amino acid residues of the *GRF1* polypeptide starting 15 from residue 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 of the *GRF1* polypeptide, preferably from residue 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 or 95. Preferably, the distance between the starting amino acid residue of amino acid stretch matching PFAM domain PF08880 and the starting 20 amino acid residue of amino acid stretch matching PFAM domain PF08879 is 60 to 82 amino acids within the *GRF1* polypeptide, more preferably 60 to 75 amino acids, more preferably 61 to 75 amino acids, even more preferably 62 to 73 amino acids within the *GRF1* polypeptide.

25 In a further embodiment, the *GRF1* polypeptide as used herein comprises the indicator motif: [D]-[P]-[E]-[P]-[G]-[R]-[C]-[R]-[R]-[T]-[D]-[G]-[K]-[K]-[W]-[R]-[C]-[AS]-[RK]-[ED]-[A]-[AH]-[PQS]-[D]-[S]-[K]-[Y]-[C]-[E]-[KR]-[H]-[M]-[H]-[R]-[G]-[R]-[N]-[R] (SEQ ID NO: 25), wherein it is allowable/tolerable that the *GRF1* polypeptide exhibits a maximum number of two mismatches in comparison with the indicator motif, i.e., up to two mismatches may appear in a sequence alignment between the 30 respective *GRF1* polypeptide and the indicator motif, preferably a maximum number of one mismatch in comparison with the indicator motif, i.e., only one mismatch may appear in a sequence alignment between the respective *GRF1* polypeptide and the indicator motif, and more preferably no mismatch in comparison with the indicator motif, and more preferably no mismatch in comparison with the indicator motif, 35 motif. A mismatch means that the amino acid at a certain position according to the indicator motif is replaced by different amino acid or is deleted or shifted by the insertion of at least one additional amino acid. The letters of the indicator motif

within square brackets indicate the amino acid residue (one-letter code) and the motif represents the order of the amino acid residues in direction from N-terminus to C-terminus as present in any *GRF1* polypeptide of the present invention. If there are two or three letters within one square bracket they represent alternatives. The motif

5 has been deduced from a comprehensive comparison of the sequences of GRF1 polypeptides derived from different plant species including monocotyledonous and dicotyledonous plants and allows to distinguish GRF1 polypeptides from other members of the GRF protein family like GRF5. Preferably, the motif is located in the N-terminal half of the GRF1 polypeptide, more preferably the motif is located in the

10 N-terminal half of the GRF1 polypeptide and contains a sub-region of amino acid stretch matching PFAM domain PF08879, i.e. the motif has a sequential overlap with the amino acid stretch matching PFAM domain PF08879. Preferably the indicator motif consists of any of the amino acid sequences SEQ ID NO: 26 to SEQ ID NO: 35. Correspondingly the *GRF1* polypeptide preferably comprises one

15 contiguous motif consisting of any of the amino acid sequences SEQ ID NO: 26 to SEQ ID NO: 35.

According to one aspect, the invention provides a method for transforming a plant cell comprising the steps

- 20 (a1) introducing into a plant cell
- (i) at least one nucleotide sequence of interest; and
 - (ii) an expression cassette comprising a polynucleotide encoding a GRF1 polypeptide, mRNA(s) encoding GRF1 polypeptide or a GRF1 polypeptide, wherein (i) and (ii) can be introduced in parallel or
- 25 sequentially in any order, or
- (a2) introducing into a plant cell at least one nucleotide sequence of interest; and inducing in said plant cell in parallel or sequentially an enhanced expression level of an endogenous gene encoding a GRF1 polypeptide; and
- (b) optionally cultivating the plant cell of (a1) or (a2) or a plant cell derived from
- 30 the plant cell of (a1) or (a2) under conditions, where in the plant cell the GRF1 polypeptide is expressed from the expression cassette, the GRF1 polypeptide is translated from introduced mRNA(s), GRF1 polypeptide(s) is enhanced/increased expressed from the endogenous gene, or the GRF1 polypeptide(s) is (are) present, preferably in an enhanced amount compared
- 35 to the amount in a wild type plant cell or a plant cell into which the expression cassette comprising a polynucleotide encoding a GRF1 polypeptide, mRNA(s) encoding GRF1 polypeptide or the GRF1 polypeptide(s) has (have)

not been introduced according to step (a1) or in which the enhanced expression level of an endogenous gene encoding the GRF1 polypeptide has not been induced according to step (a2).

- 5 The method according to the present invention yields a modified or transformed plant cell having an improved ability of regeneration due to the presence of GRF1 or the presence of GRF1 in an enhanced amount. It is preferred, however, that in the modified plant cell the presence of GRF1 or the presence of GRF1 in an enhanced amount is transient.

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Transformation of a plant cell means introducing a nucleic acid molecule into a plant cell in a manner to cause stable integration into the genome of the plant cell or transient appearance in the plant cell leading to expression of the nucleic acid sequence for example constitutively, temporally or specifically related to particular
15 tissue(s) or certain developmental stage(s) et cetera. Transformation and regeneration of both monocotyledonous and dicotyledonous plant cells is now routine, and the selection of the most appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant or genotype to be transformed; those skilled in the art will recognize the suitability of
20 particular methods for given plant types or genotypes. Suitable methods can include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene glycol (PEG) mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and Agrobacterium-mediated
25 transformation.

Step (a1) (i) or (a2) of introducing the at least one nucleotide sequence of interest can be performed using any suitable method commonly known in the art. A number of methods is available to transfer nucleic acids of interest into plant cells. An
30 exemplary vector mediated method is Agrobacterium-mediated transformation, as described, for example, by Lindsay & Gallois, 1990, Journal of Experimental Botany, and Kischenko et al., 2005, Cell Biology International for sugar beet, by Ishida et al., 2007, ("Agrobacterium-mediated transformation of maize." *Nature protocols*, 2(7), 1614-1621) for corn, or by the PureWheat Technology from Japan Tobacco
35 company for wheat. Other suitable techniques include particle bombardment and electroporation.

The nucleotide sequence of interest according to the invention may be a DNA or RNA sequence, e.g. mRNA, siRNA, miRNA etc. More particularly, the nucleotide sequence of interest encodes at least one phenotypic trait. Preferably, the phenotypic trait conferred by the DNA or RNA can be selected from the group consisting of resistance/tolerance to biotic stress, including pathogen resistance/tolerance, wherein the pathogen can be a virus, bacterial, fungal or animal pathogen, resistance/tolerance to abiotic stress including chilling resistance/tolerance, drought stress resistance/tolerance, osmotic resistance/tolerance, heat stress resistance/tolerance, cold or frost stress resistance/tolerance, oxidative stress resistance/tolerance, heavy metal stress resistance/tolerance, salt stress or water logging resistance/tolerance, lodging resistance/tolerance, shattering resistance/tolerance, or resistance/tolerance against one or more herbicides like glyphosate, glufosinate, 2,4-D, Dicamba, ALS inhibitors et cetera. The at least one phenotypic trait of interest can also be selected from the group consisting of the modification of a further agronomic trait of interest including yield increase, flowering time modification, seed color modification, endosperm composition modification, nutritional content modification or metabolic engineering of a pathway of interest.

In context of the present invention, GRF1 can be introduced as an expression cassette comprising a polynucleotide encoding a GRF1 polypeptide, as mRNA encoding a GRF1 polypeptide (including also pre-mRNA or precursor mRNA) or as a GRF1 polypeptide. Exemplary techniques for introducing a nucleic acid molecule are described above. Alternatively, GRF1 can be provided in the plant cell by activating the expression of the endogenous gene encoding for GRF1 polypeptide. This would lead to an enhanced expression level of the endogenous *GRF1* gene, i.e. to the presence or occurrence of GRF1 polypeptide in an enhanced amount in the plant cell. The activation of the expression of the endogenous gene can be achieved by modifying the activity or structure of the promoter of the endogenous gene encoding the *GRF1* polypeptide. For instances, enhancer elements can be introduced into the promoter by means of gene editing; or either an enhancer element regulating the promoter can be further strengthened or a silencer element regulating the promoter can be weakened by e.g. targeted mutagenesis/modification; or modifications can be introduced into the epigenome related to enhancers by means of gene editing tools like CRISPR systems (Hilton et al. (2015). Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nature biotechnology*, 33(5), 510-517); or

synthetic transcription factors based on e.g. TALE activators or dCas9 activators can be introduced into the cell where they are able to bind targeted recognition sites on or near by the promoter und activate transcription of the *GRF1* gene (Cheng et al. (2013). Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell research*, 23(10), 1163.); or the amount of microRNA (miRNA) in the plant cell regulating the expression of the *GRF1* gene by post-transcriptional inhibition can be reduced by e.g. knock out (null mutant) or knock down in order to increase the amount of translated GRF1 polypeptide in the plant cell.

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Dependent on the plant species as well as on the cell type different levels of gene or expression activation are needed in order to have adequate amount of *GRF1* polypeptide present in the plant cell at the time when regeneration takes place. There are various techniques available to a person skilled in the art in order to measure the actual expression level of an endogenous or an introduced gene, e.g., qPCR, RT-PCR, Northern blot, or microarrays. These methods allow those skilled in the art by routine work to adjust the level of expression of the *GRF1* gene which effects improved regeneration ability from diverse tissues or somatic and reproductive cells (e.g. microspores). In a preferred embodiment, in the plant cell the expression level of an endogenous gene encoding a *GRF1* polypeptide is increased at least by the factor of 2, the factor of 3, or the factor of 5, preferably by the factor of 10, the factor of 25 or factor of 50, more preferred by the factor of 100, the factor of 200, or the factor of 500.

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As described further above the induction of an enhanced expression level of an endogenous gene in a plant cell can be carried out by the application of one or more activators or a precursor thereof. These can be applied to the medium in which the plant cells are cultivated and is then actively or passively absorbed by the plant cell. Furthermore, the one or more activator or a precursor thereof can be directly introduced into the plant cell by microinjection, electroporation or biolistic bombardment. Beside the above synthetic transcription activators, a number of further activators are known from the state of the art that can be used for increasing the expression level of an endogenous gene, in particular the expression level of the endogenous *GRF1* gene: In the recent years, the technical fields of chemical plant genetics and chemical plant biology emerged where biological systems are treated with small molecules to specifically perturb cellular functions. Small molecules are used commercially as drugs, herbicides, and fungicides in different systems, but in

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recent years they are increasingly exploited also as tools for genetic regulation. For instance, chemical genetics involves the discovery of small-molecule effectors of various cellular functions through screens of compound libraries (Dejonghe & Russinova (2017). *Plant Chemical Genetics: From Phenotype-Based Screens to Synthetic Biology*. *Plant Physiology*, pp-01805; Kawasumi, M., & Nghiem, P. (2007). Chemical genetics: elucidating biological systems with small-molecule compounds. *Journal of Investigative Dermatology*, 127(7), 1577-1584.). Such small molecule effectors suitable for the activation of the expression of a target gene like *GRF1*, can be identified by chemical screens following different strategies (Dejonghe & Russinova, 2017). Comprehensive compound libraries are available which allow the simple screening of countless small molecules and the identification of effectors which can be used for activation of the gene expression of genes like *GRF1*. As mentioned above another approach to enhance the expression level of an endogenous gene like *GRF1* is the application of so-called synthetical transcription activators. They are typically designed by the fusion of a recognition domain and at least one activator domain. The recognition domain can be derived from known systems like Zinc finger, TAL effectors or CRISPR; for activation, fusing for instances the herpes simplex virus derived VP-16 or VP-64 activation domains to a recognition domain can cause an increase in transcription. Weaker activation domains such as the AD of human NF- κ B add to the variety of options for gene activation. Furthermore, as shown on endogenous promoters, combinations of activators can be used to introduce synergistic effects (Moore et al. (2014). "Transcription activator-like effectors: a toolkit for synthetic biology." *ACS synthetic biology*, 3(10), 708-716.; US 2002/0046419 A1; Lowder et al. (2017). "Multiplexed transcriptional activation or repression in plants using CRISPR-dCas9-based systems." *Plant Gene Regulatory Networks: Methods and Protocols*, 167-184.). The synthetical transcription activator can be delivered to the plant cell or introduced into the plant cell also as precursor, i.e. as DNA or RNA molecule encoding such artificial or synthetical transcription activator or a domain thereof or as inactive form of transcription activator which is activated later in the cell or in a specific compartment of the cell. Finally, enhancing expression of *GRF* genes can be also achieved by the inactivation of upstream negative regulators or by the creation of a mutant version of the *GRF1* gene that is resistant to such negative regulators.

Preferably, the *GRF1* polypeptide of the present invention comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 2, 37, 39, 41, 43, 45, 47, 49, 51 and 53, or an amino acid sequence having at least 70% identity to a

sequence selected from the group consisting of SEQ ID NO: 2, 37, 39, 41, 43, 45, 47, 49, 51 and 53 preferably at least 80%, at least 85%, at least 90%, more preferably at least 95%, at least 98% or at least 99% identity to a sequence selected from the group consisting of SEQ ID NO: 2, 37, 39, 41, 43, 45, 47, 49, 51 and 53.

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An exogenous (heterologous) or endogenous polynucleotide encoding the GRF1 polypeptide of the invention comprises

- (i) a coding nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 36, 38, 40, 42, 44, 46, 48, 50, and 52;
- 10 (ii) a coding nucleotide sequence comprising a sequence being at least 70%, preferably at least 80%, at least 85%, at least 90%, more preferably at least 95%, at least 98% or at least 99% identical to a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 36, 38, 40, 42, 44, 46, 48, 50, and 52;
- 15 (iii) a nucleotide sequence encoding the GRF1 polypeptide as defined above or a nucleotide sequence encoding a polypeptide encoded by (i) and/or (ii) within the scope of the degeneracy of the genetic code;
- (iv) a nucleotide sequence complementary to the nucleotide sequence of (i), (ii) or (iii); or/and
- 20 (v) a nucleotide sequence hybridizing with a nucleotide sequence of (iv) under stringent condition.

Preferably, the polynucleotide encoding the *GRF1* polypeptide, particularly the polynucleotide encoding the *GRF1* polypeptide, may comprise the coding nucleotide sequence of SEQ ID NO: 1.

