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(54) **Title:** METHODS FOR IMPROVED REGENERATION OF TRANSGENIC PLANTS USING GROWTH-REGULATING FACTOR (GRF), GRF-INTERACTING FACTOR (GIF), OR CHIMERIC GRF-GIF GENES AND PROTEINS

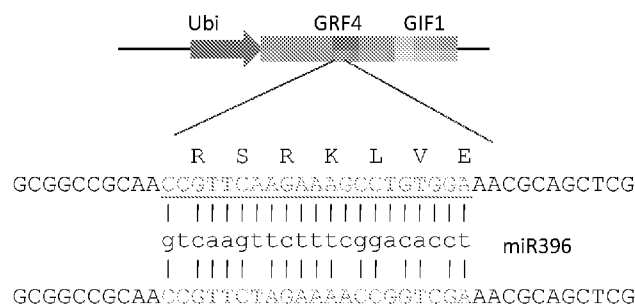


FIG. 8

(57) **Abstract:** Disclosed are methods of producing plants with an improved regeneration efficiency using Growth-Regulating Factor (GRF), GRF-Interacting Factor (GIF), or chimeric GRF-GIF genes and proteins. The disclosure also provides plants with an improved regeneration efficiency that are produced by the disclosed methods, methods of reducing the use of exogenous cytokinins in the regeneration of plants, and methods of improving the regeneration efficiency of plants.



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Methods for improved regeneration of transgenic plants using Growth-Regulating Factor (*GRF*), *GRF*-Interacting Factor (*GIF*), or chimeric *GRF-GIF* genes and proteins

REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to previously filed and co-pending application USSN 62/873,123, filed July 11, 2019, the contents of which are incorporated herein by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant No. 2017-67007-25939 awarded by the United States Department of Agriculture, National Institute of Food and Agriculture. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 27, 2020 is named P1322WO00_SEQLISTING_06-17-2020_ST25 and is 385,059 bytes in size.

BACKGROUND

[0004] Plant breeding and genetic engineering have been used now for many decades to improve plant performance and provide agricultural improvement of plants especially crop plants. A drawback in plant transformation techniques is the low regeneration efficiency of certain plants, including low efficiency in select plant genus and species and even particular genotypes within a plant species. Therefore, there is a need to improve regeneration efficiency of plants.

SUMMARY

[0005] The present methods are to improving plant regeneration efficiency. The methods employ introducing into one or more plant cells a Growth Regulating Factor (*GRF*) and/or *GRF*-Interacting Factor protein (*GIF*) that are introduced into a plant cell, or a *GRF-GIF* chimera introduced into a plant cell. Introducing a nucleic acid molecule encoding the polypeptides or

introducing the polypeptides increases regeneration efficiency. In certain embodiments, the methods increase regeneration efficiency of low regeneration efficiency plants. Other embodiments provide for accelerating the time to produce transgenic plants. Embodiments provide for mutating the miR396 target region of the *GRF* sequence, to reduce repression of the *GRF* protein by miR396 in plant cells. Still further embodiments provide that the presence of the *GRF*, and *GIF* or *GRF-GIF* chimera polypeptide in the plant cell allows the cell to be regenerated on media that has cytokinin concentration too low for regeneration of plant cells that do not comprise the polypeptides so introduced. Plants may be selected on such media without the need of an additional marker sequence. In certain embodiments, plants transformed with *GRF* and/or *GIF* or *GRF-GIF* that have high regeneration efficiency are used for subsequent transformation experiments. Operably linking the *GRF*, *GIF* or *GRF-GIF* chimera to an inducible promoter or domain also allows for selection of timing of expression or activity of the polypeptides. In certain embodiments, plants transformed with *GRF* and/or *GIF* or *GRF-GIF* that have high regeneration efficiency are used for subsequent transformation experiments. Still further embodiments provide that the *GRF*, *GIF* or *GRF-GIF* chimera are combined with gene editing technologies, and that the *GRF*, *GIF* or *GRF-GIF* chimera together with the gene editing constructs may be removed by segregation in the progeny of the edited plant.

TECHNICAL FIELD

[0006] This disclosure relates generally to methods of plant regeneration. More specifically, this disclosure relates to methods for improving plant regeneration efficiency by transformation with a Growth-Regulating Factor (*GRF*) and/or a *GRF*-Interacting Factor (*GIF*), or a *GRF-GIF* chimera.

BRIEF DESCRIPTION OF THE FIGURES

[0007] FIG. 1. A-C) Molecular Phylogenetic analysis using the Neighbor-Joining method. A) *Arabidopsis*, rice and wheat *GRF* proteins. B) *Arabidopsis*, rice and wheat *GIF* proteins. C) Scheme of the wheat *GRF4-GIF1* chimera expressed by the maize UBIQUITIN promoter.

[0008] FIG. 2. Callus in cytokinins regeneration media. A higher number of shoots regenerated during Kronos transformation in the presence of the Ubi::*GRF4-GIF1* chimera (SEQ ID NO:5,

middle) than in Kronos transformation using the same vector without the *GRF-GIF* chimera (shown at both sides).

[0009] FIG. 3. A) Validation of 26 independent transformation events by PCR. PCR products obtained with primers Fw-*GRF4b* and Rev-*GIF1b* using genomic DNA isolated from 26 independent T₀ plants as template. Each number correspond to an independent transformation event. L= DNA ladder. B) Schematic representation of the *GRF4-GIF1* chimera and the primers used for genotyping. The amplified fragment is 1.087Kb.

[0010] FIG. 4. Callus in cytokinins regeneration media. Shoot regeneration during wheat transformation in the presence of the Ubi::*GIF1* (SEQ ID NO:2) and in wheat transformation using the same vector without the *GIF1* gene.

[0011] FIG. 5. Callus in cytokinins regeneration media. Regeneration efficiency of plant cells transformed with Ubi::*GRF4* (SEQ ID NO:1), Ubi::*GIF1* (SEQ ID NO:2), Ubi::*GRF4-GIF1* chimera (SEQ ID NO:5) compared to control.

[0012] FIG. 6. Effect of *GRF4-GIF1* chimera on regeneration from leaf explants.

[0013] FIG. 7. A) Schematic representation of the different steps of wheat transformation. B) Effect of *GRF4-GIF1* chimera on induction of embryogenesis in the absence of cytokinins.

[0014] FIG. 8. Scheme of the wheat *GRF4-GIF1* chimera expressed by the maize UBIQUITIN promoter (SEQ ID NO: 59). Below is the sequence of the wild-type miR396 target site (top (SEQ ID NO: 53), a miR396-resistant site with silent mutations (bottom (SEQ ID NO: 54), and their interaction with miR396 (middle (SEQ ID NO: 55).

[0015] FIG. 9. A) Shoot regeneration of plant cells transformed with a construct comprising *GRF4-GIF1* chimera, Cas9, and a guide RNA (gRNA) targeting Gene Q. B) Region of the gene Q targeted with the guide RNA (SEQ ID NO: 68). C) Confirmation of the editing of the target gene.

[0016] FIG. 10. Shoot regeneration of plant cells transformed with *GRF4-GIF1* chimera grown in one-tenth of the normal cytokinin concentration.

[0017] FIG. 11. Coding sequences of wheat chimeric clones. A) The nucleotide sequence of wheat *GRF4-GIF1*. Sequences are from tetraploid wheat Kronos. B) The sequence of the wheat *GRF4-GIF1*-encoded protein. Sequences in blue are from *GRF4*, in black for the spacer, and in green for *GIF1*.

[0018] FIG. 12. The sequence of pLC41- Ubi::*GRF4-GIF1* (SEQ ID NO: 5). The *GRF4* sequence is in blue letters, the spacer in black, and the *GIF* sequence is in green letters. The Ubi promoter is highlighted in gray, the HA tag in red, the NOS terminator in pink, the 35S promoter in green, and HPT in yellow. LB (GTTTACACCACAATATATCCTGCCA) (SEQ ID NO: 57) and RB (GTTTACCCGCCAATATATCCTGTCA) (SEQ ID NO: 58) sequences are underlined.

[0019] FIG. 13. The sequences of the *Vitis vinifera GRF4-GIF1* chimera. A) The nucleotide sequence of *Vitis GRF4-GIF1* (SEQ ID NO:6). B) The sequence of the *Vitis GRF4-GIF1*-encoded protein (SEQ ID NO:7). Sequences in blue are from *GRF4*, in black for the spacer, and in green for *GIF1*.

[0020] FIG. 14. A) Example sequence of a concatenated QLQ-WRC domain used for a BLASTP search and the phylogenetic analysis. B) The sequences of predicted proteins of the five selected wheat *GRFs* (SEQ ID NO: 9-13). Conserved QLQ and WRC domains are highlighted in yellow and green respectively.

[0021] FIG. 15. The sequences of predicted proteins of the five rice *GRF* orthologs (SEQ ID NO: 14 – 18). Conserved QLQ and WRC domains are highlighted in yellow and green respectively.

[0022] FIG. 16 shows A) the predicted sequences of the closest *Arabidopsis GRFs* (SEQ ID NO: 19 -22) and B) the predicted protein of the closet *Vitis vinifera GRF* (SEQ ID NO: 23). Conserved QLQ and WRC domains are highlighted in yellow and green respectively.

[0023] FIG. 17 shows A) the predicted sequences of the closest *Arabidopsis GIFs* (SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26) and B) shows the predicted wheat *GIF* sequences (SEQ ID NO:27, SEQ ID NO:28, and SEQ ID NO:29). Conserved SNH domains are highlighted in yellow.

[0024] Fig. 18 are graphs showing wheat transformation efficiency using single wheat *GRF4* or *GIF1*. A) Schemes of the different cassette used to express wheat *GIF1* and *GRF4* alone, *GRF4* and *GIF1* alone in the same T-DNA, and the *GRF4-GIF1* chimera. B to D) Regeneration frequency of transgenic Kronos plants transformed with the wheat *GRF4-GIF1* chimera relative to Kronos plants transformed with: B) empty vector (n=14, ****P < 0.0001). C) Control vector and vectors including only *GIF1* or only *GRF4* (n=5, different letters indicate significant differences at P < 0.05, Tukey Test). D) Vector including *GRF4* and *GIF1* under separate Ubi promoters (not fused, n=5, ** P < 0.0144).

[0025] FIG. 19 shows phylogenetic trees of *GRF* and *GIF* families for wheat (yellow highlight), rice, Arabidopsis, citrus and grape. The closest homologs to wheat *GRF4* and *GIF1* are highlighted in orange for citrus and in violet for grape. We will refer to those genes and their encoded proteins as citrus *GRF4* (Ciclev10032065m), citrus *GIF1* (Ciclev10022144m), and grape *GRF4* (GSVIVT01024326001) and grape *GIF1* (GSVIVT01036262001). **A)** We used the QLQ and WRC domains for the analysis of the *GRF* proteins and **B)** the SNH domain for the analysis of the *GIF* proteins. The evolutionary history was inferred by using the Maximum Likelihood method. We show the tree with the highest log-likelihood. The percentage of trees in which the associated taxa clustered together is shown next to the branches. We conducted the evolutionary analysis in the program MEGA X. Yellow highlight: wheat. Orange highlight: selected Citrus homolog. Violet highlight: selected Vitis homolog.

[0026] FIG. 20. Shows wheat transformation with chimeras including other *GRF* and/or *GIF* sequences. **A)** Scheme of a *GRFs-GIF1* chimeras tested. **B)** Regeneration efficiency induced by chimeras combining different wheat *GRF* genes with *GIF1*. Means are based on three experiments, except for *GRF5* (2 experiments available). Different letters over the bars indicate significant differences with the control in a Tukey test ($P < 0.05$). Only *GRF4-GIF1* and *GRF5-GIF1* were significantly different from the control. The asterisk indicates a significant difference in regeneration efficiency ($P = 0.0368$) in a contrast comparing the combined *GRF4-GIF1* and *GRF5-GIF1* chimeras (evolutionary related) with the combined *GRFGRF1-GIF1* and *GRF9-GIF1* chimeras (more distantly related, Figure 19A). **C)** Scheme of a *GRF4-GIFs* chimeras tested. **D)** Regeneration frequency of transgenic Kronos plants transformed *GRF4* chimeras fused to either *GIF1*, *GIF2* or *GIF3* (3 experiments, contrast chimeras with *GIF1* vs. *GIF2* and *GIF3* $P = 0.0046$). Different letters above bars indicate significant differences ($P < 0.05$, Tukey test). Error bars are s.e.m. The number of inoculated embryos is indicated below the constructs. Normality of residuals was confirmed by Shapiro-Wilk's test and homogeneity of variances by Levene's test (raw-data available in Table 7).

[0027] FIG. 21. Shows effect of the *GRF4-GIF1* chimera in regeneration efficiency in different genotypes. **A)** Representative transformations showing higher frequency of regenerated shoots in the presence of the *GRF4-GIF1* chimera than in the control (empty vector) in different wheat and Triticale genotypes. **B)** Regeneration efficiency of *GRF4-GIF1* vs control in different cultivars of tetraploid and hexaploid wheat and Triticale breeding line UC3190. The number of

inoculated embryos is indicated below the names and the frequencies are indicated on top of the bars. The raw data is in Table 9.

[0028] FIG. 22. Shows **A)** Scheme of the inducible wheat *GRF4-GR-GIF1* chimera with a rat Glucocorticoid Receptor (GR) in the middle of *GRF4-GIF1* (SEQ ID NO:32, 33). **C)** Picture of plates containing transformed Kronos embryos in regeneration media in absence of DEX (-dex) or in the presence of 10 uM DEX (+dex). The presence of DEX induces *GRF4-GR-GIF1* activity and significantly increases regeneration efficiency.

[0029] FIG. 23 shows the *GRF4-GIF1* chimera induces embryogenesis in the absence of cytokinins. **A)** Schematic representation of the different steps of wheat transformation. **B).** Representative callus in auxin media with no hygromycin. Note growing green shoots in callus transformed with the wheat *GRF4-GIF1* chimera in the absence of cytokinins (red arrows). Control: pLC41. **C)** Transgenic specific PCR product (arrow) shows no transgenic plants among four plants regenerated from the control and five transgenic plants among nine regenerated from the *GRF4-GIF1* chimera. A pair of primers specific for the T-DNA was used in the PCR.

[0030] FIG. 24 shows the effect of the *GRF4-GIF1* chimera in regeneration efficiency in the absence of exogenous cytokinin. Immature wheat embryos from a *GRF4-GIF1* transgenic Kronos T₁ plant and a segregating non-transgenic T₁ sister line were treated following the standard transformation protocol, excluding the *Agrobacterium* inoculation and the addition of hygromycin to the plates. In the last step, the calli were transferred to regeneration media in the absence of cytokinin. The number of calli regenerating green shoots was significantly higher in the *GRF4-GIF* transgenic plant (21 out of 27) than in the non-transgenic sister control (3 out of 26). Pictures of representative plates showing calli in regeneration media without cytokinin.

[0031] FIG. 25 shows accelerated wheat transformation protocol using the *GRF4-GIF1* chimera relative to normal protocol of wheat transformation at the UC Davis transformation facility. The protocol with the *GRF4-GIF1* chimera is faster, reducing the overall process by 5 weeks.

[0032] FIG. 26 shows transformation of citrus with *GRF4-GIF1* chimeras. **A)** *Citrus* epicotyls transformed with an empty vector and the *Citrus GRF4-GIF1* chimera (60 d after *Agrobacterium* inoculation). **B)** *Citrus* epicotyls transformed with an empty vector and the *Vitis GRF4-GIF1* and miR396-resistant *GRF4-GIF1* (*rGRF4-GIF1*) chimeras (120 d after *Agrobacterium* inoculation). **C)** Scheme of a *Vitis GRF4-GIF1* chimera showing the miR396 target site and its interaction with

miR396 below. In the miR396-resistant *rGRF4-GIF1* version, we introduced silent mutations (in red) to reduce interactions with miR396. **D)** Statistical comparison of the three *Citrus* experiments. Different letters indicate significant differences in a Tukey test ($P < 0.05$). Horizontal lines on top indicate a significant contrast between the control and the *GRF-GIF* constructs ($P = 0.0153$).

[0033] Fig. 27 shows transformation of grape with *rGRF4-GIF1* chimera. The photo is of grape regenerating calli transformed with an empty vector and the grape *rGRF4-GIF1* chimera.

[0034] Fig. 28 shows transformation of pepper (*Capsicum annuum*) cultivar R&C Cayenne with a *GRF4.1-GIF1.1* chimera including *Capsicum annuum* closest homologs to wheat *GRF4* = pepper LOC107869915 (SEQ ID NO: 138) and to wheat *GIF1* = pepper LOC107870303 (SEQ ID NO: 139) respectively. The picture corresponds to Experiment # 201027/28, showing a >4-fold increase in regeneration efficiency in the pepper cotyledon pieces transformed with the *GRF4.1-GIF1.1* chimera (10 in 42= 23.8%) compared with those transformed with the empty control vector (2 in 40= 5.0%) (Table 11, Method 6).