For the purpose of this invention, the “*sequence identity*” of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other, is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970). The computer-assisted sequence alignment above, can be conveniently performed using standard software program such as program NEEDLE as implemented in the The European Molecular Biology Open Software Suite (EMBOSS), e.g. version 6.3.1.2 (*Trends in Genetics* 16 (6), 276 (2000)), with its

default parameter, e.g. for proteins matrix = EBLOSUM62, gapopen = 10.0 and gapextend = 0.5.

The terms "*stringent conditions*" or "*hybridization under stringent conditions*" refer to conditions under which nucleotide sequences with sufficient complementarity to one another usually remain hybridized. These stringent conditions are known to the skilled person and described, for example, in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989) 6.3.1-6.3.6. The skilled person knows how to determine the required hybridization conditions on the basis of, for example, Sambrook et al., Molecular Cloning, Cold Spring Harbour Laboratory, 1989. The term "*hybridization conditions*" in this respect refers not only to the actual conditions prevailing during actual agglomeration of the nucleic acids, but also to the conditions prevailing during the subsequent washing steps. Examples of stringent hybridization conditions are conditions under which primarily only those nucleic acid molecules that have at least at least 80%, preferably at least 85%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity undergo hybridization. Stringent hybridization conditions are, for example: 4×SSC at 65°C and subsequent multiple washes in 0.1×SSC at 65°C. for approximately 1 hour. The term "stringent hybridization conditions" as used herein may also mean: hybridization at 68°C in 0.25 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA and 1% BSA for 16 hours and subsequently washing twice with 2×SSC and 0.1% SDS at 68°C. Preferably, hybridization takes place under stringent conditions.

The expression "*operably linked*" means that said elements of the chimeric gene are linked to one another in such a way that their function is coordinated and allows expression of the coding sequence, i.e. they are functionally linked. By way of example, a promoter is functionally linked to another nucleotide sequence when it is capable of ensuring transcription and ultimately expression of said other nucleotide sequence. Two proteins encoding nucleotide sequences are functionally or operably linked to each other if they are connected in such a way that a fusion protein of first and second protein or polypeptide can be formed.

A gene is said to be expressed when it leads to the formation of an expression product. An expression product denotes an intermediate or end product arising from the transcription and optionally translation of the nucleic acid, DNA or RNA, coding for such product, e. g. the second nucleic acid described herein. During the transcription process, a DNA sequence under control of regulatory regions,

particularly the promoter, is transcribed into an RNA molecule. An RNA molecule may either itself form an expression product or be an intermediate product when it is capable of being translated into a peptide or protein. A gene is said to encode an RNA molecule as expression product when the RNA as the end product of the expression of the gene is, e.g., capable of interacting with another nucleic acid or protein. Examples of RNA expression products include inhibitory RNA such as e.g. sense RNA (co-suppression), antisense RNA, ribozymes, miRNA or siRNA, mRNA, rRNA and tRNA. A gene is said to encode a protein as expression product when the end product of the expression of the gene is a protein or peptide.

A nucleic acid (molecule) or nucleotide (sequence) or polynucleotide, as used herein, refers to both DNA and RNA. DNA also includes cDNA and genomic DNA. A nucleic acid molecule can be single- or double-stranded, and can be synthesized chemically or produced by biological expression *in vitro* or even *in vivo*.

It will be clear that whenever nucleotide sequences of RNA molecules are defined by reference to nucleotide sequence of corresponding DNA molecules, the thymine (T) in the nucleotide sequence should be replaced by uracil (U). Whether reference is made to RNA or DNA molecules will be clear from the context of the application.

As used herein "*comprising*" or the like is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined may comprise additional DNA regions etc.

By means of GRF1, a plant cell or other plant precursor tissues can be provided having an improved ability of regeneration. This is particularly helpful for genetically modified plant cells, in particular plant cells with an edited genome.

According to a preferred embodiment of the invention, step (a1) of introducing the at least one nucleotide sequence of interest and GRF1 or the step (a2) of introducing the at least one nucleotide sequence of interest and inducing an enhanced expression level of an endogenous gene encoding the GRF1 yields in transient

transformation of the plant cell. In terms of the invention, "*transient transformation*" means that the inserted sequence is not (stably) integrated into the genome of the plant cell. In another embodiment, a stable transformation is effected, wherein the nucleotide sequence of interest in step (a1) and (a2) of the method for transforming
5 disclosed here and/or the polynucleotide encoding an GRF1 polypeptide in step (a1) of the method of modifying the genome disclosed here is inserted into the genome of the plant cell. According to an especially preferred embodiment of the invention, the nucleotide sequence encoding GRF1 is transformed transiently into the cell while the nucleotide sequence of interest is stably transformed into the genome of
10 the cell.

Modifying the genome of the plant cell can be accomplished by means of a single stranded DNA break (SSB) or double stranded DNA break (DSB) inducing enzyme or a base editor enzyme which preferably recognizes a predetermined site in the
15 genome of said cell.

Thus, another embodiment of the present invention is a method for modifying the genome of a plant cell comprising the steps

- (a1) introducing into a plant cell an expression cassette comprising a
20 polynucleotide encoding a GRF1 polypeptide, mRNA(s) encoding GRF1 polypeptide (including pre-mRNA(s)), or GRF1 polypeptide(s); or
- (a2) inducing in a plant cell an enhanced expression level of an endogenous gene encoding a GRF1 polypeptide; and
- (b) cultivating the plant cell of (a1) or (a2) or a plant cell derived from the plant
25 cell of (a1) or (a2) under conditions where in the plant cell the GRF1 polypeptide is expressed from the expression cassette, GRF1 polypeptide is translated from introduced mRNA(s), GRF1 polypeptide is enhanced/increased expressed from the endogenous gene, or GRF1 polypeptide(s) are present, preferably in an enhanced amount compared to
30 the amount in a wild type plant cell or a plant cell into which the expression cassette comprising a polynucleotide encoding a GRF1 polypeptide, mRNA(s) encoding GRF1 polypeptide or the GRF1 polypeptide has not been introduced according to step (a1) or in which the enhanced expression level of an endogenous gene encoding a GRF1 polypeptide has not been induced
35 according to step (a2);
- (c) modifying the genome of the plant cell of (b) by means of a single stranded DNA break (SSB) or double stranded DNA break (DSB) inducing enzyme or

a base editor enzyme which preferably recognizes a predetermined site in the genome of said cell, and optionally by means of a repair nucleic acid molecule,

wherein the modification of said genome at said predetermined site is selected from

- 5 i. a replacement of at least one nucleotide;
 ii. a deletion of at least one nucleotide;
 iii. an insertion of at least one nucleotide; or
 iv. any combination of i. – iii.; and

wherein step (c) is conducted simultaneously with step (a1)/(a2) and/or (b), before
10 step (a1)/(a2), between step (a1)/(a2) or (b) or after step (b).

As used herein, a "*double-stranded DNA break inducing enzyme*" or "*DSBI enzyme*" is an enzyme capable of inducing a double-stranded DNA break at a particular nucleotide sequence, called the "*recognition site*". Accordingly, a "*single-stranded*
15 *DNA or RNA break inducing enzyme*" or "*SSBI enzyme*" is an enzyme capable of inducing a single-stranded DNA or RNA break at a particular nucleotide sequence.

In order to enable a break at a predetermined target site, the enzymes preferably include a binding domain and a cleavage domain. Particular enzymes capable of
20 inducing double or single-stranded breaks are nucleases as well as variants thereof, no longer comprising a nuclease function but rather operating as recognition molecules in combination with another enzyme. In recent years, many suitable nucleases, especially tailored endonucleases have been developed comprising meganucleases, zinc finger nucleases, TALE nucleases, Argonaute nucleases,
25 derived, for example, from *Natronobacterium gregoryi*, and CRISPR nucleases, comprising, for example, Cas, Cpf1, CasX or CasY nucleases as part of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system. Thus, in a preferred aspect of the invention, the DSB or SSB inducing enzyme is selected from CRISPR systems like CRISPR/Cas9, CRISPR/Cpf1, CRISPR/CasX,
30 CRISPR/CasY, CRISPR/Csm1 or CRISPR/MAD7a, preferably a CRISPR/Cas9 endonuclease or a CRISPR/Cpf1 endonuclease, a zinc finger nuclease (ZFN), a homing endonuclease, a meganuclease and a TAL effector nuclease.

Rare-cleaving endonucleases are enzymes that have a recognition site of preferably
35 about 14 to 70 consecutive nucleotides, and therefore have a very low frequency of cleaving, even in larger genomes such as most plant genomes. Homing endonucleases, also called meganucleases, constitute a family of such rare-

cleaving endonucleases. They may be encoded by introns, independent genes or intervening sequences, and present striking structural and functional properties that distinguish them from the more classical restriction enzymes, usually from bacterial restriction-modification Type II systems. Their recognition sites have a general asymmetry which contrast to the characteristic dyad symmetry of most restriction enzyme recognition sites. Several homing endonucleases encoded by introns or inteins have been shown to promote the homing of their respective genetic elements into allelic intronless or inteinless sites. By making a site-specific double strand break in the intronless or inteinless alleles, these nucleases create recombinogenic ends, which engage in a gene conversion process that duplicates the coding sequence and leads to the insertion of an intron or an intervening sequence at the DNA level. A list of other rare cleaving meganucleases and their respective recognition sites is provided in Table I of WO 03/004659 (pages 17 to 20) (incorporated herein by reference).

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Furthermore, methods are available to design custom-tailored rare-cleaving endonucleases that recognize basically any target nucleotide sequence of choice. Briefly, chimeric restriction enzymes can be prepared using hybrids between a zinc-finger domain designed to recognize a specific nucleotide sequence and the non-specific DNA-cleavage domain from a natural restriction enzyme, such as FokI. Such methods have been described e.g. in WO 03/080809, WO 94/18313 or WO 95/09233 and in Isalan et al. (2001). A rapid, generally applicable method to engineer zinc fingers illustrated by targeting the HIV-1 promoter. *Nature biotechnology*, 19(7), 656; Liu et al. (1997). Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. *Proceedings of the National Academy of Sciences*, 94(11), 5525-5530.).

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Another example of custom-designed endonucleases includes the TALE nucleases (TALENs), which are based on transcription activator-like effectors (TALEs) from the bacterial genus *Xanthomonas* fused to the catalytic domain of a nuclease (e.g. FokI or a variant thereof). The DNA binding specificity of these TALEs is defined by repeat-variable di-residues (RVDs) of tandem-arranged 34/35-amino acid repeat units, such that one RVD specifically recognizes one nucleotide in the target DNA. The repeat units can be assembled to recognize basically any target sequences and fused to a catalytic domain of a nuclease create sequence specific endonucleases (see e.g. Boch et al. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science*, 326(5959), 1509-1512; Moscou & Bogdanove (2009). A

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simple cipher governs DNA recognition by TAL effectors. *Science*, 326(5959), 1501-1501; and WO 2010/079430, WO 2011/072246, WO 2011/154393, WO 2011/146121, WO 2012/001527, WO 2012/093833, WO 2012/104729, WO 2012/138927, WO 2012/138939). WO 2012/138927 further describes monomeric
5 (compact) TALENs and TALEs with various catalytic domains and combinations thereof.

Recently, a new type of customizable endonuclease system has been described; the so-called CRISPR/Cas system. A CRISPR system in its natural environment
10 describes a molecular complex comprising at least one small and individual non-coding RNA in combination with a Cas nuclease or another CRISPR nuclease like a Cpf1 nuclease (Zetsche et al., „Cpf1 Is a Single RNA-Guides Endonuclease of a Class 2 CRISPR-Cas System", *Cell*, 163, pp. 1-13, October 2015) which can produce a specific DNA double-stranded break. Presently, CRISPR systems are
15 categorized into 2 classes comprising five types of CRISPR systems, the type II system, for instance, using Cas9 as effector and the type V system using Cpf1 as effector molecule (Makarova et al., *Nature Rev. Microbiol.*, 2015). In artificial CRISPR systems, a synthetic non-coding RNA and a CRISPR nuclease and/or optionally a modified CRISPR nuclease, modified to act as nickase or lacking any
20 nuclease function, can be used in combination with at least one synthetic or artificial guide RNA or gRNA combining the function of a crRNA and/or a tracrRNA (Makarova et al., 2015, supra). The immune response mediated by CRISPR/Cas in natural systems requires CRISPR-RNA (crRNA), wherein the maturation of this guiding RNA, which controls the specific activation of the CRISPR nuclease, varies
25 significantly between the various CRISPR systems which have been characterized so far. Firstly, the invading DNA, also known as a spacer, is integrated between two adjacent repeat regions at the proximal end of the CRISPR locus. Type II CRISPR systems code for a Cas9 nuclease as key enzyme for the interference step, which system contains both a crRNA and also a trans-activating RNA (tracrRNA) as the
30 guide motif. These hybridize and form double-stranded (ds) RNA regions which are recognized by RNaseIII and can be cleaved in order to form mature crRNAs. These then in turn associate with the Cas molecule in order to direct the nuclease specifically to the target nucleic acid region. Recombinant gRNA molecules can comprise both the variable DNA recognition region and also the Cas interaction
35 region and thus can be specifically designed, independently of the specific target nucleic acid and the desired Cas nuclease. As a further safety mechanism, PAMs (protospacer adjacent motifs) must be present in the target nucleic acid region;

these are DNA sequences which follow on directly from the Cas9/RNA complex-recognized DNA. The PAM sequence for the Cas9 from *Streptococcus pyogenes* has been described to be "NGG" or "NAG" (Standard IUPAC nucleotide code) (Jinek et al, "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity", Science 2012, 337: 816-821). The PAM sequence for Cas9 from *Staphylococcus aureus* is "NNGRRT" or "NNGRR(N)". Further variant CRISPR/Cas9 systems are known. Thus, a *Neisseria meningitidis* Cas9 cleaves at the PAM sequence NNNNGATT. A *Streptococcus thermophilus* Cas9 cleaves at the PAM sequence NNAGAAW. Recently, a further PAM motif NNNNRYAC has been described for a CRISPR system of *Campylobacter* (WO 2016/021973 A1). For Cpf1 nucleases it has been described that the Cpf1-crRNA complex, without a tracrRNA, efficiently recognize and cleave target DNA proceeded by a short T-rich PAM in contrast to the commonly G-rich PAMs recognized by Cas9 systems (Zetsche et al., supra). Furthermore, by using modified CRISPR polypeptides, specific single-stranded breaks can be obtained. The combined use of Cas nickases with various recombinant gRNAs can also induce highly specific DNA double-stranded breaks by means of double DNA nicking. By using two gRNAs, moreover, the specificity of the DNA binding and thus the DNA cleavage can be optimized. Further CRISPR effectors like CasX and CasY effectors originally described for bacteria, are meanwhile available and represent further effectors, which can be used for genome engineering purposes (Burstein et al., "New CRISPR-Cas systems from uncultivated microbes", Nature, 2017, 542, 237-241).