DETAILED DESCRIPTION

[0035] The present disclosure provides methods of using Growth-Regulating Factor proteins (*GRFs*) and *GRF*-Interacting Factor proteins (*GIFs*), either alone or in combination or as a fused chimera, to improve plant regeneration efficiency. The *GRF* genes belong to a conserved plant-specific family of transcription factors (TFs) (van der Knaap et al., 2000; Kim et al 2003). The *GRF* family is defined by the presence of the domains QLQ and WRC, which mediate protein-protein and protein-DNA interactions respectively (Kim et al., 2003, Kim and Kende 2004, Horiguchi et al., 2005). The *GRF* TFs are highly conserved in land plants, and *GRF* genes have been identified in dicots, monocots, gymnosperms and moss (Omidbakhshfard et al., 2015). These genes are also conserved targets of the microRNA miR396 (Debernardi et al., 2012). *GRFs* can control the size of many plant organs, and act as promoters of growth by increasing cell proliferation in developing organs (Rodriguez et al., 2010). Loss-of-function mutations in *GRFs* or downregulation by overexpression of miR396, can significantly reduce plant size in species like *Arabidopsis* and rice (Horiguchi et al., 2005; Kim et al., 2003; Kim and Kende, 2004; Wang et al., 2011; Liu et al., 2009; Rodriguez et al., 2010; Li S et al., 2016). On the other hand, increased *GRF* activity can produce larger organs, including larger leaves, grain and roots in *Arabidopsis*, rice,

wheat, and brassicas, among others (Horiguchi et al., 2005; Rodriguez et al., 2010; Debernardi et al., 2014; Beltramino et al., 2018; Li, S. et al. 2018).

[0036] The *GRF* proteins can form complexes and work together with proteins encoded by members of the *GIF* gene family. *GIF* proteins do not have a DNA binding domain (Kim and Kende, 2004), but it has been demonstrated that *GIF* proteins interact with *GRFs* and with chromatin remodeling complexes in vivo (Debernardi et al., 2014; Vercruyssen et al., 2014). Based on that observation, it was proposed that *GIFs* could act as co-activators bringing chromatin remodeling complexes to DNA sequences recognized by the *GRFs* (Debernardi et al., 2014, Vercruyssen et al., 2014). Mutation in *GIF* genes mimic most of the phenotypes observed in *GRF* loss-of-function, while overexpression of *GIF* can promote organ growth and can boost the activity of *GRFs* (Kim and Kende 2004, Horiguchi et al., 2005; He et al., 2017; Shimano et al., 2018; Zhang et al., 2018; Debernardi et al., 2014). It has been previously observed that when *Arabidopsis* *GRF3* and *GIF1* are expressed together as a chimera, they can promote a larger increase of leaf size relative to the individual separate genes (patent WO 2013/102762 A1).

[0037] In an embodiment, the plant into which the *GRF* and/or *GIF* sequences are introduced is transgenic, that is, one which has had a heterologous nucleic acid molecule or polypeptide introduced, or which has had its genome edited by any of the techniques available including the examples provided herein. Such a heterologous nucleic acid molecule or polypeptide is any which is not naturally found next to the adjacent nucleic acid molecule or where the levels of polypeptide are higher or lower than that of a plant not comprising the heterologous nucleic acid molecule or polypeptide. In another example the nucleic acid molecule or polypeptide may be from another organism. When referring to a heterologous nucleic acid molecule it includes such a molecule linked to the promoter that does not naturally occur with the promoter sequence and/or is modified in genomic locus by human intervention. The nucleotide sequence in an example is heterologous to the promoter sequence, but it may be from any source, and it may be homologous or native and found naturally occurring in the plant cell, or heterologous or foreign to the plant host. Using the methods described here, a plant transformed with a *GRF-GIF* chimera or the *GRF* and/or *GIF* transgenes and the heterologous nucleic acid molecule or polypeptide has increased regeneration efficiency. The present disclosure provides methods that may significantly expand the number of plant species amenable to efficient transformation technology by significantly increasing plant regeneration efficiency. The methods provided herein may also be used to expand the genotypes

that can be transformed within a certain crop. In wheat, for example, only a few cultivars including Bobwhite, Fielder and Kronos can be transformed efficiently, and those still show low regeneration efficiency. The ability to transform different genotypes is important for breeding applications, because the ability to transform directly top producing genotypes would eliminate the need of costly and lengthy backcrossing processes. The methods provided herein may be used to accelerate the production of transgenic plants from leaf explants instead of embryos.

[0038] In certain embodiments, the present disclosure provides methods of producing a plant having an improved regeneration efficiency. This method comprises the steps of (1) transforming one or more cells of the plant with one or more nucleic acid molecules, wherein the one or more nucleic acid molecules encode a *GRF* protein, a *GIF* protein, or a *GRF-GIF* chimera; and (2) culturing the one or more plant cells in regeneration media. Embodiments provide the *GRF* and *GIF* nucleic acid molecule or polypeptide may be introduced separately or may be introduced into the plant as a chimera. Constructs may comprise nucleic acid molecules encoding the chimera or multiple constructs may be introduced in embodiments.

[0039] The one or more cells of the plant may be transiently or stably transformed. The cells may be derived from any plant tissue, for example, from a leaf explant, microspore, ovule, etc. The cells may be protoplasts.

[0040] Embodiments provide that the time to produce a transgenic plant from the time of transformation is greatly reduced. The time to produce such a plant may be accelerated such that the time to produce the transgenic plant is decreased by five days, ten days, 15 days, 20 days, 25 days, 30 days, or more, or amounts in-between, compared to time to produce a transgenic plant where the *GRF/GIF* proteins are not introduced into the plant. For example, use of the proteins and nucleic acid molecules encoding same in wheat accelerates the production of transgenic plants from 90 to 60 days.

[0041] The term plant or plant material or plant part is used broadly herein to include any plant at any stage of development, or part of a plant, including a plant cutting, a plant cell, a plant cell culture, a plant organ, a plant seed, and a plantlet. A plant cell is the structural and physiological unit of the plant, comprising a protoplast and a cell wall. A plant cell can be in the form of an isolated single cell or aggregate of cells such as a friable callus, or a cultured cell, or can be part of a higher organized unit, for example, a plant tissue, plant organ, or plant. Thus, a plant cell can be a protoplast, a gamete producing cell, or a cell or collection of cells that can regenerate into a

whole plant. As such, a seed, which comprises multiple plant cells and is capable of regenerating into a whole plant, is considered a plant cell for purposes of this disclosure. A plant tissue or plant organ can be a seed, protoplast, callus, or any other groups of plant cells that is organized into a structural or functional unit. Particularly useful parts of a plant include harvestable parts and parts useful for propagation of progeny plants. A harvestable part of a plant can be any useful part of a plant, for example, flowers, pollen, seedlings, tubers, leaves, stems, fruit, seeds, roots, and the like. A part of a plant useful for propagation includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks, and the like. The tissue culture will preferably be capable of regenerating plants. Preferably, the regenerable cells in such tissue cultures will be embryos, protoplasts, meristematic cells, callus, pollen, leaves, anthers, roots, root tips, silk, flowers, kernels, ears, cobs, husks or stalks. Still further, provided are plants regenerated from the tissue cultures.

[0042] Improved or increased regeneration efficiency refers to increasing the number of plant cells, tissue or plants regenerated from the one or more cells having introduced the *GRF*, *GIF* or *GRF/GIF* chimera. In an embodiment the number of shoot regeneration and embryogenesis is increased. Further embodiments provide the regeneration can occur on media that has cytokinin concentration that is too low for the plant cell to regenerate. Using this type of method, it is possible to select for plants comprising the *GRF*, *GIF* or *GRF-GIF* chimera without the need for a separate marker to identify transformed cells. Plants that can grow on such media will comprise the molecules or proteins. In certain embodiments, the *GRF* protein can be a wheat *GRFGRF1*, *GRF2GRF2*, *GRF3*, *GRF4*, *GRF5*, *GRF6* or *GRF9* polypeptide or the related proteins in other plant species. Embodiments provide in one example the *GRF* protein is wheat, rice, Arabidopsis, *Vitis*, *Citrus* or *Capsicum* *GRF* polypeptide. Still further embodiments provide the *GRF* protein is a wheat *GRF4* or a protein that shares significant identity with a wheat *GRF4*, and the *GIF* is wheat *GIF1* or a protein that shares significant identity with a wheat *GIF1*. Exemplary embodiments provide the *GRF* polypeptide may be as shown in any one of SEQ ID NO: 9-23, and 37-39. Examples of *GIF* sequences are any of those shown at SEQ ID NO: 24-30, 43-44, and 52. In some embodiments, the *GRF* is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to the above listed sequences. In some embodiments, the *GIF* is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or at least about 99% identical to the above listed sequences. In some embodiments, the one or

more nucleic acid molecules encode a chimeric *GRF-GIF* construct in which the *GRF* portion is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to a corresponding portion of a wheat *GRF4* or other above listed *GRF* sequences and the *GIF* portion is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to the corresponding portion of the above *GIF* sequences and in an embodiment to the wheat *GIF1* sequence. In some embodiments, the one or more nucleic acid molecules encode a chimeric *GRF4-GIF1* construct.

[0043] In some embodiments, the *GRF* protein comprises QLQ and WRC domains that are at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% identical to at least one of the domains as may be found in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, or SEQ ID NO:22. In other embodiments, the *GRF* comprises QLQ and WRC domains that are at least 70%, at least 85%, at least 90%, at least 95%, at least 99% identical to at least one of the QLQ domain of at least one of SEQ ID NO: 69 – 83 and the WRC domain of at least one of SEQ ID NO: 84 – 95 which are found in the full length *GRF* sequences of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, or SEQ ID NO:22. In examples, QLQ domains are those as shown in SEQ ID NO: 69 – 83 and WRC domains are those as shown in SEQ ID NO: 84 – 95 and having such identity thereto.

[0044] In some embodiments, the *GIF* comprises an SNH domain that is at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% identical to at least one of SEQ ID NO: 97 – 103 which are found in the full length *GIF* sequences of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, or SEQ ID NO:29 or SEQ ID NO: 133(Os11g40100); SEQ ID NO: 134 (Os12g31350) or SEQ ID NO: 135(Os03g52320). Examples of such domains are those found at SEQ ID NO: 97-103, 136 and 137nd having such sequence identity thereto.

[0045] In some embodiments, the one or more nucleic acid molecules encoding the *GRF* comprise one or more mutations in the recognition site of miR396. Further embodiments provided the mutations are silent mutations. Still further embodiments provide the mutations reduce

repression of the *GRF* protein. Yet further embodiments provide the mutations are made to a miR396 target site as shown in wild type sequence of SEQ ID NO: 53 and in one example can produce the modified miR396 of SEQ ID NO: 54.

[0046] The term nucleic acid molecule refers to a nucleic acid molecule, which can be a RNA molecule as well as DNA molecule, and can be a molecule that encodes for a desired polypeptide or protein, but also may refer to nucleic acid molecules that do not constitute an entire gene, and which do not necessarily encode a polypeptide or protein. For example, when used in a homologous recombination process, the promoter may be placed in a construct with a sequence that is similar to an area of the chromosome in the plant that may not encode a protein. If desired, the nucleotide sequence of interest can be optimized for plant translation by optimizing the codons used for plants and the sequence around the translational start site for plants. Sequences resulting in potential mRNA instability can also be avoided.

[0047] Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. The term conservatively modified variants applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are silent variations and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also, by reference to the genetic code, describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine; and UGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described polypeptide sequence and is within the scope of the products and processes described.

[0048] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" referred to herein as a "variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. See, for example, Davis et al., "Basic Methods in Molecular Biology" Appleton & Lange, Norwalk, Conn. (1994).

[0049] The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (see, e.g., see, e.g., Creighton, Proteins: Structures and Molecular Properties (WH Freeman & Co.; 2nd edition (December 1993))).

[0050] In an embodiment the *GRF* polypeptide or nucleic acid molecule encoding same is selected from a wheat *GRFGRF1*, *GRF2GRF2*, *GRF3*, *GRF4*, *GRF5*, *GRF6*, *GRF9* polypeptide or nucleic acid molecule encoding same or homolog thereof. An embodiment provides the *GIF* polypeptide or nucleic acid molecule encoding same is selected from a wheat *GIF1*, *GIF2* or *GIF3* polypeptide or nucleic acid molecule encoding same or homolog thereof. Embodiments provide the polypeptide or nucleic acid molecule encoding same is a wheat *GRFGRF1*, *GRF2GRF2*, *GRF3*, *GRF4*, *GRF5*, *GRF6*, *GRF9* polypeptide or nucleic acid molecule encoding same or a homolog of such wheat polypeptide or nucleic acid molecule encoding same. Further embodiments provide the polypeptide or nucleic acid molecule encoding same is a wheat *GIF1*, *GIF2* or *GIF3* polypeptide or nucleic acid molecule encoding same or homolog thereof. A homolog is a gene or polypeptide inherited in different species retaining the same biological function. The *GRF* family is defined herein and are transcription factors having the conserved QLQ and WRC domains which mediate protein-protein and protein-DNA interactions, are highly conserved in land plants and identified in dicots, monocots, gymnosperms and moss, as discussed herein. *GIF* proteins interact with *GRFs* and have a conserved SNH domain. Such *GRF* and *GIF* polypeptides have the property of increasing regeneration efficiency, particularly when combined in a chimeric protein. Examples of such homologs are provided herein, such as those found in

rice, *Citrus*, *Vitis*, *Capsicum* and *Arabidopsis*. A person of skill in the art can readily identify such homologs. For example, NCBI provides searching for homologs for a gene or the protein in other organisms. See ncbi.nlm.nih.gov/homologene. Further, such sequences include those that hybridize to the sequences of the *GRF* and *GIF* nucleic acid molecules or polypeptides shown herein under stringent hybridization conditions.

[0051] Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm.

[0052] Optimal alignment of sequences for comparison can use any means to analyze sequence identity (homology) known in the art, e.g., by the progressive alignment method of termed "PILEUP" (Morrison, (1997) *Mol. Biol. Evol.* 14:428-441, as an example of the use of PILEUP); by the local homology algorithm of Smith & Waterman (*Adv. Appl. Math.* 2: 482 (1981)); by the homology alignment algorithm of Needleman & Wunsch (*J. Mol. Biol.* 48:443-453 (1970)); by the search for similarity method of Pearson (*Proc. Natl. Acad. Sci. USA* 85: 2444 (1988)); by computerized implementations of these algorithms (e.g., GAP, BEST FIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.); ClustalW (CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, Calif., described by, e.g., Higgins(1988), *Gene* 73: 237-244; Corpet (1988), *Nucleic Acids Res.* 16:10881-10890; Huang, *Computer Applications in the Biosciences* 8:155-165 (1992); and Pearson (1994), *Methods in Mol. Biol.* 24:307-331); Pfam (Sonnhammer (1998), *Nucleic Acids Res.* 26:322-325); TreeAlign (Hein (1994), *Methods Mol. Biol.* 25:349-364); MEG-ALIGN, and SAM sequence alignment computer programs; or, by manual visual inspection.

[0053] Another example of algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul et al, (1990) *J. Mol. Biol.* 215: 403-410. The BLAST programs (Basic Local Alignment Search Tool) of Altschul, S. F., et al., searches under default parameters for identity to sequences contained in the BLAST "GENEMBL" database. A sequence can be analyzed for identity to all publicly available DNA sequences contained in the GENEMBL database using the BLASTN algorithm under the default parameters.

[0054] Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/; see also Zhang (1997), *Genome Res.* 7:649-656 for the "PowerBLAST" variation. This algorithm involves first identifying high

scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al (1990), *J. Mol. Biol.* 215: 403-410). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W , T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff (1992), *Proc. Natl. Acad. Sci. USA* 89:10915-10919) alignments (B) of 50, expectation (E) of 10, $M=5$, $N=-4$, and a comparison of both strands. The term BLAST refers to the BLAST algorithm which performs a statistical analysis of the similarity between two sequences; see, e.g., Karlin (1993), *Proc. Natl. Acad. Sci. USA* 90:5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

[0055] In an embodiment, GAP (Global Alignment Program) can be used. GAP uses the algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443-453, 1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. Default gap creation penalty values and gap extension penalty values in the commonly used Version 10 of the Wisconsin Package® (Accelrys, Inc., San Diego, CA) for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. A general purpose scoring system is the BLOSUM62 matrix (Henikoff and Henikoff (1993), *Proteins* 17:

49-61), which is currently the default choice for BLAST programs. BLOSUM62 uses a combination of three matrices to cover all contingencies. Altschul, J. Mol. Biol. 36: 290-300 (1993), herein incorporated by reference in its entirety and is the scoring matrix used in Version 10 of the Wisconsin Package® (Accelrys, Inc., San Diego, CA) (see Henikoff & Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915).

[0056] (c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

[0057] (d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

[0058] When referring to hybridization techniques, all or part of a known nucleotide sequence can be used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ³²P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the DNA sequences. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed (Sambrook et al., 2001).