The cleavage site of a DSBI/SSBI enzyme relates to the exact location on the DNA or RNA where the double-stranded break is induced. The cleavage site may or may not be comprised in (overlap with) the recognition site of the DSBI/SSBI enzyme and hence it is said that the cleavage site of a DSBI/SSBI enzyme is located at or near its recognition site. The recognition site of a DSBI/SSBI enzyme, also sometimes referred to as binding site, is the nucleotide sequence that is (specifically) recognized by the DSBI/SSBI enzyme and determines its binding specificity. For example, a TALEN or ZNF monomer has a recognition site that is determined by their RVD repeats or ZF repeats respectively, whereas its cleavage site is determined by its nuclease domain (e.g. FokI) and is usually located outside the recognition site. In case of dimeric TALENs or ZFNs, the cleavage site is located between the two recognition/binding sites of the respective monomers, this intervening DNA or RNA region where cleavage occurs being referred to as the spacer region.

A person skilled in the art would be able to either choose a DSB/SSB enzyme recognizing a certain recognition site and inducing a DSB or SSB at a cleavage site at or in the vicinity of the preselected/predetermined site or engineer such a DSB/SSB enzyme. Alternatively, a DSB/SSB enzyme recognition site may be introduced into the target genome using any conventional transformation method or by crossing with an organism having a DSB/SSB enzyme recognition site in its genome, and any desired nucleic acid may afterwards be introduced at or in the vicinity of the cleavage site of that DSB/SSB enzyme.

10

A "*base editor enzyme*" or "*base editor*" as used herein refers to a protein or a fragment thereof having the same catalytical activity as the protein it is derived from, which protein or fragment thereof, alone or when provided as molecular complex, referred to as base editing complex herein, has the capacity to mediate a targeted base modification, i.e., the conversion of a base of interest resulting in a point mutation of interest which in turn can result in a targeted mutation, if the base conversion does not cause a silent mutation, but rather a conversion of an amino acid encoded by the codon comprising the position to be converted with the base editor. Preferably, the at least one base editor according to the present invention is temporarily or permanently linked to at least one site-specific effector, or optionally to a component of at least one site-specific effector complex. The linkage can be covalent and/or non-covalent.

20

Any base editor or site-specific effector, or a catalytically active fragment thereof, or any component of a base editor complex or of a site-specific effector complex as disclosed herein can be introduced into a cell as a nucleic acid fragment, the nucleic acid fragment representing or encoding a DNA, RNA or protein effector, or it can be introduced as DNA, RNA and/or protein, or any combination thereof.

25

There are two major and distinct pathways to repair breaks - homologous recombination and non-homologous end-joining (NHEJ). Homologous recombination requires the presence of a homologous sequence as a template (e.g., "*donor*") to guide the cellular repair process and the results of the repair are error-free and predictable. In the absence of a template (or "*donor*") sequence for homologous recombination, the cell typically attempts to repair the break via the process of non-homologous end-joining (NHEJ).

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In a particularly preferred aspect of this embodiment, a repair nucleic acid molecule is additionally introduced into the plant cell. As used herein, a “*repair nucleic acid molecule*” is a single-stranded or double-stranded DNA molecule or RNA molecule that is used as a template for modification of the genomic DNA at the preselected site in the vicinity of or at the cleavage site. As used herein, “*use as a template for modification of the genomic DNA*”, means that the repair nucleic acid molecule is copied or integrated at the preselected site by homologous recombination between the flanking region(s) and the corresponding homology region(s) in the target genome flanking the preselected site, optionally in combination with non-homologous end-joining (NHEJ) at one of the two end of the repair nucleic acid molecule (e.g. in case there is only one flanking region). Integration by homologous recombination will allow precise joining of the repair nucleic acid molecule to the target genome up to the nucleotide level, while NHEJ may result in small insertions/deletions at the junction between the repair nucleic acid molecule and genomic DNA.

As used herein, “*a modification of the genome*”, means that the genome has changed by at least one nucleotide. This can occur by replacement of at least one nucleotide and/or a deletion of at least one nucleotide and/or an insertion of at least one nucleotide, as long as it results in a total change of at least one nucleotide compared to the nucleotide sequence of the preselected genomic target site before modification, thereby allowing the identification of the modification, e.g. by techniques such as sequencing or PCR analysis and the like, of which the skilled person will be well aware.

As used herein “*a preselected site*”, “*a predetermined site*” or “*predefined site*” indicates a particular nucleotide sequence in the genome (e.g. the nuclear genome or the chloroplast genome) at which location it is desired to insert, replace and/or delete one or more nucleotides. This can e.g. be an endogenous locus or a particular nucleotide sequence in or linked to a previously introduced foreign DNA or transgene. The preselected site can be a particular nucleotide position at(after) which it is intended to make an insertion of one or more nucleotides. The preselected site can also comprise a sequence of one or more nucleotides which are to be exchanged (replaced) or deleted.

As used in the context of the present application, the term “*about*” means +/- 10% of the recited value, preferably +/- 5% of the recited value. For example, about 100

nucleotides (nt) shall be understood as a value between 90 and 110 nt, preferably between 95 and 105.

As used herein, a "*flanking region*", is a region of the repair nucleic acid molecule
5 having a nucleotide sequence which is homologous to the nucleotide sequence of
the DNA region flanking (i.e. upstream or downstream) of the preselected site. It will
be clear that the length and percentage sequence identity of the flanking regions
should be chosen such as to enable homologous recombination between said
flanking regions and their corresponding DNA region upstream or downstream of the
10 preselected site. The DNA region or regions flanking the preselected site having
homology to the flanking DNA region or regions of the repair nucleic acid molecule
are also referred to as the homology region or regions in the genomic DNA.

To have sufficient homology for recombination, the flanking DNA regions of the
15 repair nucleic acid molecule may vary in length, and should be at least about 10 nt,
about 15 nt, about 20 nt, about 25 nt, about 30 nt, about 40 nt or about 50 nt in
length. However, the flanking region may be as long as is practically possible (e.g.
up to about 100-150 kb such as complete bacterial artificial chromosomes (BACs).
Preferably, the flanking region will be about 50 nt to about 2000 nt, e.g. about 100
20 nt, 200 nt, 500 nt or 1000 nt. Moreover, the regions flanking the DNA of interest
need not be identical to the homology regions (the DNA regions flanking the
preselected site) and may have between about 80% to about 100% sequence
identity, preferably about 95% to about 100% sequence identity with the DNA
regions flanking the preselected site. The longer the flanking region, the less
25 stringent the requirement for homology. Furthermore, to achieve exchange of the
target DNA sequence at the preselected site without changing the DNA sequence of
the adjacent DNA sequences, the flanking DNA sequences should preferably be
identical to the upstream and downstream DNA regions flanking the preselected
site.

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As used herein, "*upstream*" indicates a location on a nucleic acid molecule which is
nearer to the 5' end of said nucleic acid molecule. Likewise, the term "*downstream*"
refers to a location on a nucleic acid molecule which is nearer to the 3' end of said
nucleic acid molecule. For avoidance of doubt, nucleic acid molecules and their
35 sequences are typically represented in their 5' to 3' direction (left to right).

In order to target sequence modification at the preselected site, the flanking regions must be chosen so that 3' end of the upstream flanking region and/or the 5' end of the downstream flanking region align(s) with the ends of the predefined site. As such, the 3' end of the upstream flanking region determines the 5' end of the predefined site, while the 5' end of the downstream flanking region determines the 3' end of the predefined site.

As used herein, said preselected site being located outside or away from said cleavage (and/or recognition) site, means that the site at which it is intended to make the genomic modification (the preselected site) does not comprise the cleavage site and/or recognition site of the DSB/SSB enzyme or the base editor enzyme, i.e. the preselected site does not overlap with the cleavage (and/or recognition) site. Outside/away from in this respect thus means upstream or downstream of the cleavage (and/or recognition) site.

The modified plant cell that has been transformed or gene edited according to the methods of the present invention and possibly has a modified genome can be regenerated into a whole (fertile) plant. Due to the presence of the additional *GRF1* in the plant cell their ability to regenerate is significantly improved. Thus, in a preferred aspect of the invention, the transformation of a plant cell or the modification of a genome of a plant cell, respectively, is followed by a step of regenerating a plant. Accordingly, the present invention provides a method for producing a transgenic plant comprising

- (a) transforming a plant cell as described hereinabove and
- (b) regenerating from the plant cell of (a) or from a plant cell derived from the plant cell of (a) a plant comprising at least one plant cell which comprises the at least one nucleotide sequence of interest as a transgene.

Further, the present invention also provides a method of producing a genetically modified plant comprising

- (a) modifying the genome of a plant cell as described hereinabove and
- (b) regenerating from the plant cell of (a) or from a plant cell derived from the plant cell of (a) a plant comprising in at least one cell the modification of the genome or the modified plant cell.

As used herein, "*regeneration*" refers to a process, in which single or multiple cells proliferate and develop into tissues, organs, and eventually entire plants.

Step b) of regenerating a plant can for example comprise culturing the transformed or genetically modified plant cell from step a) on a regeneration medium.

- 5 Regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, occasionally relying on a biocide and/or herbicide marker that can be introduced together with the desired nucleotide sequence(s) of interest. Plant regeneration from cultured protoplasts is described in Evans et al., Protoplasts Isolation and Culture, Handbook of Plant Cell Culture, pp. 124-176, 10 MacMillan Publishing Company, New York, 1983; and Binding, Regeneration of Plants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, protoplasts, immature or mature embryos, embryonic tissue, meristematic tissues, organs, or parts thereof. Such regeneration techniques are described generally in Klee (1987) Ann. Rev. of Plant 15 Phys. 38:467-486. To obtain whole plants from transgenic tissues such as immature embryos, they can be grown under controlled environmental conditions in a series of media containing nutrients and hormones, a process known as tissue culture. Once whole plants are generated and produce seed, evaluation of the progeny begins.
- 20 According to the present invention, it is not only possible to improve the regeneration ability of transformed or genetically modified plant cells, but also other types of sensitive cells with poor regeneration abilities. In particular, the production of a haploid plant embryo from precursors like an immature male gametophyte or a microspore can be improved by means of *GRF1*.

25

Thus, another aspect of the present invention is a method of producing a haploid plant embryo comprising the steps

- (a1) introducing into an immature male gametophyte or a microspore an expression cassette comprising a polynucleotide encoding a GRF1 polypeptide, mRNA(s) encoding GRF1 polypeptide, or GRF1 polypeptide(s); 30 or
- (a2) inducing in an immature male gametophyte or a microspore an enhanced expression level of an endogenous gene encoding a GRF1 polypeptide; and
- (b) cultivating the immature male gametophyte or the microspore of (a) under 35 conditions where in the immature male gametophyte or the microspore the GRF1 polypeptide is expressed from the expression cassette, GRF1 polypeptide is translated from introduced mRNA(s), GRF1 polypeptide is

- enhanced expressed from the endogenous gene, or *GRF1* polypeptide(s) are present, preferably in an enhanced amount compared to the amount in a wild type plant cell or a plant cell into which the expression cassette comprising a polynucleotide encoding a *GRF1* polypeptide, mRNA(s) encoding *GRF1* polypeptide or the *GRF1* polypeptide has not been introduced according to step (a1) or in which the enhanced expression level of an endogenous gene encoding a *GRF1* polypeptide has not been induced according to step (a2); and
- (c) selecting a haploid plant embryo derived from the immature male gametophyte or the microspore of step (b).

The invention also includes a method of producing haploid seedlings comprising exposing haploid plant material to an expression cassette comprising a polynucleotide encoding a *GRF1* polypeptide, mRNA encoding *GRF1* polypeptide, or *GRF1* polypeptide(s) to produce haploid embryos and then converting (i.e. germinating) the haploid embryos into seedlings. The invention therefore includes a method of making haploid plants comprising growing a seedling produced in accordance with the aforementioned method. The invention also provides a method of producing a double haploid plant comprising culturing haploid plant material in the presence of *GRF1* for a period, stimulating or allowing a spontaneous chromosome doubling, and growing the double haploid plant material into a seedling, plantlet or plant. In certain embodiments, haploid embryogenesis and chromosome doubling may take place substantially simultaneously. In other embodiments, there may be a time delay between haploid embryogenesis and chromosome doubling. Should growth of haploid seedlings, plants or plantlets not involve a spontaneous chromosome doubling event, then a chemical chromosome doubling agent may be used, e.g. colchicine. Many procedures involve contact of plant cells with colchicine, anti-microtubule agents or anti-microtubule herbicides such as pronamide, nitrous oxide, or any mitotic inhibitor. The result is homozygous doubled haploid cells. Where colchicine is used, the concentration in the medium may be generally 0.01%-0.2%. The range of colchicine concentration may be from about 400 - 600 mg/L.

Where a microspore is exposed to *GRF1* polypeptide(s), then a callus may form and this may undergo organogenesis to form an embryo. The invention therefore includes a method of producing haploid plant callus comprising exposing an immature male gametophyte or a microspore to *GRF1* polypeptide(s) or introducing

into it an expression cassette comprising a polynucleotide encoding a *GRF1* polypeptide, mRNA encoding *GRF1* polypeptide, or *GRF1* polypeptide(s).