[0059] For example, the sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding sequences. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among the sequences to be screened and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such sequences may alternatively be used

to amplify corresponding sequences from a chosen plant by PCR. This technique may be used to isolate sequences from a desired plant or as a diagnostic assay to determine the presence of sequences in a plant. Hybridization techniques include hybridization screening of DNA libraries plated as either plaques or colonies (Sambrook et al., 2001).

[0060] Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

[0061] Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 50°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 0.1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

[0062] Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267-284 (1984): $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the

hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with ~90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel et al., eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook et al. (2001) *Molecular Cloning: A Laboratory Manual* (3rd ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.) and Haymes et al. (1985) In: *Nucleic Acid Hybridization*, a Practical Approach, IRL Press, Washington, D.C.

[0063] The term introduced in the context of inserting a nucleic acid or polypeptide into a cell, includes transfection or transformation or transduction and includes in embodiments reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA). As discussed below, introduction of proteins into plants can utilize methods such as those described in Example 8.

[0064] Various methods of transformation/transfection are available. As newer methods are available to transform crops or other host cells they may be directly applied. Accordingly, a

wide variety of methods have been developed to insert a DNA sequence into the genome of a host cell to obtain the transcription or transcript and translation of the sequence to effect phenotypic changes in the organism. Thus, any method which provides for efficient transformation/transfection may be employed. Utilizing the methods here, increased regeneration efficiency is provided.

[0065] By way of example without limitation, methods for introducing expression vectors into plant tissue available to one skilled in the art are varied and will depend on the plant selected. Procedures for transforming a wide variety of plant species are well known and described throughout the literature. (See, for example, Miki and McHugh (2004) *Biotechnol.* 107, 193-232; Klein et al. (1992) *Biotechnology* (N Y) 10, 286-291; and Weising et al. (1988) *Annu. Rev. Genet.* 22, 421-477). For example, the DNA construct may be introduced into the genomic DNA of the plant cell using techniques such as microprojectile-mediated delivery (Klein et al. 1992, *supra*), electroporation (Fromm et al., 1985 *Proc. Natl. Acad. Sci. USA* 82, 5824-5828), polyethylene glycol (PEG) precipitation (Mathur and Koncz, 1998 *Methods Mol. Biol.* 82, 267-276), direct gene transfer (WO 85/01856 and EP-A-275 069), *in vitro* protoplast transformation (U.S. Pat. No. 4,684,611), and microinjection of plant cell protoplasts or embryogenic callus (Crossway, A. (1985) *Mol. Gen. Genet.* 202, 179-185). *Agrobacterium* transformation methods of Ishida et al. (1996) and also described in U.S. Pat. No. 5,591,616 are yet another option. Co-cultivation of plant tissue with *Agrobacterium tumefaciens* is a variation, where the DNA constructs are placed into a binary vector system (Ishida et al., 1996 *Nat. Biotechnol.* 14, 745-750). The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct into the plant cell DNA when the cell is infected by the bacteria. See, for example, Fraley et al. (1983) *Proc. Natl. Acad. Sci. USA*, 80, 4803-4807. *Agrobacterium* is primarily used in dicots, but monocots including maize can be transformed by *Agrobacterium*. See, for example, U.S. Pat. No. 5,550,318. In one of many variations on the method, *Agrobacterium* infection of corn can be used with heat shocking of immature embryos (Wilson et al. U.S. Pat. No. 6,420,630) or with antibiotic selection of Type II callus (Wilson et al., U.S. Pat. No. 6,919,494).

[0066] Rice transformation is described by Hiei et al. (1994) *Plant J.* 6, 271-282 and Lee et al. (1991) *Proc. Nat. Acad. Sci. USA* 88, 6389-6393. Standard methods for transformation of canola are described by Moloney et al. (1989) *Plant Cell Reports* 8, 238-242. Corn transformation is described by Fromm et al. (1990) *Biotechnology* (N Y) 8, 833-839 and Gordon-Kamm et al. (1990)

supra. Wheat can be transformed by techniques similar to those used for transforming corn or rice. Sorghum transformation is described by Casas et al. (Casas et al. (1993) Transgenic sorghum plants via microprojectile bombardment. *Proc. Natl. Acad. Sci. USA* 90, 11212-11216) and barley transformation is described by Wan and Lemaux (Wan and Lemaux (1994) Generation of large numbers of independently transformed fertile barley plants. *Plant Physiol.* 104, 37-48). Soybean transformation is described in a number of publications, including U.S. Pat. No. 5,015,580. In certain embodiments, the regulatory sequence comprises an inducible promoter. In other embodiments the activity of *GRF*, *GIF* or the *GRF-GIF* chimera is regulated by an inducible system. The inducible system may be or may include a glucocorticoid receptor fused to the *GRF*, *GIF* or *GRF-GIF* proteins. Other inducible systems may also be used.

[0067] In certain embodiments described herein, the one of more transformed plant cells may be cultured in regeneration media that comprises a suboptimal concentration of exogenous cytokinins. The term “suboptimal concentration” is defined as any concentration that is too low to allow adequate regeneration of plant cells that are not transformed with a *GRF-GIF* chimera or the *GRF* and/or *GIF* transgenes. A suboptimal concentration may be, e.g., less than about 50%, less than about 10%, less than about 5%, less than about 1%, or less than 0.01% of the cytokinin concentration than is typically used for plant regeneration. Cytokinin concentrations can be tested to determine an optimal concentration that permits regeneration of plant cells transformed with the *GRF-GIF* chimera but that is not sufficient to induce regeneration of non-transgenic plants. This can be used as a positive selection method to identify transgenic shoots without using antibiotic markers.

[0068] In certain embodiments, the methods disclosed herein increase regeneration efficiency. Regeneration efficiency refers to the number of plant cells that can be regenerated. The methods provide that efficiency is increased compared to regeneration in which a *GRF/GIF* polypeptide or nucleotide is not introduced into the plant. The regeneration efficiency can be increased by 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 80%, 90% or more or amounts in-between. By way of example without limitation a plant having 10% regeneration efficiency without use of *GRF/GIF* can be increased to 50 – 70%. A plant having 1% efficiency can be increased to 10 – 20% and a plant that has zero regeneration efficiency can be increased to 1-5%.

[0069] In some embodiments, the one or more nucleic acid molecules used in the methods disclosed here may, in addition to encoding a *GRF* and a *GIF*, encode at least one additional polynucleotide of interest.

[0070] The methods disclosed herein may be used in a variety of plants. In certain embodiments, the methods may be used in a plant that has a low regeneration efficiency. In certain embodiments, the plant is a monocot species. In certain other embodiments, the plant is a dicot species. In yet other embodiments, the plant is neither a monocot nor a dicot species. Example species include but are not limited to corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*), Triticale (($\times$ Triticosecale, a cross of wheat and rye), *Triticale* ($\times$ Triticosecale, a cross of wheat and rye), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats (*Avena*), barley (*Hordeum*), vegetables, ornamentals, and conifers. Vegetables include tomatoes (*Lycopersicon esculentum*), pepper (*Capsicum annuum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.) and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers which may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contotta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver

fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*).

[0071] The plant in preferred embodiments may be selected from a species of *Oryza*, *Gossypium*, *Glycine*, *Vitis*, *Medicago*, *Juglans*, *Citrus*, *Capsicum*, *Sorghum*, *Zea*, *Hordeum* or *Triticum* or other appropriate plant species. In certain embodiments, the plant is *Triticum turgidum* or *Triticum aestivum*.

[0072] The present disclosure also provides plants that are produced by any of the methods described herein.

[0073] The present disclosure provides methods to modulate the expression of *GRF* and *GIF* genes in plants to improve regeneration efficiency and to allow regeneration in the absence of exogenous cytokinin. These methods may be practiced by increasing the protein levels and activities of *GRF* and/or *GIF* or *GRF-GIF* chimera in a plant, plant cell, or protoplast.

[0074] In some embodiments, the *GRF* genes and *GIF* genes may be increased in a plant or plant cell by various means, including using vectors that each have only a *GRF* gene or a *GIF* gene, or a vector with a *GRF-GIF* chimera. The *GRF* gene may be mutated to render it less sensitive to the repression by miR396. The one or more nucleic acid molecules that encode a Growth-Regulating Factor protein (*GRF*) and/or a *GRF*-Interacting Factor protein (*GIF*) may be operably linked to a regulatory sequence operable in the plant cell. The genes in the vectors may be controlled by various types of promoters, including inducible, tissue-specific, and constitutive promoters. The plant or plant cell may be transformed by various means, including by using *Agrobacterium* harboring a vector with *GRF* and *GIF* genes or bombardment.

[0075] Gene editing may be used in combination with the present methods. For example, a vector with *GRF* and/or *GIF*, or *GRF-GIF* chimera and Cas9 may be used for gene editing of a plant or plant cell. The plants or plant cells may be transformed to express the nucleic acids transiently or stably. *GRF* and/or *GIF*, or *GRF-GIF* chimera proteins may be delivered to plants or plant cells to increase regeneration efficiency using methods described by other investigators to deliver CRISPR/Cas9 preassembled ribonucleoprotein complexes (RNPs) (Woo et al., 2015; Subburaj et al., 2016; Malnoy et al., 2016; Kim et al., 2017; Liang et al., 2017; Svitashhev et al., 2016; Wolter et al., 2017).

[0076] Methods which provides for targeting of a molecule of interest (MOI) to the target site of the target gene may be utilized in the method. The following is provided by way of example

rather than limitation. A guide nucleic acid molecule is one that directs the nuclease to the specific cut site in the genome, whether via use of a binding domain, recognition domains, guide RNAs or other mechanisms. The guide nucleic acid molecule is introduced into the cell under conditions appropriate for operation of the guide nucleic acid molecule in directing cleavage to the target locus. A person of skill in the art will have available a number of methods that may be used, the most common utilizing a nuclease to cleave the target region of the gene, along with sequences which will recognize sequences at the target locus and direct cleavage to the locus. Any nuclease that can cleave the phosphodiester bond of a polynucleotide chain may be used in the methods described here. By way of example without limitation, available systems include those utilizing site specific nucleases (SSN) such as ZFNs (Zinc finger nuclease), Whyte, et al. Cell Biology Symposium: Zinc finger nucleases to create custom-designed modifications. *J Anim Sci* 90, 1111-1117 (2012)); TALENs (Transcription activator-like effector nucleases) (see, Carlson, D.F. et al. Efficient TALEN-mediated gene knockout in animals. *Proc Natl Acad Sci U S A* 109, 17382-17387 (2012); Tan, W. et al. Efficient nonmeiotic allele introgression in livestock using custom endonucleases. *Proc Natl Acad Sci U S A* 110, 16526-16531 (2013); and the CRISPR (Clustered regularly interspaced short palindromic repeats) -associated (Cas) nuclease system (Hai, T., Teng, F., Guo, R., Li, W. & Zhou, Q. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. *Cell Res* 24, 372-375 (2014)) that have permitted editing of animal genomes. The use of recombinases such as FLP/FRT as described in US Patent No. 6,720,475, or CRE/LOX as described in US Patent No. 5,658,772, can be utilized to integrate a polynucleotide sequence into a specific chromosomal site. Meganucleases have been used for targeting donor polynucleotides into a specific chromosomal location as described in Puchta *et al.*, *PNAS USA* 93 (1996) pp. 5055-5060. ZFNs work with proteins or domains of proteins binding to a binding domain having a stabilized structure as a result of use a zinc ion. TALENs utilize domains with repeats of amino acids, which can specifically recognize a base pair in a DNA sequence. For a discussion of both systems, see Voytas et al. US Patent No. 8,697,853, incorporated herein by reference in its entirety. These systems utilize enzymes prepared for each target sequence.

[0077] In referring to a target gene or molecule is meant to refer to any nucleic acid molecule within the genome desired to be modified as described or where it is desired to delete or insert a nucleic acid molecule or modify the molecule in some manner. Where the target molecule is a

nucleic acid sequence, the target molecule can, for example, be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA).

[0078] The term “regeneration” as used herein means generation of a plant or plant cells from another plant or plant cell. This includes the generation of shoots from calli generated from a plant embryo or young leaflets. In some embodiments, plants, plant cells, or protoplasts may be regenerated from plants, plant cells, or protoplasts that have been transformed to have increased *GRF* and/or *GIF* or *GRF-GIF* chimera expression. In some embodiments, plants, plant cells, or protoplasts regenerated from plants, plant cells, or protoplasts that have been transformed to have increased *GRF* and/or *GIF* or *GRF-GIF* chimera expression may be further transformed with at least one additional polypeptide.

[0079] In various embodiments, the plant or plant cell may originate from various plant species, including those resistant to transformation, having low regeneration efficiency, and those in *Triticum*, *Vitis*, *Oryza*, *Gossypium*, *Citrus*, *Capsicum*, *Sorghum*, and *Juglans* genus.

[0080] The present disclosure also provides methods to regenerate a plant or plant cell in the absence of exogenous cytokinins. In certain embodiments, such methods comprise introducing a *GRF* gene and/or a *GIF* gene, or a *GRF-GIF* chimera into plant cells and culturing the cells in medium substantially free of exogenous cytokinins to regenerate a plant. The *GRF* and *GIF* genes may be of *Triticum aestivum*, *Triticum turgidum* ssp. *durum* or of another plant species, including those that have low regeneration efficiency or are resistant to transformation.

[0081] In various embodiments, a plant may be transformed using vectors that each have only a *GRF* gene or a *GIF* gene, a vector with a *GRF* and *GIF* as a chimera, or a vector with a *GRF* and a *GIF* in a non-chimeric form.

[0082] In certain embodiments, the plant is or is considered recalcitrant to regeneration.

[0083] The nucleic acid molecule or polypeptide may be introduced into the plant along with other components. As used herein, a nucleotide segment is referred to as operably linked when it is placed into a functional relationship with another DNA segment. For example, DNA for a signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, by operably linked it is intended that the coding regions are in the same reading frame.

Alternatively, the additional gene(s) can be provided on multiple expression cassettes. Such an expression cassette is provided with a plurality of restriction sites and/or recombination sites for insertion of the polynucleotide to be under the transcriptional regulation of the regulatory regions. The expression cassette can include one or more enhancers in addition to a promoter. By enhancer is intended a cis-acting sequence that increases the utilization of a promoter. Such enhancers can be native to a gene or from a heterologous gene. Further, it is recognized that some promoters can contain one or more enhancers or enhancer-like elements. An example of one such enhancer is the 35S enhancer, which can be a single enhancer, or duplicated. See for example, McPherson et al, US Patent 5,322,938.

[0084] The terms promoter, promoter region or promoter sequence refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

[0085] Spatial and temporal control is also often important in driving gene expression in plants. For example, selectable and scoreable markers must be expressed at a suitable time and in an appropriate tissue to allow for screening and controlling enzymes and regulatory factors must be produced in metabolically active and physiologically responsive tissues, respectively. Similarly, genes conferring host protection must be expressed in the target tissues for the pathogen or pest, and plant produced protein products should be expressed in tissues suitable for protein accumulation and storage. Furthermore, since certain protein products may have detrimental effects on plant health and yield when expressed in metabolically active plant tissues that are essential for survival and growth, promoters may be favored that are active in the chosen plant storage tissues but show low or no activity in other, non-storage tissues.

[0086] In the methods, a number of promoters that direct expression of a nucleic acid molecule in a plant can be employed. Such promoters can be selected from constitutive,

chemically-regulated, inducible, tissue-specific, and seed-preferred promoters. Constitutive promoters include, for example, the core CaMV 35S promoter (Odell et al. (1985) *Nature* 313:810-812); rice actin (McElroy et al. (1990) *Plant Cell* 2:163-171); ubiquitin (European patent application no. 0 342 926; Christensen et al. (1989) *Plant Mol. Biol.* 12:619-632 and Christensen et al. (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last et al. (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten et al. (1984) *EMBO J.* 3:2723-2730), the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; to name a few.

[0087] A skilled person appreciates a promoter sequence can be modified to provide for a range of expression levels of and operably linked heterologous nucleic acid molecule. Less than the entire promoter region can be utilized and the ability to drive expression retained. However, it is recognized that expression levels of mRNA can be decreased with deletions of portions of the promoter sequence. Thus, the promoter can be modified to be a weak or strong promoter. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Conversely, a strong promoter drives expression of a coding sequence at a high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

[0088] Tissue-preferred promoters can be utilized to target enhanced transcription and/or expression within a particular plant tissue. When referring to preferential expression, what is meant is expression at a higher level in the particular plant tissue than in other plant tissue. Examples of these type of promoters include seed preferred expression such as that provided by the phaseolin promoter (Bustos et al.(1989) *The Plant Cell* Vol. 1, 839-853), and the maize globulin-1 gene, Belanger, et al. (1991) *Genetics* 129:863-972.