5 The exposure of the immature male gametophyte or the microspore to *GRF1* during cultivation step is preferably carried out for a period of time sufficient to induce haploid embryo formation. The period of time needed may depend on the species of plant concerned and these are all readily ascertainable by a person of ordinary skilled in the art. A preferred range of *GRF1* exposure is from about 1 to about 20 hours; more preferably from about 2 to about 20 hours.

10

In certain aspects of the invention, a physical stress is applied to the haploid plant material prior to the introduction of the expression cassette comprising a polynucleotide encoding a *GRF1* polypeptide, mRNA encoding *GRF1* polypeptide, or *GRF1* polypeptide(s). The physical stress may be any of temperature, darkness, 15 light or ionizing radiation, starvation or osmotic stress, for example. The light may be full spectrum sunlight, or one or more frequencies selected from the visible, infrared or UV spectrum. The stresses may be continuous or interrupted (periodic); regular or random over time.

20 In another aspect of the invention, it was found that transformation and regeneration capacity of plant material can be further improved by combining *GRF1* and *WUS2*. Accordingly, in addition to introducing an expression cassette comprising a polynucleotide encoding a *GRF1* polypeptide, mRNA encoding a *GRF1* polypeptide, or *GRF1* polypeptide(s) as described above in the method for transforming a plant 25 cell, the method for modifying the genome of a plant cell, or the method of producing a haploid plant embryo according to the present invention for step (a1), or inducing an enhanced expression level of an endogenous gene encoding a *GRF1* polypeptide as described above in the method for transforming a plant cell, the method for modifying the genome of a plant cell, or the method of producing a haploid plant embryo according to the present invention for step (a2), the approach 30 of the invention according to this aspect further provides for introducing an expression cassette comprising a polynucleotide encoding a *WUS2* polypeptide, mRNA encoding a *WUS2* polypeptide, or *WUS2* polypeptide(s) into the plant cell. If in step (a1) an expression cassette comprising a polynucleotide encoding a *GRF1* polypeptide is introduced into the cell, the polynucleotide encoding a *WUS2* 35 polypeptide can be integrated into the same expression cassette and operably linked to the same regulatory elements.

This approach has proven to be advantageous in both the transformation of plant cells, and the modification of plant cells as well as in methods of producing transgenic plants, genetically modified plants or haploid plant embryos as described
 5 above.

Preferably, the WUS2 polypeptide of the present invention comprises an amino acid sequence selected from group consisting of SEQ ID NO: 8, 10, 12, 14, 16, 18, 20, 22, and 24, or an amino acid sequence having at least 70% identity to SEQ ID NO:
 10 8, 10, 12, 14, 16, 18, 20, 22, and 24, preferably at least 80%, at least 85%, at least 90%, more preferably at least 95%, at least 98% or at least 99% identity to SEQ ID NO: 8, 10, 12, 14, 16, 18, 20, 22, and 24.

A polynucleotide encoding the WUS2 polypeptide of the invention preferably
 15 comprises

- (i) a coding nucleotide sequence selected from group consisting of SEQ ID NO: 7, 9, 11, 13, 15, 17, 19, 21, and 23,
- (ii) a coding nucleotide sequence comprising a sequence being at least 70%, preferably at least 80%, at least 85%, at least 90%, more preferably at least
 20 95%, at least 98% or at least 99% identical to a coding nucleotide sequence selected from group consisting of SEQ ID NO: 7, 9, 11, 13, 15, 17, 19, 21, and 23;
- (iii) a nucleotide sequence encoding a WUS2 polypeptide encoded by (i) or (ii) within the scope of the degeneracy of the genetic code;
- 25 (iv) a nucleotide sequence complementary to a nucleotide sequence of (i), (ii) or (iii); or/and
- (v) a nucleotide sequence hybridizing with a nucleotide sequence of (iv) under stringent condition.

30 The present invention is applicable to any plant species, whether monocot or dicot. Preferably, plants which may be subject to the methods and uses of the present invention are plants of the genus selected from the group consisting of *Hordeum*, *Sorghum*, *Saccharum*, *Zea*, *Setaria*, *Oryza*, *Triticum*, *Secale*, *Triticale*, *Malus*, *Brachypodium*, *Aegilops*, *Daucus*, *Beta*, *Eucalyptus*, *Nicotiana*, *Solanum*, *Coffea*,
 35 *Vitis*, *Erythrante*, *Genlisea*, *Cucumis*, *Marus*, *Arabidopsis*, *Crucihimalaya*, *Cardamine*, *Lepidium*, *Capsella*, *Olmarabidopsis*, *Arabis*, *Brassica*, *Eruca*, *Raphanus*, *Citrus*, *Jatropha*, *Populus*, *Medicago*, *Cicer*, *Cajanus*, *Phaseolus*,

Glycine, *Gossypium*, *Astragalus*, *Lotus*, *Torenia*, *Allium*, or *Helianthus*. More preferably, the plant is selected from the group consisting of *Hordeum vulgare*, *Hordeum bulbosum*, *Sorghum bicolor*, *Saccharum officinarum*, *Zea* spp., including *Zea mays*, *Setaria italica*, *Oryza minuta*, *Oryza sativa*, *Oryza australiensis*, *Oryza*
 5 *alta*, *Triticum aestivum*, *Triticum durum*, *Secale cereale*, *Triticale*, *Malus domestica*, *Brachypodium distachyon*, *Hordeum marinum*, *Aegilops tauschii*, *Daucus glochidiatus*, *Beta* spp., including *Beta vulgaris*, *Daucus pusillus*, *Daucus muricatus*, *Daucus carota*, *Eucalyptus grandis*, *Nicotiana sylvestris*, *Nicotiana tomentosiformis*, *Nicotiana tabacum*, *Nicotiana benthamiana*, *Solanum lycopersicum*, *Solanum*
 10 *tuberosum*, *Coffea canephora*, *Vitis vinifera*, *Erythraea guttata*, *Genlisea aurea*, *Cucumis sativus*, *Maris notabilis*, *Arabidopsis arenosa*, *Arabidopsis lyrata*, *Arabidopsis thaliana*, *Crucihimalaya himalaica*, *Crucihimalaya wallichii*, *Cardamine nexuosa*, *Lepidium virginicum*, *Capsella bursa pastoris*, *Olmarabidopsis pumila*, *Arabis hirsute*, *Brassica napus*, *Brassica oleracea*, *Brassica rapa*, *Raphanus*
 15 *sativus*, *Brassica juncacea*, *Brassica nigra*, *Eruca vesicaria* subsp. *sativa*, *Citrus sinensis*, *Jatropha curcas*, *Populus trichocarpa*, *Medicago truncatula*, *Cicer yamashitae*, *Cicer bijugum*, *Cicer arietinum*, *Cicer reticulatum*, *Cicer judaicum*, *Cajanus cajanifolius*, *Cajanus scarabaeoides*, *Phaseolus vulgaris*, *Glycine max*, *Gossypium* sp., *Astragalus sinicus*, *Lotus japonicas*, *Torenia fournieri*, *Allium cepa*,
 20 *Allium fistulosum*, *Allium sativum*, *Helianthus annuus*, *Helianthus tuberosus* and/or *Allium tuberosum*. Particularly preferred are *Beta vulgaris*, *Zea mays*, *Triticum aestivum*, *Hordeum vulgare*, *Secale cereale*, *Helianthus annuus*, *Solanum tuberosum*, *Sorghum bicolor*, *Brassica rapa*, *Brassica napus*, *Brassica juncacea*, *Brassica oleracea*, *Raphanus sativus*, *Oryza sativa*, *Glycine max*, and/or *Gossypium*
 25 sp.

Suitable plant cells according to the present invention are especially cells of a somatic tissue, callus tissue, a meristematic tissue or an embryonic tissue, or a protoplast, gametophyte, pollen, ovule or microspore. For example, cells of a callus tissue,
 30 preferably of a friable callus, of a meristematic tissue, of a reproductive tissue (e.g. microspores) or an embryonic tissue as well as protoplasts can be used.

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

Subject matter of the present invention are also the plants that are obtained or obtainable by the methods described above. Accordingly, one embodiment of the invention is a transgenic plant obtained or obtainable by the above method of transforming a plant cell and regenerating a plant from said cell, as well as progeny or parts thereof, wherein the progeny or the part comprises the at least one nucleotide sequence of interest as transgene. Another embodiment of the invention is a genetically modified plant obtained or obtainable by the above method of modifying the genome of a plant cell and regenerating a plant from said cell as well as progeny or parts thereof, wherein the progeny or the part comprises the modification in the genome introduced by the above method of modification.

Further subject matter of the present invention is a plant cell or a seed derived from the above transgenic plant or genetically modified plant. Such a plant cell preferably comprises a polynucleotide encoding a GRF1 polypeptide transiently or stably integrated and a single-stranded DNA break (SSB)- or double-stranded DNA break (DSB)-inducing enzyme or base editor enzyme, which preferably recognizes a predetermined site in the genome of said cell and optionally a repair nucleic acid molecule. The polynucleotide encoding the *GRF1* polypeptide is preferably operably linked to a suitable regulatory sequence so that the plant cell is capable of expressing the *GRF1* polypeptide. A regulatory sequence means, for example, a "promoter" which refers to a nucleotide sequence, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. "Constitutive promoter" refers to promoters that direct gene expression in nearly all tissues and at all times. Examples of constitutive promoters include CaMV 35S promoter, double CaMV 35S promoter (70S promoter), nopaline synthase (nos) promoter, *BdEF1* promoter, or ubiquitin promoter like *PcUbi4* or *ZmUbi1*. "Regulated promoter" refers to promoters that direct gene expression not constitutively but in a temporally and/or spatially regulated manner and include both tissue-specific and inducible promoters. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences. Different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. New promoters of various types useful in plant cells are constantly being discovered and are well-known to a person skilled in the art. "Tissue-specific promoter" refers to regulated promoters that are not expressed in all plant cells but only in one or more cell types in specific organs (such as leaves or

seeds), specific tissues (such as embryo or cotyledon), or specific cell types (such as leaf parenchyma or seed storage cells). These also include promoters that are temporally regulated (such as in early or late embryogenesis), during fruit ripening in developing seeds or fruit, in fully differentiated leaf, or at the onset of senescence.

5 "Inducible promoter" refers to those regulated promoters that can be turned on in one or more cell types by an external stimulus (such as a chemical, light, hormone, stress, or pathogen). Examples for inducible promoter are promoters inducible by ecdysone, dexamethasone, ethanol. Such promoters are well-known from the state of the art (e.g., Samalova et al. (2005). pOp6/LhGR: a stringently regulated and
10 highly responsive dexamethasone-inducible gene expression system for tobacco. *The Plant Journal*, 41(6), 919-935; Gatz & Lenk (1998). Promoters that respond to chemical inducers. *Trends in Plant Science*, 3(9), 352-358.).

A further subject matter of the invention is a haploid plant embryo obtained or
15 obtainable by the method of the invention.

Another subject-matter of the present invention is a plant cell comprising a polynucleotide encoding a *GRF1* polypeptide transiently or stable integrated, and a single-stranded DNA break (SSB) or double stranded DNA break (DSB) inducing
20 enzyme or base editor enzyme which preferably recognize a predetermined site in the genome of said cell, and optionally a repair nucleic acid molecule, wherein preferably the polynucleotide encoding the *GRF1* polypeptide being operatively linked to a suitable regulatory sequence, so that the plant cell is capable of expressing the *GRF1* polypeptide. Such plant cell can be obtained when conducting
25 the above described method for modifying the genome of a plant cell.

A further aspect of the present invention is the use of a polynucleotide encoding a *GRF1* polypeptide, mRNA encoding a *GRF1* polypeptide, *GRF1* polypeptide or an activator of expression of an endogenous gene encoding a *GRF1* polypeptide in a
30 method of transformation of a plant cell, preferably in the method of transformation as described above, in a method of modifying the genome of a plant cell, preferably in the method of modifying the genome as described above, in a method for the production of a plant or a haploid plant embryo, preferably in the method for the production of a plant or a haploid plant embryo as described above, or in a method
35 for regeneration of a plant, preferably in the method for regeneration of a plant as described above. According to a preferred aspect, the use is combined with a

polynucleotide encoding a *WUS2* polypeptide, mRNA encoding a *WUS2* polypeptide, or *WUS2* polypeptide(s).

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Cray, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Other references for standard molecular biology techniques include Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY, Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson et al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany.

20

All patents, patent applications, and publications or public disclosures (including publications on internet) referred to or cited herein are incorporated by reference in their entirety.

25 The invention will be further described with reference to the following Figures and Examples described herein. However, it is to be understood that the invention is not limited to such Examples.

Figures

30

Fig 1. Assessment of biolistic delivery by tDT expression (24 hours after bombardment). **A** and **C**: immature embryo bombarded with tDT construct; **A**, RFP channel and **C**, bright field; **B** and **D**: immature embryo bombarded with ZmWUS2, ZmBBM and tDT three constructs; **B**, RFP channel and **D**, bright field.

35

- Fig 2.** Assessment of biolistic delivery by target gene expression: **A:** tDT gene is expressed in the samples that were bombarded with tDT construct but not expressed in the wild type A188 embryos; **B:** ZmWUS2 gene is expressed in the samples that were bombarded with ZmWUS2 construct but not expressed in the wild type A188 embryos or the samples bombarded with only tDT construct; **C:** ZmBBM gene is expressed in the samples that were bombarded with ZmBBM construct but not expressed in the wild type A188 embryos or the samples bombarded with only tDT construct
- Fig 3.** Expression of ZmGRF1 is up-regulated in ZmWUS2 and ZmBBM expressing samples. **A:** ZmGRF1 expression is higher in the samples bombarded with WUS2/BBM/tDT compared to the samples bombarded with tDT alone; **B** and **C:** ZmGRF1 expression is dramatically increased in the transgenic WUS/BBM callus samples (**B**) and small shoots (**C**) compared with tDT the control obtained from agrobacterial transformation.
- Fig 4.** Transient delivery of ZmGRF1 enhances regeneration. Fluorescent embryonic structures appearing 1 month after bombardment in tDT only samples (**A**), ZmWUS2/ZmGRF1/tDT samples (**B**) and ZmWUS2/ZmPLT7/tDT (**C**).
- Fig 5.** Quantification of fluorescent embryonic structure from Fig 4.
- Fig 6.** Increase in transformation frequency in stable transformation of A188 corn line. Transformation frequency fold changes comparing ZmGRF1 to the standard transformation construct. In experiment 1 (left column) the transgenic plants were obtained 3 weeks earlier than in a standard transformation due to the growth of the events.
- Fig 7.** Increase in transformation frequency in stable transformation of A188 corn line. Event number and size after callus selection. Standard construct (left) ZmGRF1 (right)
- Fig 8.** Increase in transformation frequency in stable transformation of A188 corn line. ZmGRF1 overexpressing single copy lines in the GH have no phenotype (left) and they are fertile (right)

Fig 9. Effect of co-transformation of GRF1 on transformation efficiency. Standard Agrobacterium mediated transformation of 2 constructs (A and B) compared with the co-transformation with an Agrobacteria containing the GRF1 expressing binary vectors.

5

Fig 10. Vector Map of pAMK-BdUbi10-ZmBbm

Fig 11. Vector Map of pAMK-BdEF1-ZmWus2

10 **Fig 12.** Vector Map of pAMK-BdEF1-ZmGRF1

Fig 13. Vector Map of pLH-Pat5077399-70Subi-tDT

15 **Examples**

1. Biolistic delivery:

Gene cloning and construct preparation

20 Maize PLT7 (ZmPLT7; cDNA=SEQ ID NO: 4; protein=SEQ ID NO: 5), BBM (ZmBBM; cDNA=SEQ ID NO: 6; protein=SEQ ID NO: 7), WUS2 (ZmWUS2; cDNA=SEQ ID NO: 8; protein=SEQ ID NO: 9) and GRF1 (ZmGRF1; genomic DNA=SEQ ID NO: 1; cDNA=SEQ ID NO: 2; protein=SEQ ID NO: 3) genes were cloned by RT-PCR using total RNA isolated from maize A188 immature embryos.

25 The boost gene fragments are cloned into expression vector pAMK-BdUbi10 or pAMK-BdEF1 at the cloning site, and expressing under the control of BdUbi10 promoter or BdEF1 promoter. Both promoters are strong constitutive promoters from Brachypodium. The sequencing-confirmed construct maps are shown in Fig. 10 to Fig. 12.

30

Preparing maize immature embryo for bombardment

9-12 days post pollination, maize ears with immature embryos size 0.8 to 1.8 mm, preferred 1.0-1.5 mm were harvested. The ears were sterilized with 70% ethanol for 10-15 minutes. After a brief air-dry in a laminar hood, remove top ~1/3 of the kernels

35 from the ears with a shark scalpel, and pull the immature embryos out of the kernels carefully with a spatula. The fresh isolated embryos were placed onto the bombardment target area in an osmotic medium plate (see below) with scutellum-

side up. Wrap the plates with parafilm and incubated them at 25 °C in dark for 4 hours before bombardment.

Particle co-bombardment

- 5 Particle bombardment gun and gold particles size 0.4 or 0.6 micron (μm) were used to deliver DNA into the scutellum cells of maize immature embryo. The plasmids were premixed with genes of interest (GOI). For 10 shots, 1 mg of gold particle in 50% (v/v) glycerol (100 μg of gold particles per shot) in a total volume of 100 microliter (μl) was pipetted into a clear low-retention microcentrifuge tube. Sonicate
- 10 for 15 seconds to suspend the gold particles. While vortex at a low speed, add the following in order to each 100 μl of gold particles:
 - Up to 10 μl of DNA (1.0-10.0 μg total DNA of pre-mixed, 100-1000 ng per each shot)
 - 100 μl of 2.5 M CaCl_2 (pre-cold on ice)
 - 15 - 40 μl of 0.1 M cold spermidine

- Close the lid and vortex the tube for 2-30 minutes at 0-10 °C, and spin down the DNA-coated gold particles. After washing in 500 μl of 100% ethanol for two times, the pellet was resuspended in 120 μl of 100% ethanol. While vortexing at a low
- 20 speed, pipet 10 μl of co-coated gold particles with a wide open 20 μl tip from the tube onto the center of the macrocarrier evenly since the particles tend to form clumps at this point, get the gold particles onto the macrocarriers as soon as possible. Air dry. Bombardment was conducted using a Bio-Rad PDS-1000/He particle gun. The bombardment conditions are: 28 mm/Hg vacuum, 450 or 650 psi
 - 25 rupture disc, 6 mm gap distance, the specimen platform is in the second position from the bottom in the chamber at a distance of 60 mm, three shots per sample (maize immature embryos) plate.

Post bombardment observation and embryo culture

- 30 After bombardment the embryos were remained on the osmotic medium for another 16 hours. Transient transformation was examined using a fluorescence microscope for the tDT expression at excitation maximum 554 nm and emission maximum 581 nm 16-20 hours after bombardment. The embryos with dense fluorescent signals under a fluorescence microscope were selected and transfer from N6OSM onto a
- 35 N6-5Ag plate (~ 15 embryos per plate) with scutellum-face-up for callus induction.

Osmotic medium: N6 salt, N6 vitamin, 1.0 mg/L of 2, 4-D, 100 mg/L of Caseine, 0.7 g/L of L-proline, 0.2 M Mannitol (36.4 g/L), 0.2 M sorbitol (36.4 g/L), 20 g/L sucrose, 15 g/L of Bacto-agar, pH 5.8.

- 5 N6-5Ag: N6 salt, N6 vitamin, 1.0 mg/L of 2, 4-D, 100 mg/L of Caseine, 2.9 g/L of L-proline, 20 g/L sucrose, 5g/L of glucose, 5 mg/L of AgNO₃, 8 g/L of Bacto-agar, pH 5.8.

10 2. Identification of ZmWUS2 and ZmBBM targets:

The following constructs were used in the biolistic delivery of A188 immature embryos: pAMK-BdUbi10Zm-Bbm, pAMK-BdEF1ZmWus2, pLH-Pat5077399-70Subi-tDt

15

The experiment set-up is as follows:

Category 1	Category 2	Category 3
Non bombardment	pLH-Pat5077399-70Subi-tDt (Figure 13)	pAMK-BdUbi10Zm-Bbm, pAMK--BdEF1ZmWus2, pLH-Pat5077399-70Subi-tDt

- 20 24 hour after biolistic delivery, the success of plasmid delivery was assessed by tDT expression. **Figure 1** indicates that the bombardment was successful for Category 2 and 3. Further the expression of introduced genes tDT, ZmWUS2 and ZmBBM (Category 3) has been determined (**Figure 2**).

25 3. RNAseq analysis:

24 hours after biolistic delivery, immature embryos were harvested and flash-frozen in liquid nitrogen. All the samples were subsequently sent for RNA extraction, library preparation and RNAseq that was done by external sequencing provider.

30

Several candidates were identified by comparison between the samples only containing tDT (Category 2) and samples contains ZmWUS2, ZmBBM, and tDT

(Category 3). Sixteen genes showed an up-regulated expression in response to the expression of ZmWUS2 and ZmBBM, five genes a down-regulated expression. After confirmation by qRT-PCR the candidate ZmGRF1 has been selected for further analysis. Figure 3A shows that ZmGRF1 expression is higher in the samples
 5 bombarded with Constructs containing ZmWUS2, ZmBBM and TDt (Category 3). In addition, ZmWUS2 and ZmBBM have been stably integrated by Agrobacterium-mediated transformation. The expression of ZmGRF1 has been assessed in callus samples and small shoots of corn plants. Compared to the control obtained by agrobacterial transformation solely of tDT gene the expression of ZmGRF1 was
 10 significantly increased in WUS2/BBM overexpressing plant material (**Figure 3B and C**).

4. Transient assay for testing ZmGRF1

15

A188 immature embryos were bombarded with the following constructs:

Category 1	Category 2	Category 3
pLH-Pat5077399-70Subi-tDt	pAMK--BdEF1ZmWus2 pMK-BdEF_ZmGRF1-GRF pLH-Pat5077399-70Subi-tDt	pAMK--BdEF1ZmWus2, pABM-BdEF1_ZmPLT7 pLH-Pat5077399-70Subi-tDt

Fluorescent embryonic structures were counted one month after bombardment. Such structures indicate enhanced regeneration capability. Samples only transiently transformed with tDT showed less pronounced embryonic structure (Category 1; **Figure 4 A**). From the combination of ZmWUS2 and ZmPLT7 it is known that a boosting in regeneration could be achieved. Thus, this category 3 served as positive control (**Figure 4C**). Even more impressive is that the occurrence of fluorescent
 20 embryonic structures in samples transiently expressing ZmWUS2 and ZmGRF1 together with tDT (Category 2) was more pronounced than in the positive control (**Figure 4B**). The quantification confirms the visual result and showed that the percentage of fluorescent embryonic structures in samples transiently expressing ZmWUS2 and ZmGRF1 together with tDT (Category 2) was more than 5 fold higher
 25 than in category 1 and more than 2 fold higher than in category 3 (**Figure 5**).
 30

5. Maize transformation methods

Corn transformation (line A188) was performed using *Agrobacterium* following a standard protocol (Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens* Nat Biotechnol 14:745-750). Briefly, immature embryos of the A188 line were inoculated with the *Agrobacterias* and co-cultured for 7 days. After the initial embryogenic callus production a selection media was used where a herbicide selected for the transformed events. DNA of the putative transgenic plants was extracted and analyzed through qPCR for the presence of the selectable marker gene. Transformation rate was calculated as the number of transgenic events per inoculated embryos (%). When a co-transformation was performed, equal amounts of the *Agrobacterias* containing the construct and the GRF1 gene were mixed together prior inoculation.

The results showed that compared to the standard transformation, i.e. without the additional delivery of ZmGRF1, up to 4 times higher transformation efficiencies have been reached. Of advantage was in addition that transgenic plants were obtained approximately 3 weeks earlier due to the rapid growth of the events. **Figure 6** shows the quantitative analysis of two independent experiments, **Figure 7** the visual comparison. Corn plant lines with ZmGRF1 single copy events grown in the greenhouse have no undesired phenotype and are fertile (**Figure 8**).

Further the effect of co-transformation of GRF1 on transformation efficiency has been investigated. Standard *Agrobacterium* mediated transformation of 2 constructs (A and B) has been compared with the co-transformation with an *Agrobacteria* containing the GRF1 expressing binary vector. The transformation rates were 2.3 and 1.8 fold higher when GRF1 was used (**Figure 9**). Additionally, the co-transformation experiment was 36% faster than the standard protocol.

Claims

1. A method for transforming a plant cell, comprising the steps
 - (a1) introducing into a plant cell in parallel or sequentially
 - i. at least one nucleotide sequence of interest; and
 - ii. an expression cassette comprising a polynucleotide encoding a GRF1 polypeptide, mRNA encoding a GRF1 polypeptide, or GRF1 polypeptide(s); or
 - (a2) introducing into a plant cell at least one nucleotide sequence of interest; and inducing in said plant cell in parallel or sequentially an enhanced expression level of an endogenous gene encoding a GRF1 polypeptide; and
 - (b) optionally, cultivating the plant cell of (a1) or (a2) or a plant cell derived from the plant cell of (a1) or (a2) under conditions where in the plant cell the GRF1 polypeptide is expressed from the expression cassette, GRF1 polypeptide is translated from introduced mRNA, GRF1 polypeptide is enhanced expressed from the endogenous gene, or GRF1 polypeptide(s) are present.
2. A method for modifying the genome of a plant cell, comprising the steps
 - (a1) introducing into a plant cell an expression cassette comprising a polynucleotide encoding a GRF1 polypeptide, mRNA encoding a GRF1 polypeptide, or GRF1 polypeptide(s); or
 - (a2) inducing in a plant cell an enhanced expression level of an endogenous gene encoding a GRF1 polypeptide; and
 - (b) cultivating the plant cell of (a1) or (a2) or a plant cell derived from the plant cell of (a1) or (a2) under conditions where in the plant cell the GRF1 polypeptide is expressed from the expression cassette, GRF1 polypeptide is translated from introduced mRNA, GRF1 polypeptide is enhanced expressed from the endogenous gene, or GRF1 polypeptide(s) are present;
 - (c) modifying the genome of the plant cell of (b) by means of a single stranded DNA break (SSB) or double stranded DNA break (DSB) inducing enzyme or a base editor enzyme which preferably recognizes a predetermined site in the genome of said cell, and optionally by means of a repair nucleic acid molecule,

wherein the modification of said genome at said predetermined site is selected from

- i. a replacement of at least one nucleotide;
- ii. a deletion of at least one nucleotide;
- 5 iii. an insertion of at least one nucleotide; or
- iv. any combination of i. – iii.; and

wherein step (c) is conducted simultaneously with step (a1)/(a2) and/or (b), before step (a1)/(a2), between step (a1)/(a2) and (b) or after step (b).

- 10 3. A method of producing a transgenic plant, comprising the steps
- (a) transforming a plant cell according to the method of claim 1, and
 - (b) regenerating from the plant cell of (a) or from a plant cell derived from the plant cell of (a) a plant comprising at least one cell which comprises the at least one nucleotide sequence of interest as transgene.

15

4. A method of producing a genetically modified plant, comprising the steps
- (a) modifying the genome of a plant cell according to the method of claim 2, and
 - (b) regenerating from the plant cell of (a) or from a plant cell derived from the plant cell of (a) a plant comprising in at least one cell the modification of the
- 20 genome.