[0089] The range of available plant compatible promoters includes inducible promoters. Any inducible promoter can be used in the instant process. See Ward et al. *Plant Mol. Biol.* 22: 361-366 (1993). Exemplary inducible promoters include ecdysone receptor promoters, U.S. Patent No. 6,504,082; promoters from the ACE1 system which responds to copper (Mett et al. *PNAS* 90: 4567-4571 (1993)); In2-1 and In2-2 gene from maize which respond to benzenesulfonamide herbicide safeners (US Patent No. 5,364,780; Hershey et al., *Mol. Gen. Genetics* 227: 229-237 (1991) and Gatz et al., *Mol. Gen. Genetics* 243: 32-38 (1994)) Tet repressor from Tn10 (Gatz et

al., *Mol. Gen. Genet.* 227: 229-237 (1991); or from a steroid hormone gene, the transcriptional activity of which is induced by a glucocorticosteroid hormone. Schena et al., *Proc. Natl. Acad. Sci. U.S.A.* 88: 10421 (1991); the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides; and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis et al. (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz et al. (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156).

[0090] A cold responsive regulatory element or a heat shock regulatory element, the transcription of which can be effected in response to exposure to cold or heat, respectively (Takahashi et al., *Plant Physiol.* 99:383-390, 1992); the promoter of the alcohol dehydrogenase gene (Gerlach et al., *PNAS USA* 79:2981-2985 (1982); Walker et al., *PNAS* 84(19):6624-6628 (1987)), inducible by anaerobic conditions; and the light-inducible promoter derived from the *pea rbcS* gene or *pea psaDb* gene (Yamamoto et al. (1997) *Plant J.* 12(2):255-265); a light-inducible regulatory element (Feinbaum et al., *Mol. Gen. Genet.* 226:449, 1991; Lam and Chua, *Science* 248:471, 1990; Matsuoka et al. (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590; Orozco et al. (1993) *Plant Mol. Bio.* 23(6):1129-1138), a plant hormone inducible regulatory element (Yamaguchi-Shinozaki et al., *Plant Mol. Biol.* 15:905, 1990; Kares et al., *Plant Mol. Biol.* 15:225, 1990), and the like. An inducible regulatory element also can be the promoter of the maize *In2-1* or *In2-2* gene, which responds to benzenesulfonamide herbicide safeners (Hershey et al., *Mol. Gen. Gente.* 227:229-237, 1991; Gatz et al., *Mol. Gen. Genet.* 243:32-38, 1994), and the Tet repressor of transposon *Tn10* (Gatz et al., *Mol. Gen. Genet.* 227:229-237, 1991). Stress inducible promoters include salt/water stress-inducible promoters such as *P5CS* (Zang et al. (1997) *Plant Sciences* 129:81-89); cold-inducible promoters, such as, *cor15a* (Hajela et al. (1990) *Plant Physiol.* 93:1246-1252), *cor15b* (Wilhelm et al. (1993) *Plant Mol Biol* 23:1073-1077), *wsc120* (Ouellet et al. (1998) *FEBS Lett.* 423-324-328), *ci7* (Kirch et al. (1997) *Plant Mol Biol.* 33:897-909), *ci21A* (Schneider et al. (1997) *Plant Physiol.* 113:335-45); drought-inducible promoters, such as, *Trg-31* (Chaudhary et al (1996) *Plant Mol. Biol.* 30:1247-57), *rd29* (Kasuga et al. (1999) *Nature Biotechnology* 18:287-291); osmotic inducible promoters, such as *Rab17* (Vilardell et al. (1991)

Plant Mol. Biol. 17:985-93) and osmotin (Raghothama et al. (1993) *Plant Mol Biol* 23:1117-28); and heat inducible promoters, such as heat shock proteins (Barros et al. (1992) *Plant Mol.* 19:665-75; Marrs et al. (1993) *Dev. Genet.* 14:27-41), *smHSP* (Waters et al. (1996) *J. Experimental Botany* 47:325-338), and the heat-shock inducible element from the parsley ubiquitin promoter (WO 03/102198). Other stress-inducible promoters include *rip2* (U.S. Patent No. 5,332,808 and *rd29a* (Yamaguchi-Shinozaki et al. (1993) *Mol. Gen. Genet.* 236:331-340). Certain promoters are inducible by wounding, including the *Agrobacterium pmas* promoter (Guevara-Garcia et al. (1993) *Plant J.* 4(3):495-505) and the *Agrobacterium ORF13* promoter (Hansen et al., (1997) *Mol. Gen. Genet.* 254(3):337-343).

[0091] In addition, markers that facilitate identification of a plant cell containing the polynucleotide encoding the marker may be employed. Scorable or screenable markers are useful, where presence of the sequence produces a measurable product and can produce the product without destruction of the plant cell. Examples include a β -glucuronidase, or *uidA* gene (GUS), which encodes an enzyme for which various chromogenic substrates are known (for example, US Patents 5,268,463 and 5,599,670); chloramphenicol acetyl transferase (Jefferson et al. (1987) *The EMBO Journal* vol. 6 No. 13 pp. 3901-3907); alkaline phosphatase. Other screenable markers include the anthocyanin/flavonoid genes in general (See discussion at Taylor and Briggs, (1990) *The Plant Cell* 2:115-127) including, for example, a *R*-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta et al., in Chromosome Structure and Function, Kluwer Academic Publishers, Appels and Gustafson eds., pp. 263-282 (1988)); the genes which control biosynthesis of flavonoid pigments, such as the maize *C1* gene (Kao et al., (1996) *Plant Cell* 8: 1171-1179; Scheffler et al. (1994) *Mol. Gen. Genet.* 242:40-48) and maize *C2* (Wienand et al., (1986) *Mol. Gen. Genet.* 203:202-207); the *B* gene (Chandler et al., (1989) *Plant Cell* 1:1175-1183), the *pl* gene (Grotewold et al., (1991) *Proc. Natl. Acad. Sci USA*) 88:4587-4591; Grotewold et al., (1994) *Cell* 76:543-553; Sidorenko et al., (1999) *Plant Mol. Biol.* 39:11-19); the *bronze* locus genes (Ralston et al., (1988) *Genetics* 119:185-197; Nash et al., (1990) *Plant Cell* 2(11): 1039-1049), among others. As discussed herein, when using the present invention with a culture media that has lower cytokine levels that necessary for regeneration of the plant cell, use of the *GRF* or *GIF* or *GRF-GIF* chimera may be utilized without an additional selectable marker.

[0092] A wide variety of other components such as signal sequences and polyadenylation sequences may be included with the vector as is desired.

EXAMPLES

[0093] The following examples are for illustration only. In light of this disclosure, those of skill in the art will recognize that variations of these examples and other embodiments of the disclosed subject matter are enabled without undue experimentation. In reference to experiments herein, see Figure 14 showing predicted proteins of selected wheat *GRF* genes *GRFGRF1-5* (SEQ ID NO: 9 – 13). Wheat gene names are based on Chinese Spring RefSeq v1.0. *GRF* numbers are based on rice orthologs. Only the sequences of the wheat A genome homeologs are provided (B and D genome are more than 90% identical). The encoded protein sequences of the closest wheat, rice and Arabidopsis homologs are indicated in SEQ ID NO: 9 – 22, with the conserved QLQ and WRC domains highlighted in yellow and green respectively. Predicted proteins of five rice *GRF* orthologs are shown in Figure 15 (SEQ ID NO: 13 – 18). Predicted proteins of the closest Arabidopsis *GRF3-6* are shown in Figure 16A (SEQ ID NO: 19 – 22). Predicted protein of the closest *Vitis vinifera* *GRF* used in the chimera is shown in Figure 13B (SEQ ID NO: 23).

[0094] Figure 17A shows predicted proteins of Arabidopsis *GIF1* and *GIF2* (SEQ ID NO: 24 – 26) and 17B shows *Triticum aestivum* *GIF1*, *GIF2* and *GIF3* (SEQ ID NO: 27 – 29). Wheat gene names are based on Chinese Spring RefSeq v1.0. *GIF* numbers are based on rice orthologs. Only the sequences of the wheat A genome homeologs are provided (B and D genome are more than 90% identical). The encoded protein sequences of the closest Arabidopsis and wheat homologs are indicated below, with the conserved SNH domain highlighted in yellow. *Vitis vinifera* *GIF* is shown in Figure 17C (SEQ ID NO: 30).

[0095] Example 1 – Transformation of wheat with a *GRF-GIF* chimera

[0096] In an effort to increase plant biomass to promote increases in grain yield of agriculturally important crops, we tested the effects of *GRF*, *GIF* and *GRF-GIF* chimeras on wheat growth. Transgenic plants that expressed a wheat *GRF-GIF* chimera under the maize UBIQUITIN promoter were generated. We identified 10 *GRF*s and 3 *GIF*s in the wheat genome by Molecular Phylogenetic analysis using the Neighbor-Joining method (Figure 1). To generate the wheat *GRF-GIF* chimera, we selected the wheat *GRF* homologue to the rice *OsGRF4* (Figure 1A), which promotes grain and plant growth in rice (Duan P et al., 2015; Hu J et al., 2015; Che R et al., 2015;

Sun P et al 2016; Li, S. et al. 2018). For the *GIF* partner, we selected the closest homologue of *Arabidopsis GIF1* (Figure 1B), since members of this clade has been shown to control growth in *Arabidopsis*, rice and maize (Kim and Kende 2004, Horiguchi et al., 2005; Shimano S et al., 2018; Zhang D et al., 2018). The amino acid sequences of the SNH conserved domain were used to perform the phylogenetic analysis in Figure 1B. The wheat closest homologue of *Arabidopsis GIF1* is highlighted in bold. A schematic representation of the chimeric construct is in Figure 1C. The amino acid sequence of the *GRF* and *GIF* genes from *Arabidopsis*, rice and *Brachypodium* were obtained from phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>). The amino acid sequences of wheat *GRF* and *GIF* genes were obtained from the wheat genome RefSeq v1.0. The phylogenetic analysis was performed using MEGA6. The coding sequence for the wheat chimeric *GRF4-GIF1* is provided in Figure 11A and the protein sequence in Figure 11B. The sequence of the construct used for transformation, pLC41- Ubi::*GRF4-GIF1*, is provided in Figure 12.

[0097] We then transformed the wheat *GRF4-GIF1* chimera into tetraploid wheat Kronos (*Triticum turgidum* ssp. *durum*) by *Agrobacterium*-mediated transformation. The plant cells infected with *Agrobacterium* harboring the *GRF4-GIF1* binary vector (a schematic representation of the construct shown in Figure 1C) regenerated a surprising number of shoots, which was significantly higher than the number of shoots obtained in control parallel transformations with vectors having the same pLC41 backbone (Figure 2). The *GRF* and *GIF* genes have not been previously associated with increased regeneration efficiency in transgenic plants.

[0098] We next analyzed the frequency of callus pieces regenerating shoots in two independent wheat transformation experiments using the *GRF4-GIF1* chimera and two experiments without the chimera. The statistical analysis demonstrated that the average number of callus pieces regenerating shoots was more than 15-fold higher ($P = 0.0019$) in the callus transformed with the *GRF4-GIF1* chimera ($92.8\% \pm 3.2\%$) than in the callus transformed with constructs without the *GRF4-GIF1* chimera ($6.0\% \pm 2\%$). The last numbers are representative of Kronos wheat transformations, where usually only one or two shoots are recovered from plates containing 25 callus (Figure 2, both sides). For the wheat *GRF4-GIF1* chimera (Figure 2, middle), we recovered around 50 shoots per plate containing 25 callus, a substantial increase in the efficiency of regeneration of transgenic wheat plants.

[0099] We transferred to soil 26 independent T_0 plants to verify the presence of the *GRF4-GIF1* chimera. PCR amplification of genomic DNA from the 26 independent T_0 plants using

primers Fw-*GRF4b* (5'-CCTCCGACTCCAAGTATTGC-3') (SEQ ID NO: 104) and Rev-*GIF1c* (5'-ATCATCAGGTTGGACGGGTA-3') (SEQ ID NO: 105) confirmed that 23 out of the 26 T₀ plants had the *GRF4-GIF1* transgene inserted in the genomic DNA (Figure 3). The transgenic plants were fertile and showed a normal phenotype. Without being bound by any particular theory, this may be because the endogenous miR396 controlled the levels of the overexpressed *GRF4-GIF1* chimera at later stages of plant development.

[00100] Example 2 – Transformation of wheat using only *GRF4* or *GIF1*

[00101] Wheat transformation experiments were performed using only *GRF4* (SEQ ID NO:1) or only *GIF1* (SEQ ID NO:2) in the same pLC41 vector as described for the *GRF4-GIF1* chimera. We also observed a significant increase in regeneration efficiency relative to the control (Figures 4 and 5). The increase in regeneration efficiency was lower than that observed in the transformation experiments with the chimeric *GRF4-GIF1*. All regenerated plants were confirmed as transgenic by PCR. We also confirmed that all regenerated plants overexpressed the corresponding transgene by qRT-PCR of T₁ plants

[00102] Example 3 – Transformation of other plant species or tissues using only *GRF4* or *GIF1*

[00103] In wheat, the *GRF4-GIF1* chimera resulted in significantly higher regeneration efficiency than either of the two genes alone. It is, however, possible that in other plant species or tissues transformation with only one of these two genes might be sufficient to improve regeneration efficiency to a similar level as for plants transformed with the chimera. For example, if one of these genes is expressed at sufficiently high levels in a particular plant, transformation with the other gene alone could be sufficient to achieve significantly higher regeneration efficiency in that plant.

[00104] Example 4 – Regeneration from leaf explants

[00105] Clones of five independent *GRF4-GIF1* T₀ wheat lines and one DsRed T₀ control line were generated and maintained in culture. Very young leaf blades were aseptically dissected under a stereomicroscope and the outer 1-2 whorls of leaves removed. The base of the inner laminae were removed and plated onto a callus induction medium containing auxins to induce callus formation. After 14 days, tissue was sub-cultured to fresh callus induction medium. After an additional 21 days, calli were transferred to shoot regeneration media containing cytokinin. The number of calli regenerating shoots was scored after 28 days.

[00106] Leaf explants transformed with the *GRF4-GIF1* chimera showed a regeneration efficiency of 55%, which was 2.8-fold higher than the regeneration efficiency in leaf explants transformed with constructs without the *GRF4-GIF1* chimera (Figure 6).

[00107] Example 5 - Regeneration of plants in suboptimal levels of exogenous cytokinins

[00108] In regular transformations, immature embryos were first inoculated with *Agrobacterium*, and then placed through different media containing different hormones to regenerate the transgenic plants. Initially, a medium containing auxin induces callus formation. Next, the calli are transferred to media containing cytokinin, which promotes shoot development (Figure 7A). Interestingly, we observed that embryos inoculated with *Agrobacterium* with the wheat *GRF4-GIF1* chimera (SEQ ID NO:5) were able to regenerate green shoots in the auxin media without being exposed to cytokinin (Figure 7B). Without being bound by any particular theory, a possible explanation for this result is the promotion of production of endogenous cytokinins by the *GRF4-GIF1* chimera. This observation indicates that the *GRF4-GIF1* chimera is able to promote embryogenesis and shoot regeneration without the addition of exogenous cytokinins, which can be used as a positive selection method to identify transgenic shoots without using antibiotic-based markers.

[00109] Example 6 – Constructing a miR396-resistant *GRF*

[00110] The results from Figure 6 show that increased expression of the *GRF4-GIF1* chimera improved regeneration efficiency from wheat leaf explants, which indicates the potential of this method for different plant species and tissues. However, in some plant species or in particular tissues of some species, high levels of miR396 can cleave the *GRF* RNA and may limit the expression of high levels of the *GRF* protein or the *GRF-GIF* chimera.

[00111] To avoid this potential problem, we generated a modified *GRF* gene (alone and in the *GRF-GIF* chimera, SEQ ID NO:34) which has silent mutations in the miR396 target site that render them less sensitive to the repression by miR396 (Figure 8). Mutations in the miR396 binding site in *Arabidopsis GRF3* (SEQ ID NO:19) or rice *GRF4* (SEQ ID NO:17) have been shown to result in higher *GRF* activity and increased organ size (Debernardi et al., 2014; Beltramino et al., 2018; Duan et al., 2015; Hu et al., 2015; Che et al., 2015; Sun et al. 2016; Li et al. 2018).

[00112] Example 7 – Regulation of expression using an inducible system

[00113] In wheat, we did not observe deleterious effects of the *GRF4-GIF1* chimera on the plant phenotype, likely because the presence of endogenous miR396 was sufficient to cleave any excess *GRF4* generated by expression of the transgene. However, it is possible that in other species the presence of a *GRF-GIF* chimera could affect the phenotype in undesirable ways. These effects might be more significant when using a *GRF* variants with mutations in the miR396 target site that render them less sensitive to the repression by miR396.

[00114] The ability to control the expression and/or activity of this chimera in plants by using an inducible system could address this possibility. Debernardi and Palatnik (patent application WO 2016/098027 A1) have demonstrated that in *Arabidopsis* the activity of a similar *GRF-GIF* chimera can be controlled exogenously by cloning a rat Glucocorticoid Receptor (GR) in the middle of *GRF-GIF*. The new *GRF-GR-GIF* chimera is activated only in the presence of the synthetic hormone dexamethasone. Using this strategy, we generated *GRF-GR-GIF* chimeras for *Vitis vinifera* (SEQ ID NO:31) and wheat (SEQ ID NO:32 and SEQ ID NO:33), which can be selectively induced only in the transformation media, and will not be active later in the transformed T₀ plants or in subsequent generations, avoiding potential effects on plant development.