20

5. A method of producing a haploid plant embryo, comprising the steps
- (a1) introducing into an immature male gametophyte or a microspore an expression cassette comprising a polynucleotide encoding a GRF1 polypeptide, mRNA encoding a GRF1 polypeptide, or GRF1 polypeptide(s); or
 - 25 (a2) inducing in an immature male gametophyte or a microspore an enhanced expression level of an endogenous gene encoding a GRF1 polypeptide; and
 - 30 (c) cultivating the immature male gametophyte or the microspore of (a) under conditions where in the immature male gametophyte or the microspore the GRF1 polypeptide is expressed from the expression cassette, GRF1 polypeptide is translated from introduced mRNA, GRF1 polypeptide is enhanced expressed from the endogenous gene, or GRF1 polypeptide(s) are present; and
 - 35 (d) selecting haploid plant embryo derived from the immature male gametophyte or the microspore of step (b).

35

6. The method of any one of the claims 1 to 5, wherein the GRF1 polypeptide comprises a PFAM domain PF08880 and a PFAM domain PF08879, preferably wherein the PFAM domain PF08880 finds a match of at least 90% coverage at or near the N-terminus of the GRF1 polypeptide and the PFAM domain PF08879 finds a match of at least 90% C-terminally located to the PFAM domain PF08880 in the GRF1 polypeptide.
7. The method of claims 6, wherein both matching amino acid stretches are located in the N-terminal half of the GRF1 polypeptide.
8. The method of any one of the claims 1 to 7, wherein the GRF1 polypeptide comprises the motif [D]-[P]-[E]-[P]-[G]-[R]-[C]-[R]-[R]-[T]-[D]-[G]-[K]-[K]-[W]-[R]-[C]-[AS]-[RK]-[ED]-[A]-[AH]-[PQS]-[D]-[S]-[K]-[Y]-[C]-[E]-[KR]-[H]-[M]-[H]-[R]-[G]-[R]-[N]-[R] (SEQ ID NO: 25) with a maximum number of two mismatches, wherein preferably the motif consists of any of the amino acid sequences SEQ ID NO: 26 to SEQ ID NO: 35, and/or wherein preferably the motif contains a sub-region of amino acid stretch matching PFAM domain PF08879.
9. The method of any one of the claims 1 to 8, wherein the GRF1 polypeptide comprises
 - (i) an amino acid sequence selected from the group consisting of SEQ ID NO: 2, 37, 39, 41, 43, 45, 47, 49, 51 and 53; or
 - (ii) an amino acid sequence comprising a sequence being at least 70% identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, 37, 39, 41, 43, 45, 47, 49, 51 and 53.
10. The method of any one of the claims 1 to 9, wherein the polynucleotide encoding the GRF1 polypeptide comprises
 - (i) a coding nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 36, 38, 40, 42, 44, 46, 48, 50 and 52;
 - (ii) a coding nucleotide sequence comprising a sequence being at least 70% identical to a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 36, 38, 40, 42, 44, 46, 48, 50 and 52;
 - (iii) a nucleotide sequence encoding the polypeptide encoded by (i) or (ii) within the scope of the degeneracy of the genetic code;

- (iv) a nucleotide sequence complementary to the nucleotide sequence of (i), (ii) or (iii); or
- (v) a nucleotide sequence hybridizing with a nucleotide sequence of (iv) under stringent condition.

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11. The method of any one of the claims 1 to 10, wherein introducing into a plant cell the expression cassette comprising a polynucleotide encoding a GRF1 polypeptide results in a stable integration thereof into the genome of the plant cell, or wherein introducing into a plant cell the expression cassette comprising
- 10 a polynucleotide encoding a GRF1 polypeptide, mRNA encoding GRF1 polypeptide, or GRF1 polypeptide(s) or inducing in a plant cell the enhanced expression level of an endogenous gene encoding a GRF1 polypeptide results in a transient occurrence of GRF1 polypeptide(s) in the plant cell or in a progeny cell thereof.

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12. The method of any one of the claims 1 to 11, wherein the polynucleotide encoding the GRF1 polypeptide is in operative linkage to at least one regulatory sequence suitable for expression of the GRF1 polypeptide in a plant cell.
13. The method of any one of claims 1 to 4, wherein the plant cell of step (a1) or (a2) is a cell of a somatic tissue, callus tissue, a meristematic tissue or an embryonic tissue, a protoplast, gametophyte, pollen, ovule or microspore.
14. A plant or part thereof obtained or obtainable by the method of claim 3 or 4, or a
- 25 progeny plant thereof.
15. A plant cell or a seed of the plant of claim 14, wherein the plant cell or the seed comprises the at least one nucleotide sequence of interest as transgene or comprises the modification in the genome.

30

FIG 1

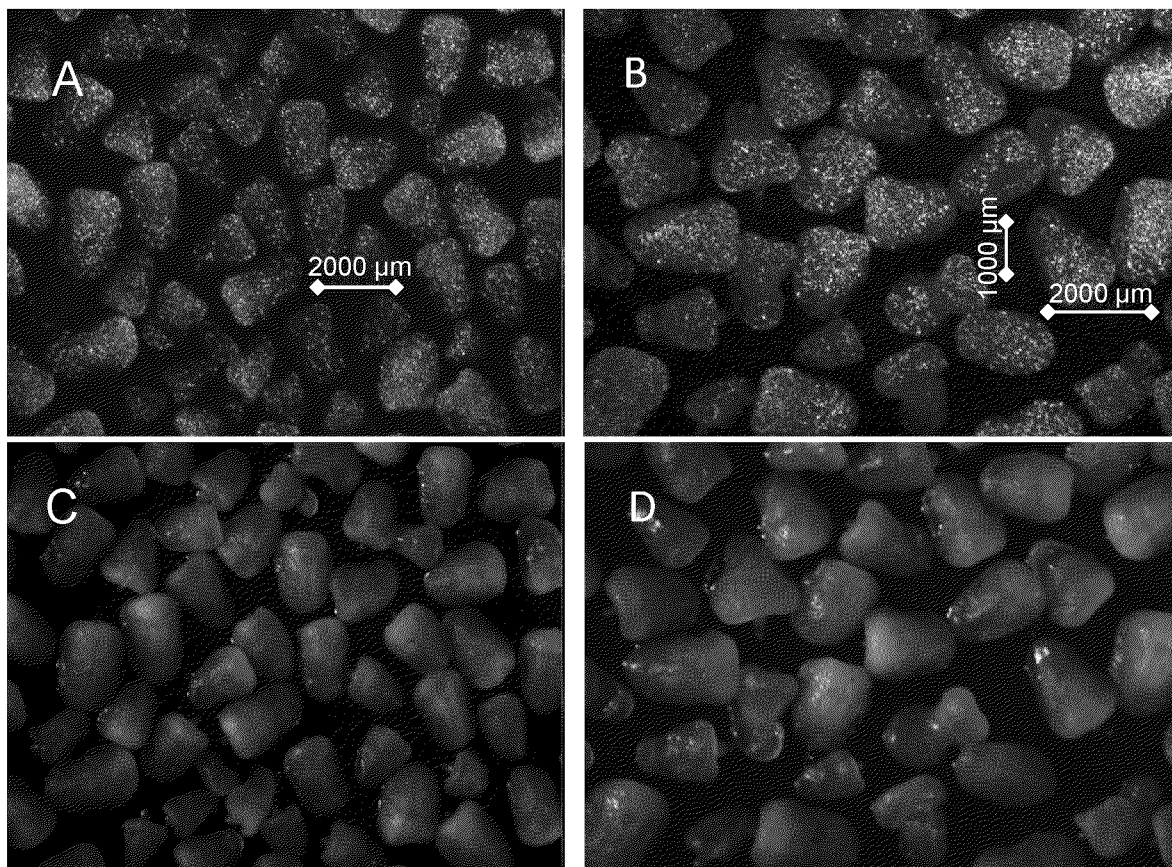


FIG 2

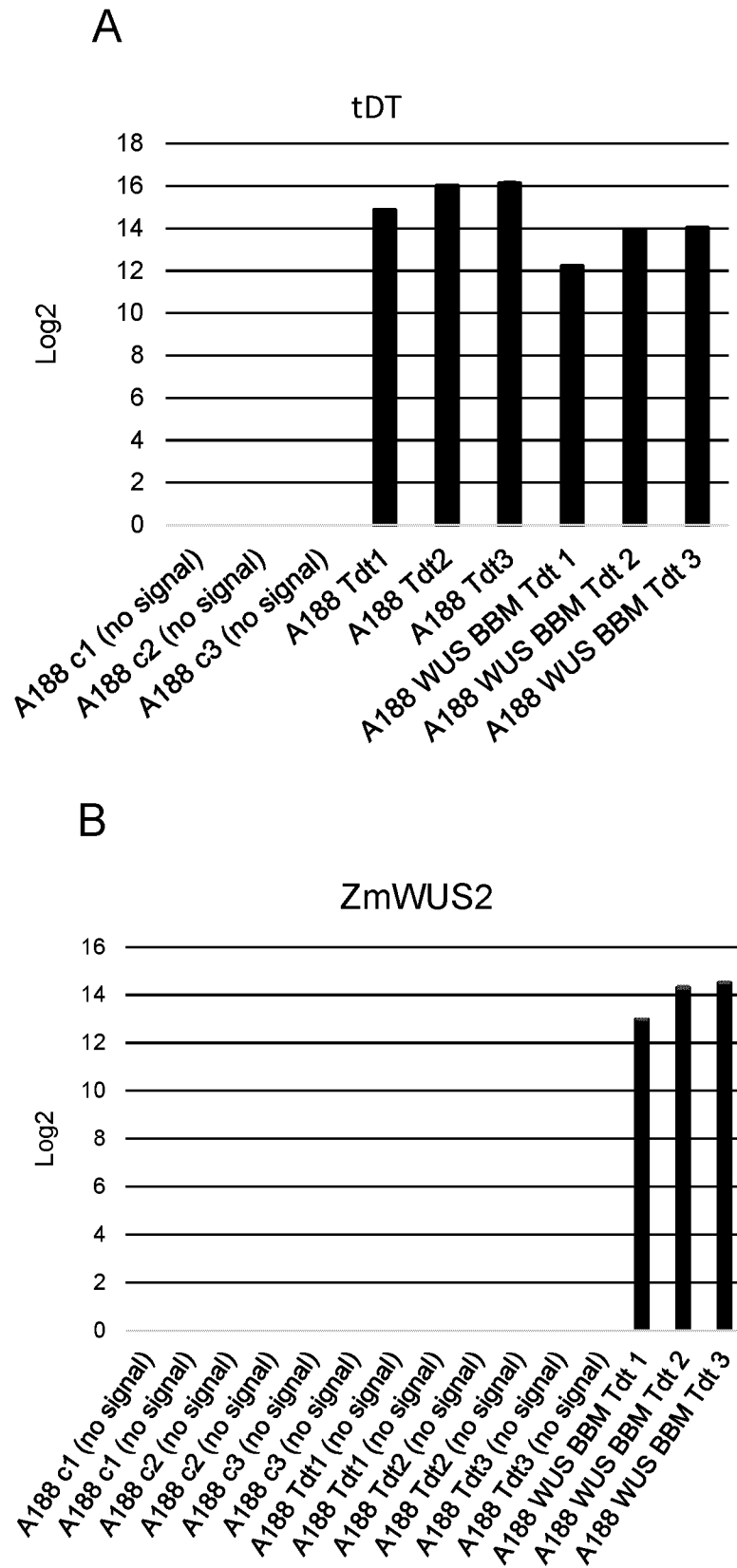


FIG 2

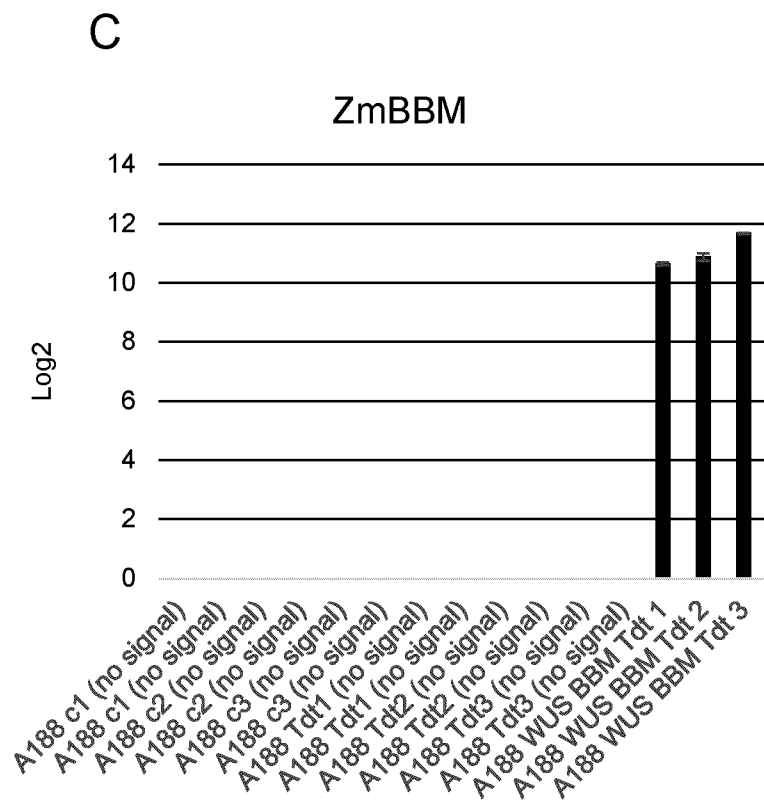


FIG 3

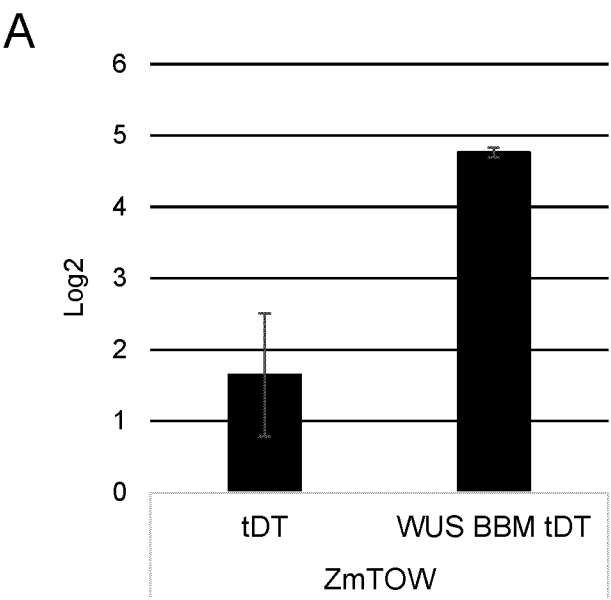


FIG 3

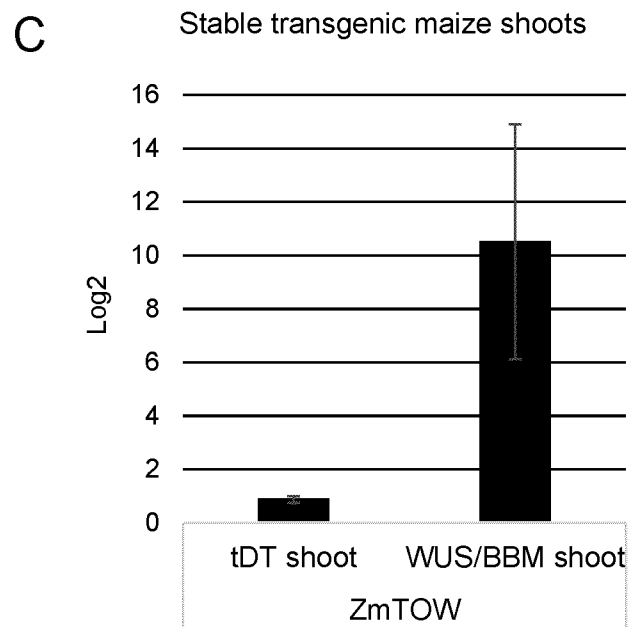
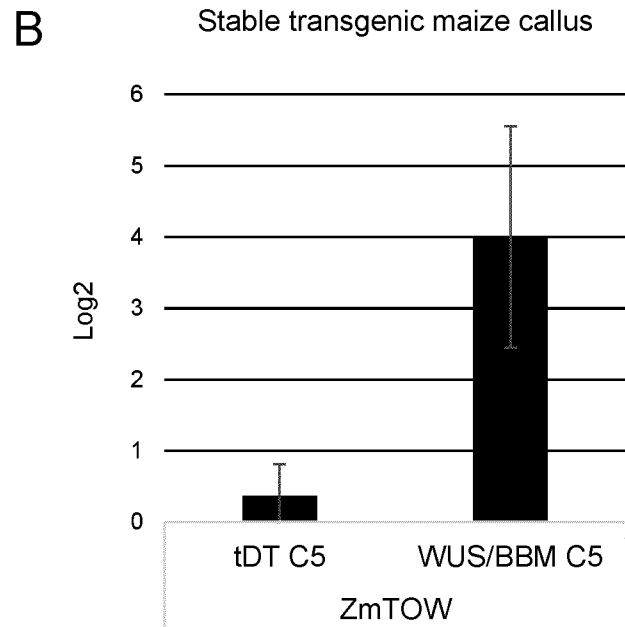