[00115] Additionally, the inducible system would allow the use of hyperactive versions of the *GRF4-GIF1* chimera, while limiting potential effects on plant development. For example, the incorporation of synonymous mutations in the miR396 target site of the *GRF* eliminates the miR396-mediated posttranscriptional repression of the chimera, boosting its expression and probably its activity even in tissues where miR396 is expressed (e.g., leaves). Through the inducible system, the *GRF4-GIF1* chimera could be overexpressed only during regeneration. Another option is the use of tissue specific promoters.

[00116] Example 8 – Methods using protein delivery

[00117] It is possible to deliver proteins to plant cells. Targeted mutagenesis was obtained in many plant species by delivering CRISPR/Cas9 preassembled ribonucleoprotein complexes (RNPs) (Woo et al., 2015; Subburaj et al., 2016; Malnoy et al., 2016; Kim et al., 2017; Liang et al., 2017; Svitashchev et al., 2016; Wolter et al., 2017). The methods previously described could be performed by delivering *GRF*, *GIF* or *GRF-GIF* chimeric proteins into plants cells. As an example, *GRF*, *GIF* or *GRF-GIF* chimeras could be delivered together with CRISPR/Cas9 RNPs to boost the regeneration of the edited plant cells. After protein delivery, the plant tissues could then be cultured in regeneration media.

[00118] Example 9 – Transformation with additional vectors

[00119] Once a plant is transformed with a *GRF*, *GIF* or *GRF-GIF* chimera and acquires high regeneration ability, cells from this plant or its progeny could be further used for transient or stable transformation with vectors other than the *GRF*, *GIF* or *GRF-GIF* chimera vectors. The plant would still have high regeneration ability. The plant cells could then be cultured in regeneration media with either normal or reduced levels of cytokinins.

[00120] Example 10 – Transformation with other *GRF* and/or *GIF* sequences

[00121] The methods previously described could be performed with other *GRF* genes or related nucleotide sequences, e.g., in which the encoded amino acid sequences of QLQ and WRC domains of the *GRF* proteins are at least about 70%, about 80%, about 90%, or about 95% identical to the sequences of the QLQ and WRC domains of wheat proteins Ta*GRFGRF1* (SEQ ID NO:9), Ta*GRF2GRF2* (SEQ ID NO:10), Ta*GRF3* (SEQ ID NO:11), Ta*GRF4* (SEQ ID NO:12), or Ta*GRF5* (SEQ ID NO:13), or to *Arabidopsis* proteins At*GRF3* (SEQ ID NO:19), At*GRF4* (SEQ ID NO:20), At*GRF5* (SEQ ID NO:21), or At*GRF6* (SEQ ID NO:22).

[00122] The methods may also be performed using a *GRF* gene in which the encoded amino acid sequence of the QLQ and WRC domains is at least about 80% similar, about 90% similar, or about 95% similar to the sequences provided in the preceding paragraph.

[00123] The percent identity and similarity are calculated by BLASTP using only the concatenated QLQ (underlined below) and WRC domains (in bold below), in the protein sequences presented in this disclosure. An example of a concatenated QLQ-WRC domain (SEQ ID NO: 96) is presented below for wheat Ta*GRF4* (for which the complete protein sequence is SEQ ID NO:12):

>Ta*GRF4* TraesCS6A01G269600

PFTAAQYEELEHQALIYKYLVAGVSVPPDLVLP**IRDPEPGRCRRTDGKKWRCAKEAA**
SDSKYCERHMHGRNRSRKPVE

[00124] Pairwise comparison of the concatenated QLQ-WRC domains among the five wheat proteins are presented in Table 1. All pairwise comparisons are higher than 70% identical and 80% similar, confirming their similarity and potential overlapping functions.

[00125] As an additional example of the conservation of these proteins, we included the comparison between the QLQ-WRC domains from the wheat *GRF* proteins and the corresponding domains in the orthologous rice (*Oryza sativa*) proteins. All pairwise comparisons show >70% identity and >80% similar (Table 2). These criteria exclude distantly related *GRFs*, such as *OsGRFGRF10* (LOC_Os02g45570.1), *OsGRFGRF11* (LOC_Os07g28430.1), and *OsGRFGRF12* (LOC_Os04g48510.1).

[00126] As a reference for dicot species, we compared the concatenated QLQ-WRC domains from the closest *Arabidopsis* *GRF* protein sequences *AtGRF3* (SEQ ID NO:19), *AtGRF4* (SEQ ID NO:20), *AtGRF5* (SEQ ID NO:21), and *AtGRF6* (SEQ ID NO:22) (Table 3). Table 3 shows that these *Arabidopsis* proteins are at least 70% identical or 80% similar to at least one of the wheat *GRF* proteins. The relatively high level of similarity found between the critical domains of this group of proteins in highly divergent dicot and monocot plants confirms the conservation of these proteins and the potential overlap in their functions. This potential is also supported by the observation that higher activity of *Arabidopsis* *GRF3* (SEQ ID NO:19) or rice *GRF4* (SEQ ID NO:17) due to mutations in the site recognized by miR396 increase seed size in these two distantly related species (Beltramino et al., 2018; Duan et al., 2015; Hu et al., 2015; Che et al., 2015; Sun et al. 2016; Li et al. 2018).

Table 1. Comparison among five wheat (*Triticum aestivum*) paralogous proteins (TaGRF) included in this patent. The percent identity (first number) and percent similarity (second number) of concatenated QLQ-WRC conserved domains were obtained by BLAST P.

%ID/%Sim QLQ-WRC	TaGRF4	TaGRF3	TaGRF5	TaGRFGRF1
TaGRF3	94/96			
TaGRF5	86/91	90/92		
TaGRFGRF1	80/92	82/92	82/91	
TaGRF2GRF2	78/91	77/89	75/88	82/94

Table 2. Comparison between five wheat TaGRF proteins included in this patent (sequences are available at the end of the document) and the closest rice orthologs (*Oryza sativa*). The percent

identity and percent similarity of concatenated QLQ-WRC conserved domains were obtained by BLAST P.

%ID/%Sim	TaGRF4	TaGRF3	TaGRF5	TaGRFGRF1	TaGRF2GRF2
QLQ-WRC					
OsGRF4	95/96				
OsGRF3		97/97			
OsGRF5			96/97		
OsGRFGRF1				96/98	
OsGRF2GRF2					87/96

Table 3. Comparison between five wheat TaGRF proteins included in this patent and the closest *Arabidopsis thaliana* homologs (AtGRF). The percent identity and percent similarity of concatenated QLQ-WRC conserved domains were obtained by BLAST P. Sequences are available at the end of this document.

%ID/%Sim	TaGRF4	TaGRF3	TaGRF5	TaGRFGRF1	TaGRF2GRF2
QLQ-WRC					
AtGRF3	72/89	74/85	73/83	71/85	72/87
AtGRF4	69/89	71/87	70/83	67/84	71/86
AtGRF5	75/88	76/88	76/89	79/91	72/86
AtGRF6	72/84	76/84	76/84	73/88	71/83

[00127] Likewise, the methods of this disclosure could be performed with other *GIF* genes or related nucleotide sequences, e.g., in which the encoded amino acid sequences of SNH domains of the *GIF* proteins are at least about 70% identical, about 80%, about 90%, or about 95% to the sequences of the SNH domains of wheat protein TaGIF1 (SEQ ID NO:27), TaGIF2 (SEQ ID NO:28), or TaGIF3 (SEQ ID NO:29), or to the *Arabidopsis* proteins AtGIF1 (SEQ ID NO:24), AtGIF2 (SEQ ID NO:25), or AtGIF3 (SEQ ID NO:26).

[00128] The methods may also be performed using a *GIF* gene in which the encoded amino acid sequence of the SNH domain is at least about 80% similar, about 90% similar, or about 95% similar to the sequences provided in the preceding paragraph.

[00129] A mutation in *Arabidopsis GIF1*, also known as *ANGUSTIFOLIA3 (AN3)*, causes smaller, narrower leaves and petals, with a reduced number of cells, similar to the phenotypes seen in multiple *grf* mutants (Kim and Kende 2004; Horiguchi et al. 2005). Single *gif2* and *gif3* mutants are similar to wild type plants, suggesting that *GIF1* has a larger impact on leaf development than other members of the *GIF* family (Lee et al. 2009). However, the phenotypes of double mutants *gif1,2* or *gif1,3* and of the triple mutant *gif1,2,3* are more severe, indicating that *GIF* genes have redundant functions (Lee et al. 2009). In addition, ectopic overexpression of all three *GIFs* can rescue a mutation in *GIF1* (Lee et al. 2009).

[00130] Based on the overlapping *GIF* functions described above, we predict that different *GIF* paralogs in wheat can have similar effects on the improvement of regeneration efficiency of transgenic plants either alone or as protein chimeras with different *GRFs*. Therefore, we include in this application *Arabidopsis* proteins At*GIF1* (SEQ ID NO:24), At*GIF2* (SEQ ID NO:25), At*GIF3* (SEQ ID NO:26) as well as the three closest wheat homologous proteins Ta*GIF1* (SEQ ID NO:27), Ta*GIF2* (SEQ ID NO:28), and Ta*GIF3* (SEQ ID NO:29), and any other plant protein that shows at least 70% identity or 80% similarity for the conserved SNH domain highlighted in the protein sequences described at the end of this document. Comparisons among the SNH domains of the three wheat *GIF* proteins showed identities of at least 70% and similarities of at least 89% (Table 4).

Table 4. Comparison among the three wheat (*Triticum aestivum*) paralogous proteins (Ta*GIF*) included in this patent. The percent identity and percent similarity of the conserved SNH domain were obtained by BLAST P.

%ID/%Sim SNH	TaGiF2	TaGIF3
TaGIF1	75/92	70/89
TaGIF2		95/98

[00131] When the wheat SNH domains were compared with the corresponding SNH domains from *Arabidopsis*, identities varied between 66% and 91% and similarities between 89 and 96% (Table 5). Each *Arabidopsis* SNH domain was at least 70% identical or 80% similar to at least one of the three wheat SNH domains (Table 5).

Table 5. Comparison between the three wheat TaGIF proteins included in this patent (sequences above) and the closest *Arabidopsis thaliana* homologs (AtGIF). The percent identity and percent similarity of the conserved SNH domain were obtained by BLAST P.

%ID/%Sim SNH	TaGIF1	TaGIF2	TaGIF3
AtGIF1	73/89	66/89	65/89
AtGIF2	71/90	89/96	84/89
AtGIF3	72/90	91/96	83/91

[00132] Example 11 – Use in combination with genome editing

[00133] The methods provided in this disclosure may be ideal for use in combination with genome editing since the transgene can be segregated-out after the editing events are completed. This eliminates the risk that the *GRF-GIF* chimera would negatively affect the plant phenotype. The high transformation efficiency of the disclosed methods could expand the list of plant varieties that would benefit from CRISPR-screens to test panels of mutants in the regulatory and coding regions of important agronomic genes. We have confirmed high regeneration efficiency (Figure 9A) of embryos transformed with vector JD635 (*GRF4-GIF1*-gRNA-GeneQ) including the *GRF4-GIF1* chimera, Cas9 and a gRNA targeting Gene Q (Figure 9B). We tested 32 callus from this transformation and confirmed editing of the target gene in 30 of them (Fig. 9C).

[00134] Example 12 - Selection of transgenic plants in absence of selectable markers

[00135] The methods disclosed herein may be used to avoid the use of selectable markers, which are objected to by people opposed to the use of transgenic plants. The ability of the *GRF4-GIF1* chimera to regenerate in the absence of cytokinins demonstrates that transgenic cells can be selected from non-transgenic ones by simply eliminating or greatly reducing the concentration of cytokinins. Figure 10 shows calluses from the same JD635 construct presented in Figure 9 (*GRF4-GIF1*-Cas9-gRNA-GeneQ) grown in a medium without hygromycin and with one-tenth of the

normal cytokinin concentration. Multiple regenerating shoots were observed in the presence of JD635 (Figure 10A, green arrows) and no regeneration was observed in the control (Figure 10B) transformed with a DsRed (without the *GRF4-GIF1* chimera).

[00136] Materials and methods for examples 1 to 12.

[00137] Vectors. All cloning PCRs were performed with Phusion High-Fidelity DNA Polymerase (NEB). Cloning reactions were done using the Gateway Cloning technology (Invitrogen). RNA was extracted from spikes using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), treated with RQ1 RNase-free DNase (Promega), and then cDNA synthesis was carried out using SuperScript II Reverse Transcriptase (Invitrogen). To clone the coding region of wheat *GRF4* encoding the protein described in (SEQ ID NO:12) and *GIF1* encoding the protein described in (SEQ ID NO:27), PCRs were performed on the cDNA generated from Kronos spike. The sequence of the primers specific for *GRF4* (Fw-*GRF4a*/Rev-*GRF4a*) and *GIF1* (Fw-*GIF1a*/Rev-*GIF1a*) are indicated in Table 6.

[00138] The PCR fragments were first cloned in pDONR by a B/P gateway reaction and transformed into chemical competent *E. coli* DH5 α . The sequence of the clones was confirmed by Sanger sequencing. The *GRF4-GIF1* chimera was generated by overlapping PCR. In a first step, *GRF4* and *GIF1* coding sequences were amplified with primers FW-*GRF4a*/Rev-*GRF4b* and Fw-*GIF1b*/Rev-*GIF1b* from the pDONR-*GRF4* and pDONR-*GIF1* clones as template. The primer Rev-*GRF4b* generates a 3' end that overlaps 12 nucleotides with the 5' end of Fw-*GIF1b*. Those 12 nucleotides generate a bridge of four alanine amino acids between *GRF4* and *GIF1*. Both PCR fragments were gel-purified and used as template in a second PCR with the primers Fw-*GRF4*/Rev-*GIF1b*. The sequence of the primers are indicated in Table 6. The resulting product was cloned in pDONR by B/P gateway reaction and transformed into chemical competent *E. coli* DH5 α . The sequence of the vector was validated by Sanger sequencing. Next, the *GRF4*, the *GIF1* and the chimera *GRF4-GIF1* genes were cloned in the binary vector pLC41 by a L/R gateway reaction under the maize UBIQUITIN promoter and transformed into chemical competent *E. coli* DH5 α . The resulting vectors for the individual genes pLC41:*GRF4* (SEQ ID NO:1) and pLC41:*GIF1* (SEQ ID NO:2), and for the chimera pLC41:*GRF4-GIF1* (SEQ ID NO:5) were verified by restriction digestion and transformed by electroporation in *Agrobacterium* EHA105.

[00139] The *Vitis vinifera* *VviGRF4-GIF1* chimera (DNA, SEQ ID NO:6), Protein, SEQ ID NO:7) was generated by gene synthesis. The DNA fragment was cloned into pDONR by B/P

gateway reaction and transformed into chemical competent *E. coli* DH5 α . The sequence of the vector was validated by Sanger sequencing. Next, the *Vitis* chimera *GRF4-GIF1* was cloned in the binary vector pGWB14 binary vector by a L/R gateway reaction under the viral 35S promoter and transformed into chemical competent *E. coli* DH5 α . The resulting vector pGWB14-Vvi*GRF4-GIF1* (DNA, SEQ ID NO:8) was verified by restriction digestion and transformed by electroporation in agrobacterium EHA105. A dexamethasone inducible version Vvi*GRF4-GR-GIF1* (SEQ ID NO:31) was generated by overlapping PCR.

[00140] The wheat dexamethasone inducible *GRF4-GR-GIF1* chimera encoding protein (SEQ ID NO:32) was generated by overlapping PCR. In a first step, *GRF4*, *GIF1* and a rat Glucocorticoid Receptor (GR) were amplified with primers FW-*GRF4a*/Rev-*GRF4b*, Fw-*GIF1-GR* /Rev-*GIF1b*, Fw-GR/Rev-GR. The primer Rev-*GRF4b* generates a 3' end that overlap 12 nucleotides with the 5' end of Fw-GR, and the Rev-GR generates a 3' end that overlap 12 nucleotides with the 5' end of Fw-*GIF1-GR*. Those 12 nucleotides generate a bridge of four alanine amino acids between *GRF4* and GR and GR and *GIF1*. The three PCR fragments were gel-purified and used as template in a second PCR with the primers Fw-*GRF4*/Rev-*GIF1b*. The sequence of the primers are indicated in Table 6. The resulting product was cloned in pDONR by B/P gateway reaction and transformed into chemical competent *E. coli* DH5 α . The sequence of the vector was validated by Sanger sequencing. Next, the chimera *GRF4-GR-GIF1* was cloned in the binary vector pLC41 by a L/R gateway reaction under the maize *UBIQUITIN* promoter and transformed into chemical competent *E. coli* DH5 α . The resulting vector pLC41:*GRF4-GR-GIF1* (SEQ ID NO:33) was verified by restriction digestion and transformed by electroporation in *Agrobacterium* EHA105.