FIG 4

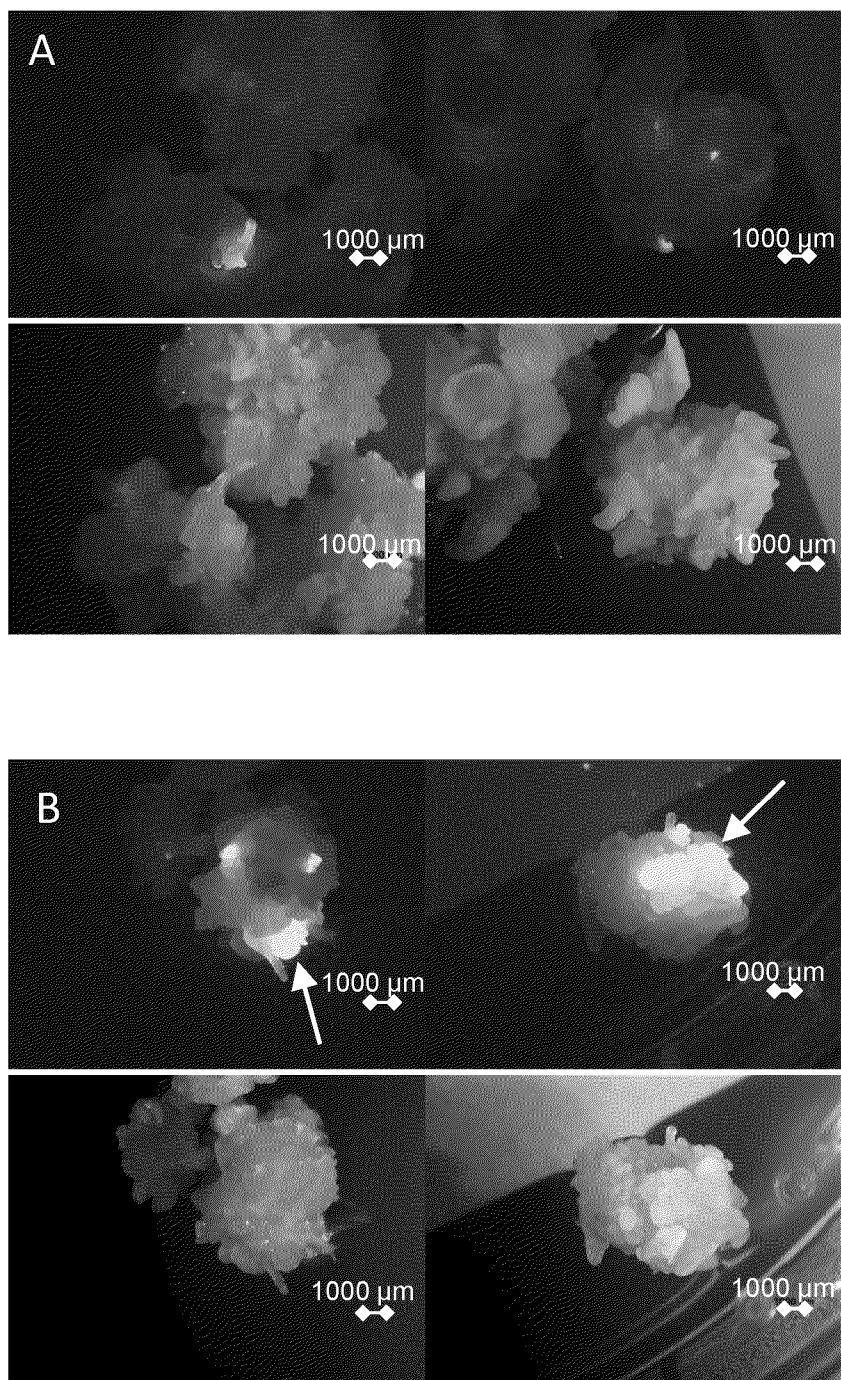


FIG 4

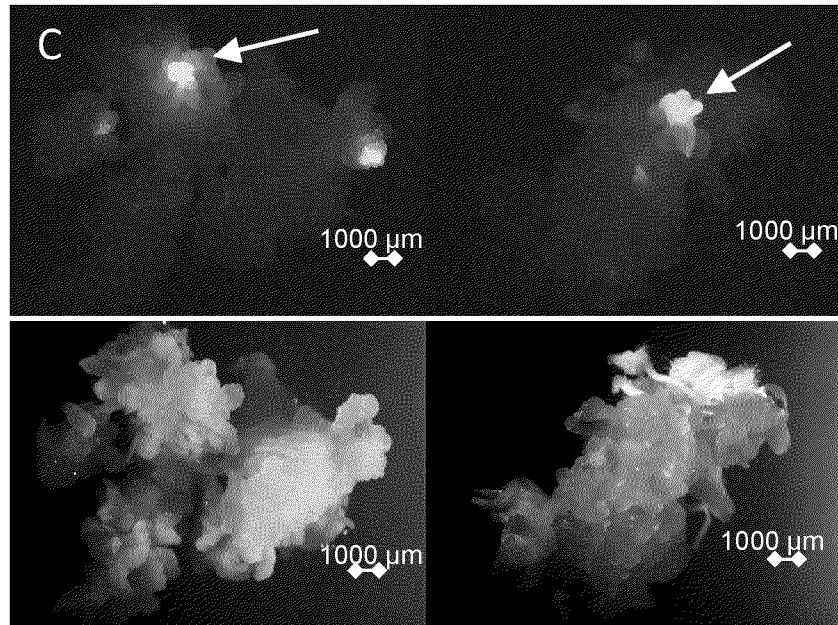


FIG 5

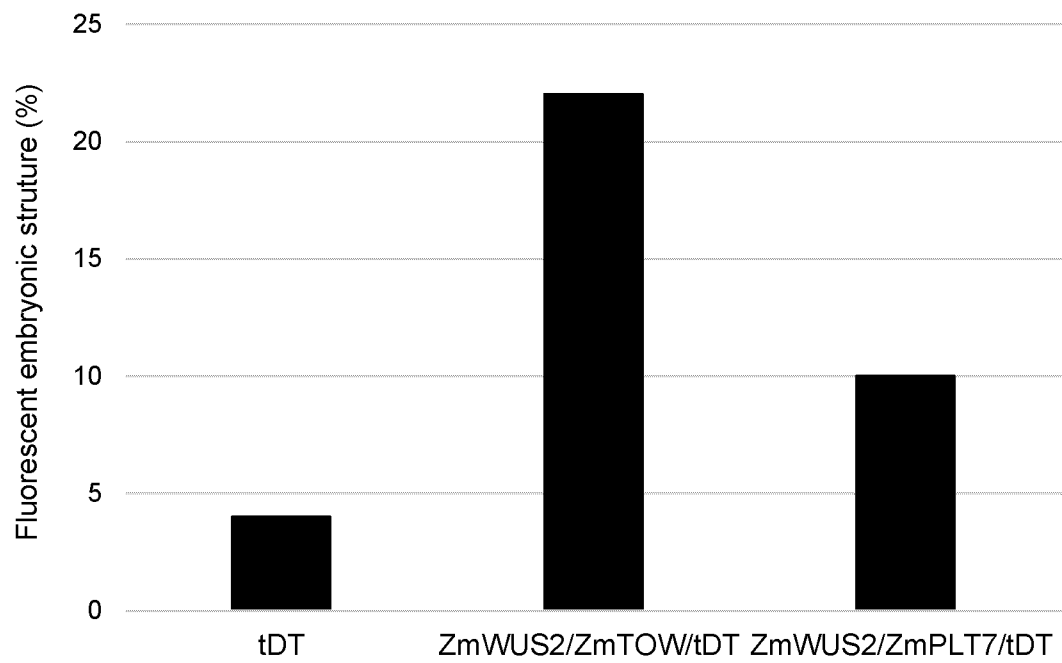


FIG 6

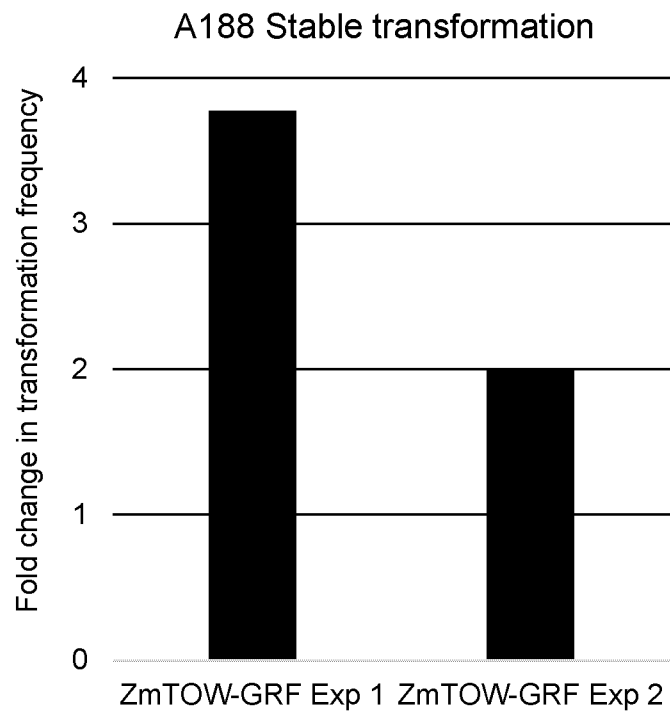


FIG 7

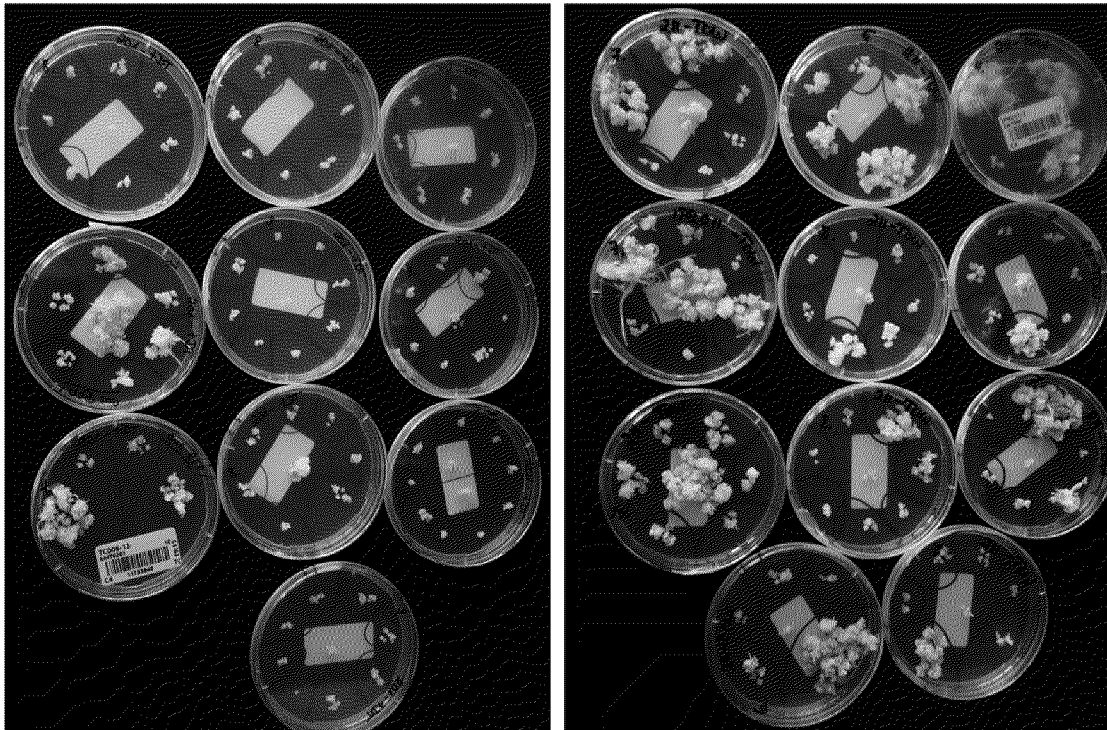


FIG 8

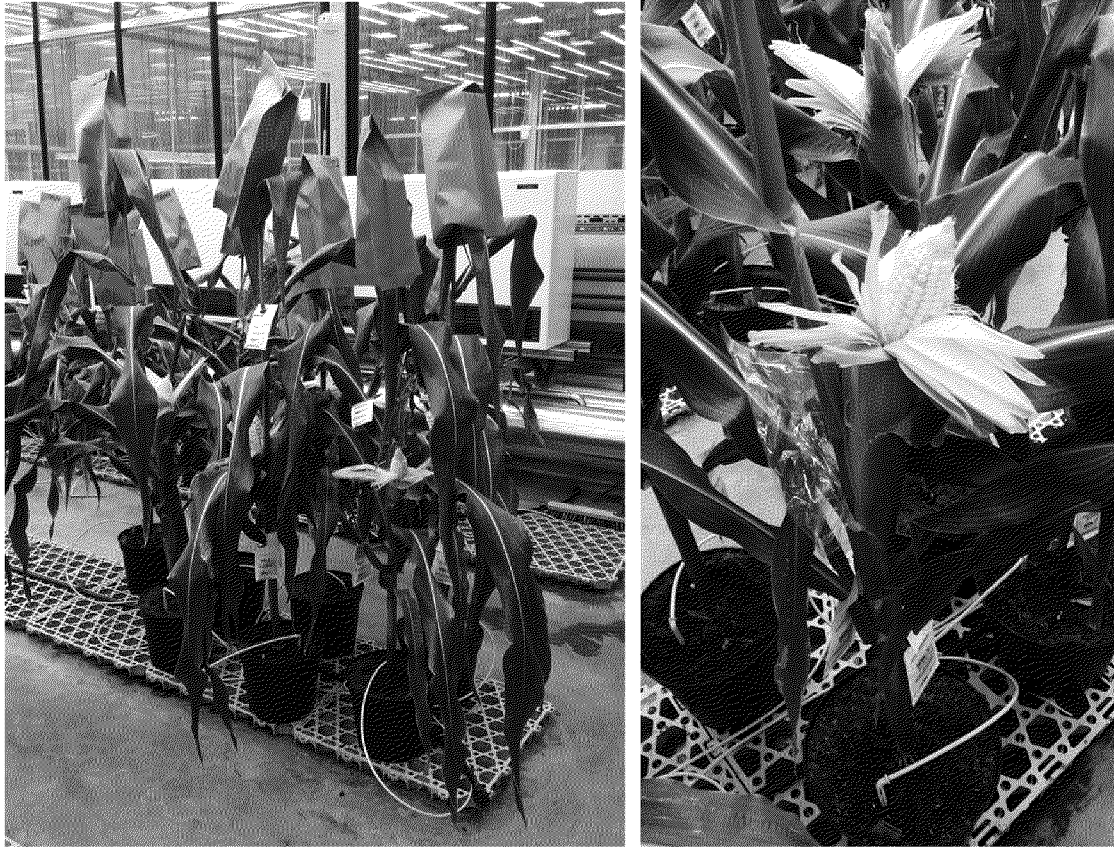


FIG 9

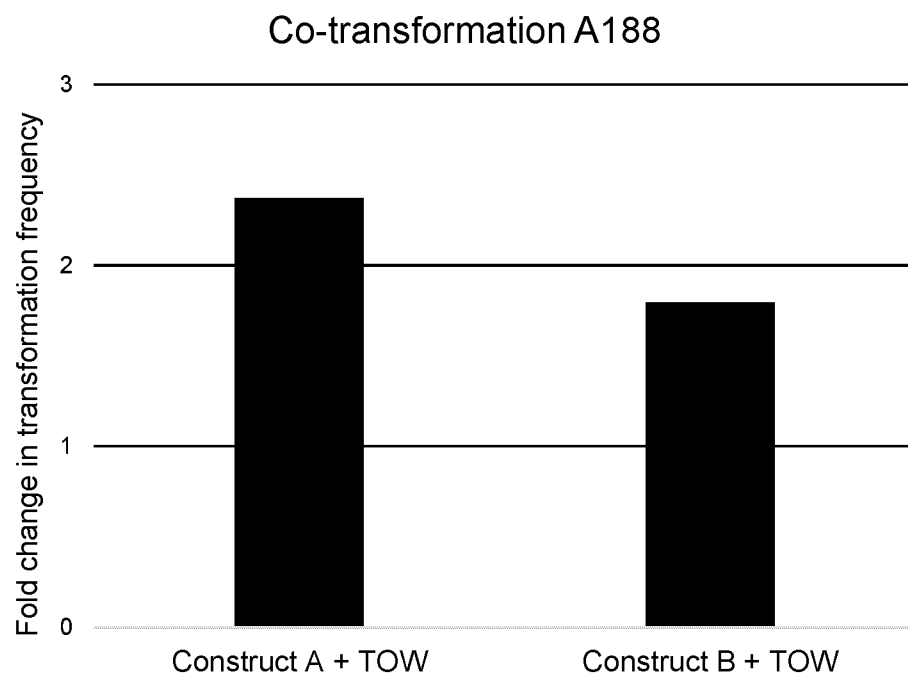


FIG 10

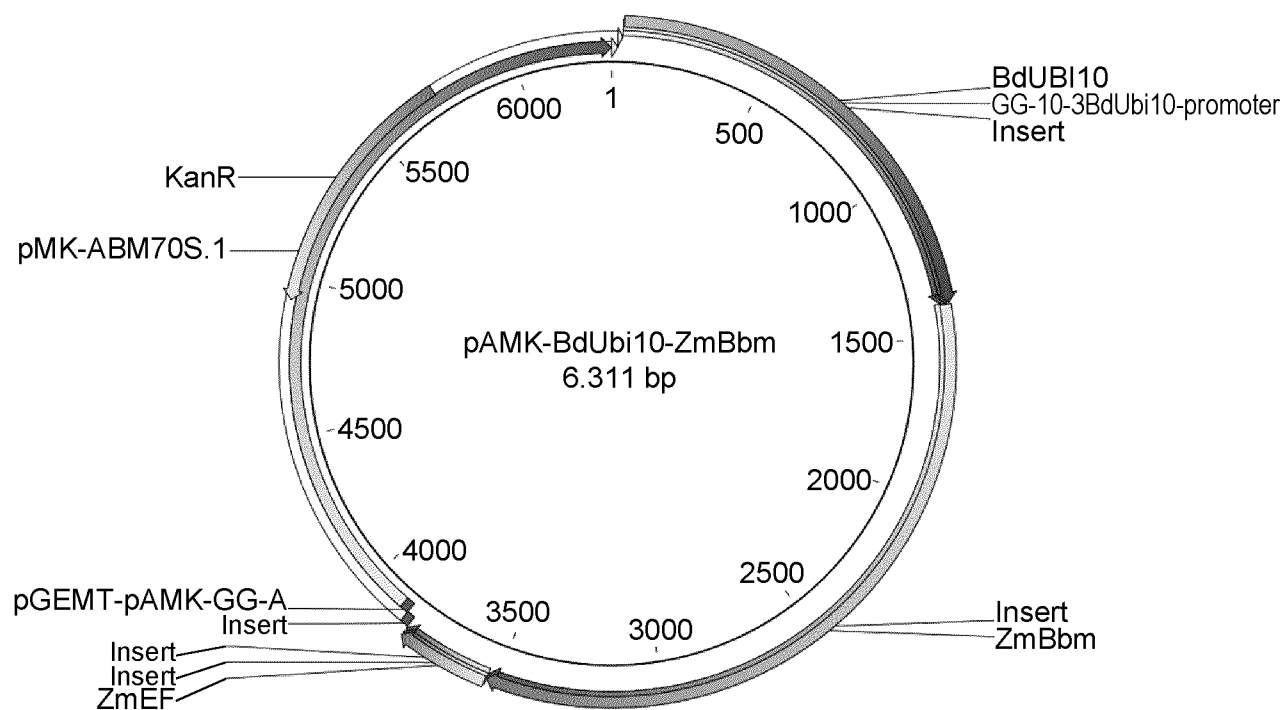


FIG 11

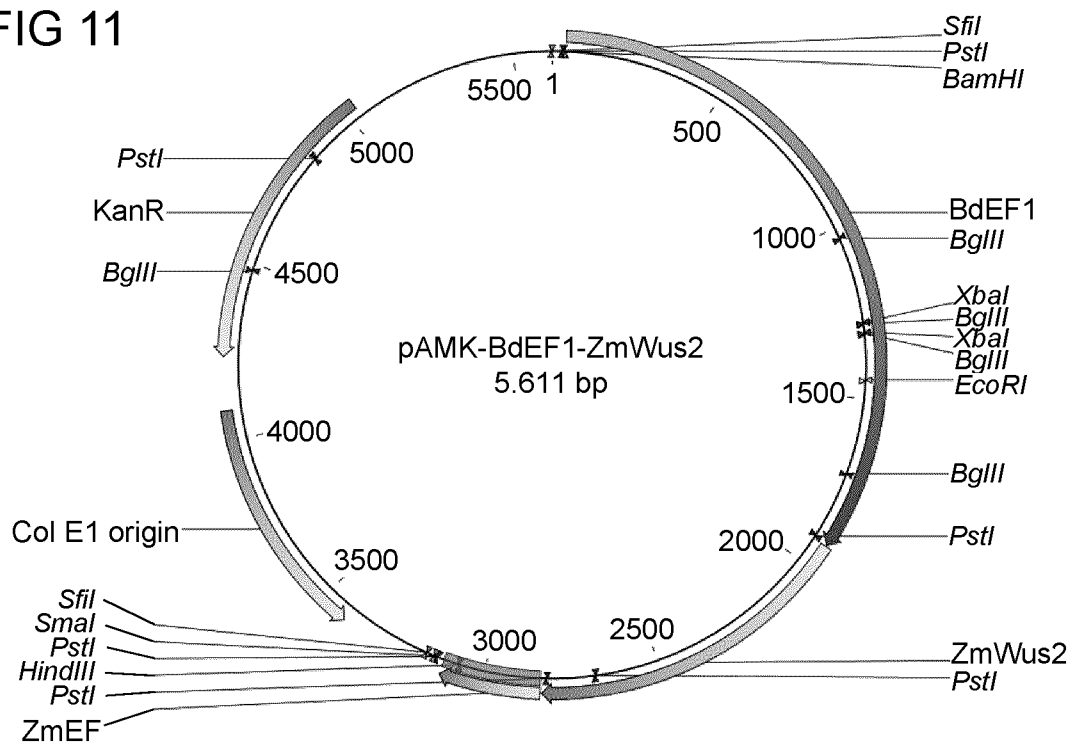


FIG 12

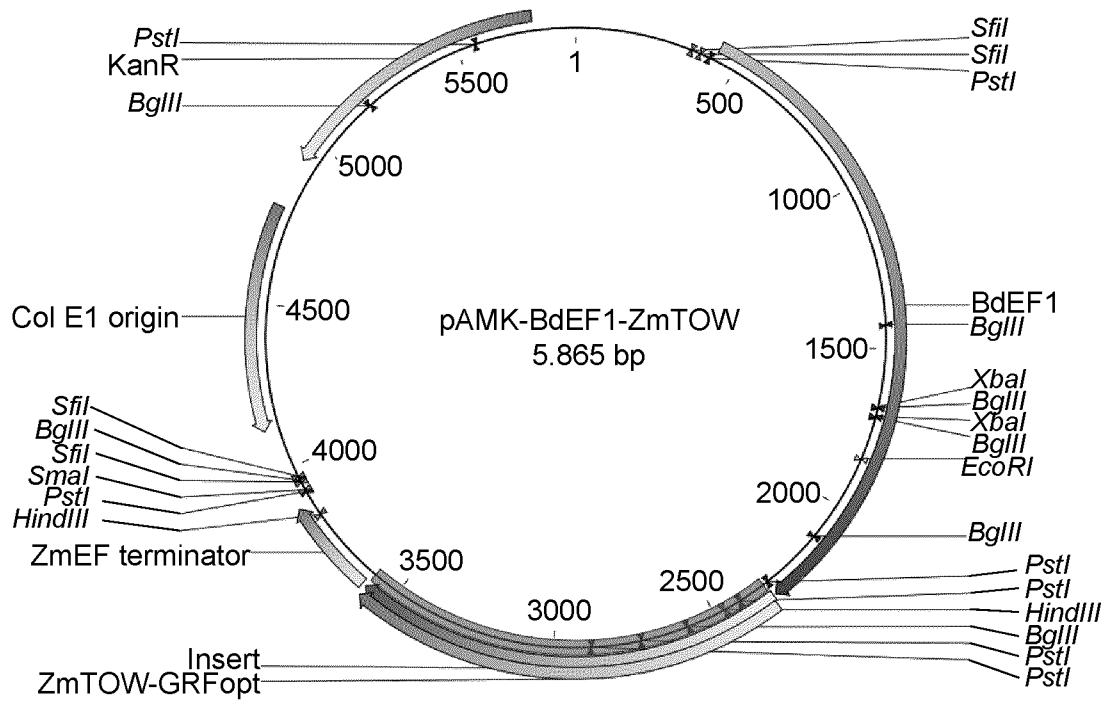


FIG 13