[00141] A miR396-resistant version of wheat *GRF4-GIF1* (r*GRF4-GIF1*) (SEQ ID NO:34) was generated by overlapping PCR. In a first step, two PCR were performed with primers Fw-*GRF4a*/r*GRF*-Rev and r*GRF*-Fw/Rev-*GIF1b* using pLC41-*GRF4-GIF1* clone as template. The primers r*GRF*-Fw and r*GRF*-Rev overlap in 17 nt and introduce silent mutations in the miR396 target site. Both PCR fragments were gel-purified and used as template in a second PCR with the primers Fw-*GRF4*/Rev-*GIF1b*. The sequence of the primers are indicated in Table 6. The resulting product was cloned in pDONR by B/P gateway reaction and transformed into chemical competent *E. coli* DH5 α . The sequence of the vector was validated by Sanger sequencing. Next, the chimera r*GRF4-GIF1* was cloned in the binary vector pLC41 by a L/R gateway reaction under the maize

UBIQUITIN promoter and transformed into chemical competent *E. coli* DH5 α . The resulting vector pLC41:rGRF4-GIF1 was verified by restriction digestion and transformed by electroporation in *Agrobacterium* EHA105. A dexamethasone inducible version rGRF4-GR-GIF1 was generated by overlapping PCR.

[00142] To generate the JD635-GRF4-GIF1-Cas9- gRNA-GeneQ vector, a cassette including the maize *UBIQUITIN* promoter, the *GRF4-GIF1* chimera and the Nos terminator was amplified by PCR. The PCR product was gel-purified and cloned by In-fusion (Takara Bio USA, Inc.) into the *AscI* site of the pYP25F binary vector containing a wheat codon optimized Cas9 (TaCas9) and transformed into chemical competent *E. coli* DH5 α . The vector sequence was validated by Sanger sequencing. Next, a guide RNA construct targeting the coding region of Gene Q was cloned by Golden Gate reaction into two *AarI* sites of the vector and transformed into chemical competent *E. coli* DH5 α . The resulting JD635-GRF4-GIF1-Cas9-gRNA-GeneQ vector (SEQ ID NO:35) was validated by Sanger sequencing and transformed by electroporation into *Agrobacterium* EHA105.

[00143] Control vectors: The JD518 vector, in pLC41 backbone, contains the GLOSSY promoter driving the expression of LhG4, which is an artificial transcription factor with no targets in wheat. The pOp:FT1 vector, in pLC41 backbone, contains the FT1 coding sequence cloned downstream of pOp, which is an artificial promoter. The pLC41-DsRed in pLC41 backbone, contains the DsRed coding sequence cloned downstream of maize *UBIQUITIN* promoter.

[00144] Wheat transformation. Transgenic wheat plants were initially generated using technology from Japan Tobacco (JT) technology. However, cytokinin concentrations were reduced in some of the later experiments. Two independent experiments were performed for each vector. In each experiment, at least 25 immature embryos from Kronos were transformed using *Agrobacterium* EHA105 with the pLC41 constructs described before. Selection of transgenic plants was conducted using hygromycin, except in the experiments with reduced cytokinins (Figure 10) where no hygromycin or any other antibiotic was used. Regenerated plants were transferred to soil and transgene insertion was validated by DNA extraction and PCR. The primers used to genotype the T₀ plants are indicated in Table 6.

Table 6. Sequence of primers used in cloning and genotyping.

Cloning		GGGGacaagtttgtacaaaaagcTGCCACCATGGCGATGCCGTATG
pDONOR	Fw-GRF4a	CCTCT (SEQ ID NO: 106)

Overlapping PCR	Rev-GRF4a	GGGGACCACTTTGTACAAGAAAGCTGAACGGTACATYTC GCCGGCGAACAG (SEQ ID NO: 107)
	Fw-GIF1a	GGGGacaagtttgtaaaaaaagcTGCCACCATGCAGCAGCAACACC TGATG (SEQ ID NO: 108)
	Rev-GIF1a	GGGGACCACTTTGTACAAGAAAGCTGAACGGCTTCCTTC CTCCTCGGT (SEQ ID NO: 109)
	Fw-GRF4a	GGGGacaagtttgtaaaaaaagcTGCCACCATGGCGATGCCGTATG CCTCT (SEQ ID NO: 110)
	Rev-GRF4b	GGCAGCGGCCGCGTACATYTCGCCGGCGAACAG (SEQ ID NO: 111)
	Fw-GIF1b	gcggccgctgccATGCAGCAGCAACACCTGATG (SEQ ID NO: 112)
	Rev-GIF1b	GGGGACCACTTTGTACAAGAAAGCTGAACGCTAGCTTCC TTCCTCCTCGGT (SEQ ID NO: 113)
	Fw-GIF1- GR	<u>aagcTgccgcGgcc</u> ATGCAGCAGCAACACCTG (SEQ ID NO: 114)
	Fw-GR	<u>TACgcggccgctgccgaagctcgaaaaacaaag</u> (SEQ ID NO: 115)
	Rev-GR	<u>CATGGCCGCGGCAGCTTTTTGATGAAACAGAAG</u> (SEQ ID NO: 116)
Genotyping	rGRF-Fw	<u>TCTAGAAAACCGGTCGAAACGCAGCTCG</u> (SEQ ID NO: 117)
	rGRF-Rev	TCGACCGGTTTTCTAGAACGGTTGCGG (SEQ ID NO: 118)
	Fw-GRF4b	CCTCCGACTCCAAGTATTGC (SEQ ID NO: 104)
	Rev-GIF1c	ATCATCAGGTTGGACGGGTA (SEQ ID NO: 105)

[00145] Plant growth conditions. Transgenics plants were grown in PGR15 growth chambers (Convion) adjusted to 16 h of light (22°C) and 8 h of darkness (18 °C). Intensity of the sodium halide lights measured at plant heads height was ($\sim 260 \mu\text{M m}^{-2} \text{s}^{-1}$).

[00146] Phylogenetic analysis. The amino acid sequence of the *GRF* and *GIF* genes from *Arabidopsis* and rice were obtained from phytozome (phytozome.jgi.doe.gov/pz/portal.html). The

amino acid sequences of wheat *GRF* and *GIF* genes were obtained from the wheat genome RefSeq v1.0. The phylogenetic analysis was performed using MEGA6.

[00147] Example 13 - Transformation of wheat using single *GRF4* or *GIF1* genes

[00148] Wheat transformation experiments were performed using *GRF4-GIF1* chimera (SEQ ID NO:5), *GRF4* (SEQ ID NO:1) or *GIF1* (SEQ ID NO:2) alone or within the same construct (FIG. 18A). Across 14 experiments (Table 7), the regeneration efficiency of the *GRF4-GIF1* chimera ($65.8 \pm 5.3\%$) was >7-fold higher than the empty vector control ($8.7 \pm 2.0\%$, $P < 0.0001$, Figure 18B). In five separate transformation experiments (Table 7), we observed significantly lower regeneration efficiencies in embryos transformed with the *GRF4* gene alone ($20.4 \pm 11.4\%$) or the *GIF1* gene alone ($17.2 \pm 6.6\%$) relative to the *GRF4-GIF1* chimera ($54.6 \pm 9.8\%$, Tukey $P < 0.05$, FIG. 18C). The regeneration efficiency of the calli transformed with the individual genes was approximately 3-fold higher than the control ($6.0 \pm 3.0\%$) but the differences were not significant in the Tukey test (FIG. 18C).

[00149] We then compared the effect on regeneration efficiency of having the *GRF4* and *GIF1* fused in a chimera or expressed separately within the same construct by individual *Ubi* promoters (not fused, SEQ ID NO: 36) (FIG. 18A, Table 7). In five different experiments, the average regeneration efficiency of the separate *GRF4* and *GIF1* genes ($38.6 \pm 12.9\%$) was significantly lower ($P < 0.0144$) than the regeneration efficiency with the *GRF4-GIF1* chimera ($62.6 \pm 10.3\%$, FIG. 18D). This result demonstrated that the forced proximity of the two proteins in the chimera increased its ability to induce regeneration.

[00150] Example 14 – Wheat transformation with chimeras including other *GRF* and/or *GIF* sequence combinations

[00151] The *GRF* and *GIF* gene families contain several members (FIG. 19A, B), which display overlapping functions and redundancies. Therefore, we expected that other *GRFs* and/or *GIFs* besides *GRF4* and *GIF1* could also promote regeneration.

[00152] We tested *GRF* genes from different clades (FIG. 19A), including *GRF5* (SEQ ID NO:13, 37) that is in the same clade as *GRF4*, *GRFGRF1* (SEQ ID NO:9, 38) that is in the closest clade (which includes Arabidopsis *GRF5*, SEQ ID NO: 21), and *GRF9* (SEQ ID NO:39) that is in a more distal clade. Pairwise comparison of concatenated QLQ-WRC domains among eight wheat proteins, including the four tested in this patent, are presented in Table 8. All pairwise comparisons

for *GRFGRF1-GRF6* and *GRF9* are $\geq 70\%$ identical or $>80\%$ similar, confirming their similarity and potential overlapping functions.

[00153] We generated chimeras for all the selected *GRFs* with *GIF1* (FIG. 20A) (SEQ ID NO: 40-42) and tested transformation frequencies in Kronos. The regeneration efficiency induced by chimeras including the closely related *GRF4* and *GRF5* genes fused with *GIF1*, was significantly higher than the regeneration observed for chimeras including the more distantly related *GRFGRF1* and *GRF9* genes fused with *GIF1* (contrast $P = 0.0368$, FIG. 20B). Interestingly, chimeras including *GRFGRF1* and *GRF9* showed higher regeneration frequencies than the control, but the differences were not significant (Table 7). Therefore, *GRF* from different clades can promote regeneration, but *GRFs* from the *GRF4*-clade appear to have higher regeneration activity.

[00154] Next, we compared the activity of the three wheat *GIF* genes included in the phylogenetic tree (FIG. 19B) by generating chimeras with *GRF4* (FIG. 20C). Like *GIF1*, chimeras including *GIF2* (SEQ ID NO: 28, 43, 45) and *GIF3* (SEQ ID NO: 29, 44, 46) also increased regeneration frequency compared to calli transformed with the empty vector (FIG. 20D, Table 7). However, the *GRF4-GIF1* combination resulted in higher regeneration efficiency than the *GRF4-GIF2* and *GRF4-GIF3* combinations (contrast $P = 0.0046$), and all three chimeras showed higher regeneration efficiency than the control (Tukey test $P < 0.05$).

[00155] Example 15. *GRF4-GIF1* improves transformation of wheat and Triticale cultivars with low regeneration efficiency or recalcitrant.

[00156] We then tested the potential of the *GRF4-GIF1* chimera to generate transgenic plants from commercial durum, bread wheat and Triticale lines that were previously recalcitrant to *Agrobacterium*-mediated or had low regeneration efficiency at the UCD Plant Transformation Facility (FIG. 21). With the *GRF4-GIF1* chimera we observed a 20 to 25-fold increase in regeneration frequencies in tetraploid wheat elite line Desert King ($63.0 \pm 17.0\%$ vs. $2.5 \pm 2.5\%$) and hexaploid wheat Fielder ($54.0 \pm 4.0\%$ vs. $2.5 \pm 2.5\%$) relative to the control. For the hexaploid wheat varieties Hahn and Cadenza and Triticale breeding line UC3190, for which we were not able to generate transgenic plants before, we observed regeneration frequencies of 9 to 19% with the *GRF4-GIF1* chimera versus 0% with the control (FIG. 21 and Table 9A, B).

[00157] Example 16. Wheat *GRF4-GIF1* improves transformation of rice variety Kitaake.

[00158] We also tested the wheat *GRF4-GIF1* chimera in the rice variety Kitaake. In two independent transformation experiments, we observed a 2 to 3-fold increase in rice regeneration

efficiency in the calli transformed with the wheat *GRF4-GIF1* chimera (average 40.1 ± 5.4 %) compared with those transformed with the control vectors (17.6 ± 5.9 %, Table 9C). These results suggest that the wheat *GRF4-GIF1* chimera is effective in enhancing regeneration in another agronomically important monocotyledonous species.

[00159] Example 17 – Regulation of expression using an inducible system

[00160] We generated an inducible version of the wheat *GRF4-GIF1* chimera by cloning a rat Glucocorticoid Receptor (GR) in the middle of *GRF4-GIF1* (SEQ ID NO:32, 33) (FIG. 22A). This chimera is activated only in the presence of the synthetic hormone dexamethasone (Dex). Immature embryos from Kronos were transformed using this vector, and then plated in regular transformation media or in media supplemented with 10 μ M DEX. In the absence of DEX we recovered 1 shoot out of 23 embryos, while in the presence of DEX 4 shoots out of 24 embryos were recovered (FIG. 22B). This result indicated that a *GRF-GR-GIF* chimera is functional and can be controlled exogenously by the addition of DEX.

[00161] Example 18 - Regeneration of transgenic plants without exogenous cytokinins

[00162] In many plant transformation systems cytokinins are required to regenerate shoots (FIG. 23A). Interestingly, we observed that embryos inoculated with *Agrobacterium* transformed with the wheat *GRF4-GIF1* chimera were able to rapidly regenerate green shoots in auxin media without cytokinin (FIG. 23B). We then tested the regeneration efficiency of immature embryos from stable *GRF4-GIF1* transgenics (n=27) and non-transgenic (n=26) T₁ sister lines in the absence of cytokinin and hygromycin. Under these conditions, the regeneration efficiency of the *GRF4-GIF1* transgenic plants (77.8 %) was significantly higher than the non-transgenic sister lines (11.5 %, FIG. 24). These results indicated that the *GRF4-GIF1* chimera can promote embryogenesis and shoot regeneration in wheat without the addition of exogenous cytokinin.

[00163] Based on the previous results, we developed a protocol to select transgenic shoots in auxin media without using antibiotic-based markers. In three experiment we recovered 40 shoots using a *GRF4-GIF1* marker-free vector (SEQ ID NO:47) and 15 for the empty vector. Genotyping revealed that 10 out of the 40 (25 %) *GRF4-GIF1* shoots were transgenic, while none of the control was positive (FIG. 23C). These high-regenerating transgenic plants overexpressing the *GRF4-GIF1* chimera without selection markers can be used for future transformation experiments to incorporate other genes using selectable markers. This strategy generates separate insertion sites

for the *GRF4-GIF1* and the second transgene, which facilitates the segregation of the *GRF4-GIF1* insertion in the next generation.

[00164] Example 19. *GRF4-GIF1* accelerates wheat transformation.

[00165] The wheat *GRF4-GIF1* chimera accelerates the regeneration process, which allowed us to develop a faster wheat transformation protocol that takes 56 days instead of the 91 days required for all the wheat experiments presented in this manuscript (FIG. 25).

[00166] Example 20. *GRF-GIF* improves transformation of dicot species.

[00167] We performed a series of *Citrus* transformation experiments to test the effect of the *GRF-GIF* technology in a dicot crop with limited regeneration efficiency. We generated heterologous citrus (SEQ ID NO:48) and grape (SEQ ID NO: 6-8) *GRF4-GIF1* chimeras using the closest homologs to wheat *GRF4* (citrus *Ciclev10032065m* and grape *GSVIVT01024326001*), and *GIF1* (citrus *Ciclev10022144m* and grape *GSVIVT01036262001*), in both species (FIG. 19A and B). In three independent transformation experiments in the citron rootstock Carrizo, epicotyls were transformed with the citrus and the grape *GRF4-GIF1* chimeras. Epicotyls transformed with the citrus *GRF4-GIF1* chimera showed a 4.8-fold increase in regeneration frequency relative to those transformed with the empty vector control (FIG. 26A and Table 10). The heterologous grape *GRF4-GIF1* chimera produced similar increases in citrus regeneration efficiency as the citrus chimera (FIG. 26B and Table 10).

[00168] We also tested the effect of a miR396-resistant grape *GRF4-GIF1* version (henceforth, *rGRF4-GIF1*, SEQ ID NO:50), in which we introduced silent mutations in the *GRF* binding site for miR396 to avoid cleavage (FIG. 26B-C). In three independent experiments, we observed that the grape *rGRF4-GIF1* chimera produced the highest frequency of transgenic events (8.4-fold increase compared to the control, $P < 0.05$). A statistical analysis comparing the control versus the three combined *GRF-GIF* constructs was also significant ($P = 0.0153$, FIG. 26D and Table 10). In spite of its higher-regeneration frequency, the *rGRF-GIF* construct would require additional optimization (e.g. an inducible system) because some of the transgenic events produced large calli that were unable to generate shoots (FIG. 26B).

[00169] We tested the grape *rGRF4-GIF1* chimera in grape transformation. The grape transformation process is slow and very laborious requiring 8 to 12 months for generating transgenic plant lines. Pro-embryogenic callus generated from anther filaments of immature flowers were inoculated with agrobacterium harboring the empty vector and the *rGRF4-GIF1*

vector. After 6 month of culture we observed that 87% of calli transformed with *rGRF4-GIF1* regenerated shoots, while only 12% of the control calli produce shoots (FIG. 27). Interestingly, in contrast to citrus *rGRF4-GIF1* transgenics, all grape *rGRF4-GIF1* developed shoots that produced leaves.

[00170] We then tested the *GRF-GIF* technology in pepper (*Capsicum annuum*) transformation using the cultivar “R&C cayenne”. In *Capsicum annuum*, there are two close paralogs of *GRF4*, designated here as *GRF4.1* and *GRF4.2*, and two close paralogs of *GIF1*, designated here as *GIF1.1* and *GIF1.2*. We used the *GRF4.1* (LOC107869915) and *GIF1.1* (LOC107870303) paralogs in the construction of the *GRF4.1-GIF1.1* chimera (SEQ ID NO: 138-139), which was cloned into the binary vector pEarleyGate100 that carries BAR selection. For the transformation experiment, we used the basal portion of the cotyledons proximal to the shoot apex, with the petiole still attached. The 40 cotyledon pieces transformed with the pepper *GRF4.1-GIF1.1* chimera showed a 23.8% regeneration efficiency, which was 4.76-fold higher than the regeneration efficiency of cotyledon pieces transformed with the empty vector pEarleyGate100 (5.0% regeneration efficiency, Table 11, FIG. 28).

[00171] Methods for examples 13 to 20.

[00172] Method 1. Vectors used in the transformation experiments.

[00173] *Wheat vectors.* To generate the vector expressing both *GRF4* and *GRFGRF1* but not fused (Ubi::*GRF4*-term Ubi::*GIF1*-term, SEQ ID NO: 36), the complete Ubi::*GRF4*-term cassette was amplified by PCR using pLC41:*GRF4* (SEQ ID NO:1) as template with primers

[00174] Fw_HindIII gccactcagcaagctttgcagcgt (SEQ ID NO: 119) and

[00175] Rev-HindIII TCACGCTGCAAAGCTCTAATTCCCGATCTAGTAAC (SEQ ID NO: 120). We cloned the PCR fragment in pGEMT-easy and we sub-cloned the Ubi::*GRF4*-term fragment into the *HindIII* site of pLC41:*GIF1* (SEQ ID NO:2).

[00176] To generate the different wheat *GRF-GIF* chimeras, the coding sequences of *GRFGRF1* (SEQ ID NO: 38), *GRF5* (SEQ ID NO:37), *GRF9* (SEQ ID NO: 39), *GIF2* (SEQ ID NO: 43) and *GIF3* (SEQ ID NO: 44) were obtained by gene synthesis. Then, the different chimeras (*GRFGRF1-GIF1*, *GRF5-GIF1*, *GRF9-GIF1*, *GRF4-GIF2*, *GRF4-GIF3*) were generated by overlapping PCR following the same strategy described to generate *GRF4-GIF1*. We cloned all

the chimeras in pLC41 vector by L/R reaction and verified the vectors by restriction digestion and transformed by electroporation in *Agrobacterium* strain EHA105.

[00177] We generated the citrus *GRF4-GIF1* chimera (SEQ ID NO: 48) by gene synthesis and cloned in pDONR. After checking the sequence by Sanger sequencing, we cloned it by L/R reaction into pGWB14 binary vector under the viral 35S promoter and transformed it into chemical competent *E. coli* DH5 α . We verified the resulting vector pGWB14-citrus *GRF4-GIF1* (DNA, SEQ ID NO:49) by restriction digestion and transformed by electroporation in *Agrobacterium* EHA105.

[00178] We generated a miR396-resistant version of *Vitis GRF4-GIF1* (*rGRF4-GIF1*) by overlapping PCR. Two PCR reactions were performed with primers

[00179] Fw-*GRF* GGGGacaagttgtacaaaaaagcTGCCACCATGAAGCAAAGCTTTGTGG (SEQ ID NO: 121)

[00180] /*rGRF*-Rev TCGACCGGTTTTCTAGAACGGTTGCGG (SEQ ID NO: 122) and

[00181] *rGRF*-Fw TCTAGAAAACCGGTCGAATCACAACTA (SEQ ID NO: 123)

[00182] /Rev-*GIF*

GGGGACCACTTTGTACAAGAAAGCTGAACGTCAATTCCCATCTTCAGCA (SEQ ID NO: 124) using pGBW14-*vitis GRF4-GIF1* clone as template (SEQ ID NO: 8). The primers *rGRF*-Fw and *rGRF*-Rev overlap in 17 nucleotides and introduce silent mutations in the miR396 target site (FIG. 26C). Both PCR fragments were gel-purified and used as template in a second PCR with the primers Fw-*GRF*/Rev-*GIF*. We cloned the resulting product in pDONR by B/P gateway reaction. Next, we cloned the chimera *rGRF4-GIF1* in the binary vector pGWB14 by a L/R gateway reaction under the viral 35S promoter. The resulting vectors were transformed by electroporation in *Agrobacterium* strain EHA105.

[00183] We generated the pepper *GRF4.1-GIF1.1* chimera (SEQ ID: NO: 140) by gene synthesis and cloned it into the binary vector pEarleyGate100 under the 35S promoter. pEarleyGate100 has a BAR selection marker. We transformed the construct, designated pTH1903, in *Agrobacterium* strain EHA105 by electroporation. As a control, we used the empty vector pEarleyGate100.

[00184] Plant transformation

[00185] **Method 2. Wheat transformation protocol.** Wheat transformation followed previously published protocols. Briefly, we grew the different wheat and triticales cultivars in a

growth chamber under long-day photoperiod (16 h of $380 \mu\text{M m}^{-2} \text{s}^{-1}$ light, 26 °C day and 18 °C night). We harvested immature grains from spikes approximately 2 weeks after anthesis, and surface sterilized for 1 minute in 70 % ethanol followed by 10 minutes in 1.2% (v/v) sodium hypochlorite solution plus 5 μl tween. After surface sterilization, we washed the seeds three times with sterilized water and isolated immature embryos under stereoscopic microscope.

[00186] We centrifuged the isolated immature embryos in liquid medium and then inoculated with *Agrobacterium*. We transferred the embryos to co-cultivation medium with the scutellum-side up and incubated at 23 °C in the dark. After 2-3 days, we excised the embryo axis, and transferred them to callus induction medium without selection, where we incubated them at 25 °C in the dark. After 5 days, we transferred the embryos to selection medium with 30 mg/l of hygromycin and incubated them at 25 °C in the dark.

[00187] After 3 weeks, we transferred the calli to selection medium that contained 100 mg/l of hygromycin. After an additional 3 weeks, we transferred the proliferating tissue to regeneration medium containing 50 mg/l of hygromycin and incubated them at 25 °C under continuous light ($30 \mu\text{M m}^{-2} \text{s}^{-1}$) for 2 weeks. We transferred the regenerated shoots into rooting medium contained 50 mg/l of hygromycin. Rooted plants were acclimated to soil by transferring them to a 1020 tray containing a 36 sheet inserts filled with Sunshine potting mix and covered with an 11 x 21 x 2 inch clear plastic dome for 10 days under 16 hour of 100 μM light and 26 °C. More recently, we developed a shorter transformation protocol to generate *GRF4-GIF1* transgenic wheat plants that is summarized in FIG. 25.

[00188] Method 3. Rice transformation protocol. Rice transformation followed previously published protocols (Ishida et al. 2015, Proc. 12th Int. Wheat Genet. Symp. 167-173). Briefly, we selected fresh rice seeds, de-husked them and surface sterilized them in a rotating flask containing 20 % (v/v) bleach for 30 min. Then, rinsed the seeds 3 times with sterile water. We placed about 25-50 seeds per plate on callus induction media (MSD, 1x Murashige and Skoog with vitamins medium containing 30 g/l sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid, 1.2% (w/v) agar, pH 5.6-5.8) without letting the embryo touch the media, wrapped plate with surgical tape and incubated under 16 h light/ 8 h dark at 28 °C. After 10-14 d, we separated the callus from the rest of the germinating seed and transferred to fresh MSD agar plates for another 5 d before co-cultivation.

[00189] *Agrobacterium culture:* We prepared a glycerol freezer stock from a single bacterial colony isolated from a plate. We then inoculated 1 ml LB containing the appropriate antibiotics to maintain the *Agrobacterium* and the plasmid, and we incubated it overnight at 28 °C at 250 rpm. The following day we added 300 µl of the *Agrobacterium* culture to 20 ml TY (pH 5.5) containing the appropriate antibiotics and 200 µM acetosyringone. We incubated the culture 28 °C for in a shaking incubator set at 250 rpm until the culture reached an OD₆₀₀ between 0.1 - 0.2 (approximately 2-4 h).

[00190] *Transformation and co-cultivation:* We placed the calli in *Agrobacterium* suspension for 30 min and shook the suspension to ensure uniform access to the calli. After the shaking incubation, we dried the calli on sterile Whatman paper to remove excess bacterial suspension. We transferred the calli onto co-cultivation medium (MSD + S + AS, 1x Murashige and Skoog with vitamins medium containing 30 g/l sucrose, 5% sorbitol, 2mg/l 2,4-dichlorophenoxyacetic acid, 200 µM acetosyringone, 1.6 % (w/v) agar, pH 5.6-5.8) and incubated for 3 d in the dark at 22 °C.

[00191] *Selection:* We transferred the co-cultivated calli to selection media (MSD + CH + PPM, 1x Murashige and Skoog with vitamins medium containing 30 g/l sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid, 400 mg/L carbenicillin, 200 mg/l timentin, 1ml/l Plant Preservative Mixture, 80 mg/L hygromycin, 1.2% agar, pH 5.6-5.8) and incubated the plates under continuous light at 28 °C . We subcultured these calli onto fresh selection media every 8-9 d.

[00192] *Regeneration and Rooting:* After 4-5 weeks on selection media, resistant micro-calli of approximately 2- 5 mm wide started to appear. We picked these off the original callus and transferred them to Petri dishes with regeneration media (BN + S + CH, 1x Murashige and Skoog with vitamins medium containing 30 g/l sucrose, 5 % sorbitol, 3 mg/l BAP, 0.5 mg/l NAA, 400 mg/l carbenicillin, 200 mg/l timentin, 1 ml/l Plant Preservative Mixture, 50 mg/l hygromycin, 1.6 % (w/v) agar, pH 5.6-5.8), and incubated under continuous light at 28 °C. We subculture these calli onto fresh regeneration media every 8-9 days. After 4-5 weeks, when calli started to turn green, the regenerated plants were transferred to rooting media (MS + H, 1x Murashige and Skoog with vitamins medium containing 50 mg/l hygromycin, 1.2 % (w/v) agar, pH 5.6-5.) and incubated in 16 h light/ 8 h dark 28 °C. When the roots were well developed, we transferred the plants to soil.

[00193] **Method 4. Citrus transformation protocol.** We placed seeds of Carrizo citrange rootstock in water to imbibe and then peel off the seed coats making sure not to remove the integument. We surface sterilized seeds in 0.6% (v/v) sodium hypochlorite solution plus 5-µl

tween 20 by placing them in a 50 ml centrifuge tube and shaking at 100 rpms for 20 minutes. We rinse the seeds 3x in 150-200 ml of sterile distilled water. We placed seeds on agar solidified 1/2x Murashige and Skoog minimal organics medium (1/2x MSO) containing 15 g/l sucrose, 7 gm TC agar (pH 5.6-5.8), and push seeds slightly into the medium for more uniform germination. Incubate in the dark at 26 °C.

[00194] *Agrobacterium culture:* We prepared a glycerol freezer stock from a single bacterial colony isolated from a plate. We then used 40 µl of the stock to inoculate 20 ml of MGL medium (pH 7.0) containing the appropriate antibiotics to maintain the *Agrobacterium* and the plasmid, and we incubated overnight at 28 °C at 250 rpm. The following day, we removed 5 ml of the overnight growth and transferred it to 15 ml of TY medium (pH 5.5) containing the appropriate antibiotics and 200 µM acetosyringone. We incubated the culture overnight at 28 °C at 250 rpm and then diluted the overnight culture grown in TY medium to an O.D_{600 nm} of 0.1 to 0.2.

[00195] *Co-cultivation:* We collected 2-5 week old etiolated epicotyls and place in a petri dish containing 10 ml of the *Agrobacterium* solution prepared above (0.1-0.2 OD₆₀₀). We cut submerged epicotyls into 0.5 cm sections and soak for 10 min. We transferred the epicotyl sections onto co-cultivation medium consisting of Murashige and Skoog minimal organics medium (MSO) modified with 30 g/l sucrose 3.0 mg/l BAP, 0.1 mg/l NAA, and 200-µM acetosyringone pH 5.6-5.8. Incubate at 23 °C in the dark.

[00196] *Induction:* After 2-3 days, we transferred the epicotyl pieces to induction medium consisting of MSO modified with 30 g/l sucrose, 3.0 mg/l BAP, 0.1 mg/l NAA, 400 mg/l carbenicillin, 150 mg/l timentin and 100mg/l kanamycin sulfate, and incubated them in the dark. After 10 days, we subcultured the epicotyl sections to fresh medium of the same formulation and then subcultured them every 21 d. After the second 21-day cycle in the dark, we transferred cultures to light under a 30 µM light and a photoperiod of 16 h light 8 h dark. We continued to transfer every 21 d to fresh medium of the same media until organogenic shoot buds develop at the cut ends.

[00197] *Elongation:* Once shoots began to form, we transferred the developing shoots to elongation medium consisting of MSO modified with 30 g/l sucrose, 0.1mg/l BA, 400 mg/l carbenicillin, 150 mg/l timentin, and 100 mg/l kanamycin sulfate. We incubated as above and subcultured the cultures every 21 d as needed until shoots elongated.

[00198] *Rooting:* Once a shoot reached 2-4 cm in height, we harvested the shoots and transferred them to rooting medium consisting of MSO modified with 30 g/l sucrose, 5 mg/l NAA, 250 mg/l cefotaxime, and 100 mg/l kanamycin. After three to five days, we transferred shoots to MSO modified with 30 g/l sucrose, 0.0 mg/l NAA, 400 mg/l carbenicillin and 100 mg/l kanamycin. Shoots started rooting in 14 days.

[00199] **Method 5. Grape transformation protocol.** For generating transgenic grape (Thompson Seedless), we transferred somatic embryos to 5 ml of liquid Lloyd and McCown WPM supplemented with 20 g/l sucrose, 1g/l casein, 1mM MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 200 μ M acetosyringone and 12.5 μ l pluronic F68. We heat-shocked the embryos by placing the tube in a 45 °C bath for 10 min.

[00200] We added *Agrobacterium* to the solution to an OD of 0.1 to 0.2, and then transferred the embryos to an empty 100 x 20 mm petri dish containing a 7 mm Whatman filter paper disk. We wrapped the petri dish with parafilm, and incubated it in the dark at 23 °C. After 2-3 days, we harvested the embryos and transferred them to WPM selection medium. We supplemented the medium with 20 g/l sucrose, 1g/l casein, 1mM MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin (or 25 mg/l hygromycin), 50 g/ sorbitol, 14 g/l agar and 4 ml plant preservative mixture (PPM).

[00201] We incubate the embryos in the dark at 26 °C and after 7 days, we subcultured the embryos to fresh medium of the same formulation. After 14 and 28 days, we subcultured the embryos to fresh medium. After an additional 14 days, we transferred the embryos to the same WPM supplemented medium but without sorbitol and with 8 g/l agar. We divided the embryos on the plate into independent clusters and incubated them under 16 h light at 26 °C, with subcultures every two to three weeks. We continue subculturing every 14-21 days until plants form.

[00202] To harvest the germinating plants, we excised the root and placed the shoot on WPM supplemented with 30 g/l sucrose, 0.01 mg/l IBA, 150 mg/l timentin, 400 mg/l carbenicillin and 100 mg/l kanamycin (or 25mg/l hygromycin). Once shoots rooted, we transferred them to a 2-inch pot containing moistened soil (1 part sunshine mix 2 part vermiculite) and placed the pot into a sealed Ziploc bag for 7 days. Incubate at 26 °C under 16 h of 100 μ M m-2 s-1 light covering the flat with a plastic dome. After one week, we opened the Ziploc bag to reduce humidity. Once new growth was evident, we removed the plants from the Ziploc bag and place them under the dome for an additional 7 days to complete acclimatization.

[00203] Method 6. Pepper transformation protocol.

[00204] We surface sterilized *Capsicum annuum* cultivar “R&C cayenne” seeds in a 1.2 % (v/v) sodium hypochlorite solution containing 5ul tween 20. We agitated the seeds on an orbital shaker at 100 rpms for 20 m followed by one rinse in 150-200 ml of sterile distilled water. We transferred them to agar-solidified ½ strength Murashige and Skoog minimal organics medium (1/2x MSO) supplemented with 16 g/l glucose, 600 mg/l MES, 8 gm PhytoAgar (Plantmedia cat number 40100072-4) (pH 5.6-5.8). We incubated the seeds at 26 °C under a 16 h photoperiod and 30 µM m² s⁻¹ light.

[00205] After 10 d, we placed the seedlings in a solution of *Agrobacterium tumefaciens* (strain EHA 105) adjusted to an O.D. 600 of 0.1-0.2. While submersing the seedlings in the *Agrobacterium* solution, we removed 2/3 of the tip of the cotyledons and then remove the cotyledons from the seedling taking care to retain the petiole of the cotyledon but not the apical meristem. After 5 minutes, we transferred the explants into 100 x 20 mm petri dishes containing co-cultivation medium consisting of MSO medium modified with 16 g/l glucose, 600 mg/l MES, 10 mg/l benzlaminopurine (BAP), 1.0 mg/l indole-3-acetic acid (IAA), and 200 µM acetosyringone pH 5.6-5.8. We incubated the tissue in the dark at 23 °C.

[00206] After 2-3 d, we transferred the cotyledon pieces to induction medium consisting of MSO medium supplemented with 16 g/l glucose, 600 mg/l MES, 10mg/l BAP, 1.0 mg/l IAA, 300 mg/l timentin, 400 mg/l carbenicillin, 8mg/l glufosinate (Sigma-Aldrich catalog number 45520). After 10 d, we sub-cultured the cotyledons to fresh medium and then subsequently every 21 d.

[00207] Once buds began to form, we transferred the developing buds to elongation medium. This medium consisted of Driver and Kuniyaki Walnut (DKW) medium, supplemented with 30 g/l glucose, 1.3 g/l ca gluconate, 2.0 mg/l meta-topolin, (MT) 10 mg/l gibberellic acid (GA3), 2 ml/l of a silver thiosulfate stock (STS) (1.0 ml of a 100 mg/ml stock of AgNO₃ added to 1.0 ml of a 12 mM (95 mg/50 ml water stock), 300 mg/l timentin, 400 mg/l carbenicillin, 4 ml/l PPM and 8 mg/l glufosinate.

[00208] As shoots begin to develop from the buds, we transferred them to elongation medium consisting of DKW supplemented with 30 g/l glucose, 1.3 g/l ca gluconate, 0.5 mg/l MT, 10 mg/l GA3, 2.0 ml /l STS, 300 mg/l timentin, 400 mg/l carbenicillin, 4ml/l PPM and 8 mg/l glufosinate. We incubated as above transferring to fresh medium every 14 days. We removed any developing callus at the base of the shoots at each subculture (Figure 28).

[00209] After two consecutive, 14 d subcultures, we transferred elongating shoots to DKW medium supplemented with 30 g/l glucose, 1.3 g/l ca gluconate, 0.1 mg/l MT, 10 mg/l GA3, 1.0 ml/l STS, 300 mg/l timentin, 400 mg/l carbenicillin, 4 ml/l PPM and 8 mg/l glufosinate. When shoots reached a size of 2-3 cm, we transferred them to rooting medium consisting of DKW medium supplemented with 30 g/l sucrose, 0.1 mg/l NAA, 2 ml/l STS, 300 mg/l timentin 400 mg/l carbenicillin 4 ml/L PPM and 4 g/l glufosinate.

[00210] **Table 7.** Regeneration frequencies for different *GRF-GIF* combinations compared with empty vector in tetraploid wheat Kronos (17 experiments). The number of embryos (n) per genotype inoculated with each specific construct. Regeneration frequencies were estimated as the number of calluses showing at least one regenerating shoot / total number of inoculated embryos. This is a conservative estimate since calli generated from embryos transformed with the *GRF4-GIF1* construct usually generate multiple independently transformed shoots. The blue “x” indicate the experiments included in the statistical analyses presented in the different figures. All experiments in this Table were done with the regular 91 d protocol.

	No inoc.		<i>GRF4-</i>	Fig.	<i>GRF4</i> & <i>GIF1</i>	Fig.			Fig.	<i>GRF4-</i>		Fig.	<i>GRF5-</i>	<i>GRFGRF1</i> - <i>GIF1</i>	<i>GRF9-</i>	Fig.
	embryos	pLC41	<i>GIF1</i>	1B	<i>GIF1</i>	1D	<i>GIF1</i>	<i>GRF4</i>	1C	<i>GIF2</i>	<i>GIF3</i>	20D		<i>GIF1</i>		20B
Exp1-a	n=25	0.04	0.90	x												
Exp1-b	n=25	0.08	0.96	x												
Exp2	n=30		0.27		0.06	x										
Exp3	n=80		0.91		0.77	x										
Exp3b ^a	n=40		0.60		0.14	x										
Exp4	n=50	0.13	0.70	x	0.46	x	0.57									
Exp6	n=20	0.20	0.65	x	0.50	x	0.35									
Exp22	n=24	0.16	0.82	x			0.16	0.64	x							
Exp25	n=15	0.00	0.70	x			0.40	0.06	x							
Exp25b ^a	n=15	0.00	0.35	x			0.00	0.00	x							
Exp26	n=20	0.08	0.55	x			0.20	0.20	x							
Exp26b ^b	n=16	0.06	0.31	x			0.10	0.12	x							
Exp12	n=10	0.00	0.50	x						0.20	0.20	x				
Exp13	n=25	0.17	0.72	x						0.50	0.46	x				
Exp24	n=10	0.10	0.50	x						0.50	0.30	x	.	0.10	0.30	x
Exp17	n=20	0.00	0.67	x									0.57	0.16	0.19	x
Exp18	n=24	0.20	0.88	x									0.70	0.79	0.76	x

[00211] **Table 8.** Comparison among the eight wheat (*Triticum aestivum*) paralogous proteins (TaGRF). The percent identity (first number) and percent similarity (second number) of concatenated QLQ-WRC conserved domains were obtained by BLAST P.

%ID/%Sim QLQ-WRC	TaGRF4	TaGRF3	TaGRF5	TaGRFGRF1	TaGRF2GRF2	TaGRF9	TaGRF6
TaGRF3	94/96						
TaGRF5	86/91	90/92					
TaGRFGRF1	80/92	82/92	82/91				
TaGRF2GRF2	78/91	77/89	75/88	82/94			
TaGRF9	70/83	72/82	73/82	71/84	68/83		
TaGRF6	72/87	73/83	74/83	70/86	73/85	76/84	
TaGRFGRF12	68/76	68/77	67/74	65/79	68/79	64/76	67/74

[00212] Sequences used in the comparison (QLQ underlined, WRC bold)

[00213] >TraesCS6A01G335900 TaGRFGRF1

[00214] PFTATQWQELEHQALIYKYMASGVPIPSDLLPLRRSFDPEPGRCRRTDGKK
WRCSKEAYPDSKYCEKHMHRGKNRSRKPVE (SEQ ID NO: 125)

[00215] >TraesCS7A01G165600 TaGRF2GRF2

[00216] LFTASQWRELEHQALIYKYMAAGSOVPHLVLPLRHRDDPEPGRCRRTDG
KKWRCSREAYGESKYCDRHMHRGKNRSRKPVE (SEQ ID NO: 126)

[00217] >TraesCS6A01G269600 TaGRF4

[00218] PFTAAQYEELEHQALIYKYL VAGVSVPPDLVLPIRRIGIDPEPGRCRRTDGKK
WRCAKEAASDSKYCERHMHRGRNRSRKPVE (SEQ ID NO: 127)

[00219] >TraesCS2A01G435100 TaGRF3

[00220] TFTAAQYEELEQQALIYKYL VAGVPVPPDLLLPIRRGFDPEPGRCRRTDGKK
WRCSKEAAQDSKYCERHMHRGRNRSRKPVE (SEQ ID NO: 128)

[00221] >TraesCS7A01G049100 TaGRF5

[00222] VFTPAQWAELEQQALIYKYLMAGVPVPPDLLLPIRPHPDPEPWRCRRTDGK
KWRCSEAHPSKYCERHMHRGRNRSRKPVE (SEQ ID NO: 129)

[00223] >TraesCS4A01G291500 TaGRF9

[00224] PFTPSQWMELEHQALIYKYLNAPISGLLISISKSFDPEPGRCRRTDGKK
WRCSKEAMAEHKYCERHINRNRHRSRKPVE (SEQ ID NO: 130)

[00225] >TraesCS4A01G255000 TaGRF6

[00226] PFTPSQWMELEHQALIYKYLAANIAVPHSLLVPIRRSVLDLEPGRCRRTDGKK
WRCSRDAVADQKYCERHMNRGRHRSRKHVE (SEQ ID NO: 131)

[00227] >TraesCS6A01G257600 TaGRFGRF12

[00228] ALTFMQQQELEHQVLIYRYFAAGAPVPVHLVLPIWKSVEPEPGRCRRTDGK
KWRCSDVVQGHKYCERHVHRGRGRSRKPVE (SEQ ID NO: 132)

[00230] Table 9. Regeneration frequencies in plants transformed with the *wheat GRF4-GIF1* chimera or the empty vector. **A)** Tetraploid and hexaploid wheat commercial cultivars. **B)** Triticale breeding line and **C)** Rice cultivar Kitaake. In all experiments we used the pLC41 vector (optimized for wheat), except for the second rice experiment where we used the pCAMBIA1300 vector which is more frequently used in rice. EHA105 and AGL1 are two different *Agrobacterium* strains used for infiltration (we observed no differences between the two strains). In the number of inoculated embryos (n) the first number indicates those inoculated with the empty vector and the second number the embryos inoculated with the *GRF4-GIF1* chimera.

9A. Wheat

Desert King (4x)	Exp1 (EHA105)	Exp2 (EHA105)		
No. embryos inoc.	n=20/20	n=50/50	Average	SE
pLC41	0.05	0	0.025	0.025
<i>Ubi::GRF4-GIF1</i>	0.80	0.46	0.630	0.170

Fielder (6x)	Exp1 (EHA105)	Exp2 (EHA105)		
No. embryos inoc.	n=49/67	n=10/10	Average	SE
pLC41	0.05	0	0.025	0.025
<i>Ubi::GRF4-GIF1</i>	0.58	0.5	0.540	0.040

Cadenza (6x)	Exp1 (AGL1)	Exp2 (AGL1)	Exp3 (EHA105)		
No. embryos inoc.	n=19/12	n=23/24	n=25/25	Average	SE
pLC41	0	0	0	0.000	0.000
<i>Ubi::GRF4-GIF1</i>	0.20	0.17	0.20	0.190	0.010

Hahn (6x)	Exp1 (EHA105)	Exp2 (EHA105)	Exp3 (EHA105)	Exp4 (AGL1)		
No. embryos inoc.	n=31/37	n=48/50	n=69/51	n=25/25	Average	SE
pLC41	0	0	0	0	0.000	0.000
<i>Ubi::GRF4-GIF1</i>	0.03	0.04	0	0.28	0.088	0.087

[00231] 9B. Triticale

Triticale UC3190 (6x)	Exp9 (EHA105)	Exp11 (EHA105)	Exp15 (EHA105)		
No. embryos inoc.	n=45/45	n=21/22	n=42/43	Average	SE
pLC41	0	0	0	0.000	0.000
<i>Ubi::GRF4-GIF1</i>	0.05	0.13	0.14	0.107	0.028

[00232] 9C. Rice. Regeneration frequencies in rice (*Oryza sativa*) cultivar Kitaake. Both experiments were performed with *Agrobacterium* strain EHA105. In the first experiment, we used the wheat-optimized vector pLC41 and in experiments 2-4 the rice-optimized vector pCAMBIA1300 vector. The first number in the inoculated calli represents the calli inoculated with the empty vector and the second number the calli inoculated with the wheat *GRF4-GIF1* chimera. Experiments 2-4 are three experiments performed with the same seed stock (100 embryos each). Experiment 2 included the *Ubi::GRF4-GIF1* chimera and a sgRNA targeted to gene OsKitaake06g041700 encoding a Tyrosine Protein Sulfotransferase (TPST), and experiments 3 and 4 included pCAMBIA1300-gus without the chimera.

Rice Kitaake	Exp1	Exp2-4		
No. calli inoc.	n=85/85	n=200/100	Average	SE
No <i>GRF4-GIF1</i>	0.118	0.235	0.176	0.059
<i>Ubi::GRF4-GIF1</i>	0.353	0.460	0.406	0.054

[00233] **Table 10.** Regeneration frequencies in *Citrus*. Experiments 1 to 3 used a *GRF4-GIF1* chimera based on *Citrus* sequences whereas experiments 4 to 6 used a *GRF4-GIF1* chimera based on *Vitis* sequences. The last three experiments included a second *Vitis* construct with mutations in the miR396 binding site (*rGRF4-GIF1*) that precludes its cleavage. The first number in the inoculated calli represents the calli inoculated with the empty vector and the other numbers the calli inoculated with the different *GRF4-GIF1* chimeras.

Carrizo	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6		
No. epicotyls.	n=45/45	n=38/41	n=56/69	n=65/59/66	n=32/31/31	n=40/40	Average	SE
Empty vector	0.04	0.00	0.09	0.02	0.09	0.02	0.05	0.02

<i>Citrus GRF4-GIF1</i>	0.15	0.39	0.10	-	-	-	0.21	0.09
<i>Vitis GRF4-GIF1</i>	-	-	-	0.07	0.25	-	0.16	0.09
<i>Vitis rGRF4-GIF1</i>	-	-	-	0.20	0.61	0.30	0.37	0.12

[00234] Table 11. Regeneration frequencies in *Capsicum annuum*

[00235] Cultivar R&C cayenne (experiments No. 201027/201028) in 40 cotyledon pieces transformed with the *GRF4.1-GIF1.1* chimera (SEQ ID NO:140) and 40 with the empty vector pEarleyGate100. In the number of inoculated explants, (n) the first number indicates those inoculated with the empty vector and the second number the embryos inoculated with the *GRF4.1-GIF1.1* chimera.

R&C cayenne	Plate 1	Plate 2		
No. explants.	n=20/22	n=20/20	Average	SE
Empty vector	0.00	0.10	0.05	0.05
<i>Capsicum GRF4.1-GIF1.1</i>	0.23	0.25	0.24	0.01

[00236] LIST OF SEQUENCE IDENTIFIERS

[00237] SEQ ID NO:

[00238] 1: Full vector pLC41 having Ta*GRF4* nucleotide sequence

[00239] 2: Full vector pLC41 having Ta*GIF1* nucleotide sequence

[00240] 3: Ta*GRF4-GIF1* chimera nucleotide sequence

[00241] 4: Ta*GRF4-GIF1* amino acid sequence

[00242] 5: Full construct pLC41 wheat *GRF4-GIF1* chimera

[00243] 6: *Vitis vinifera GRF4-GIF1* chimera nucleotide sequence

[00244] 7: *Vitis vinifera GRF4-GIF1* chimera protein sequence

[00245] 8: Full construct pGWB14 having *Vitis vinifera GRF4-GIF1* chimera

[00246] 9: Ta*GRFGRF1* amino acid sequence

- [00247] 10: TaGRF2GRF2 amino acid sequence
- [00248] 11: TaGRF3 amino acid sequence
- [00249] 12: TaGRF4 amino acid sequence
- [00250] 13: TaGRF5 amino acid sequence
- [00251] 14: OsGRFGRF1 amino acid sequence
- [00252] 15: OsGRF2GRF2 amino acid sequence
- [00253] 16: OsGRF3 amino acid sequence
- [00254] 17: OsGRF4 amino acid sequence
- [00255] 18: OsGRF5 amino acid sequence
- [00256] 19: AtGRF3 amino acid sequence
- [00257] 20: AtGRF4 amino acid sequence
- [00258] 21: AtGRF5 amino acid sequence
- [00259] 22: AtGRF6 amino acid sequence
- [00260] 23: *Vitis vinifera* GRF4 (SVIVT01024326001) used in the chimera
- [00261] 24: AtGIF1 amino acid sequence
- [00262] 25: AtGIF2 amino acid sequence
- [00263] 26: AtGIF3 amino acid sequence
- [00264] 27: TaGIF1 amino acid sequence
- [00265] 28: TaGIF2 amino acid sequence
- [00266] 29: TaGIF3 amino acid sequence
- [00267] 30: *Vitis vinifera* GIF1 (GSVIVT01036262001)
- [00268] 31: Dexamethasone inducible chimera of *Vitis vinifera* GRF4-GR-GIF1 protein
- [00269] 32: Dexamethasone inducible TaGRF4-GR-GIF1

- [00270] 33: Full vector of pLC41 with wheat *GRF4-GR-GIF1* chimera
- [00271] 34: wheat *rGRF4-GIF1* with four mutation in miR396 target site
- [00272] 35: Full construct JD635 with wheat *GRF4-GIF1* chimera
- [00273] 36: Full construct pLC41 with separate Ubi::*GRF4*-Ubi::*GIF1*
- [00274] 37: Ta*GRF5*
- [00275] 38: Ta*GRFGRF1*
- [00276] 39: Ta*GRF9*
- [00277] 40: Ta*GRFGRF1-GIF1*
- [00278] 41: Ta*GRF5-GIF1*
- [00279] 42: Ta*GRF9-GIF1*
- [00280] 43: Ta*GIF2*
- [00281] 44: Ta*GIF3*
- [00282] 45: Ta*GRF4-GIF2*
- [00283] 46: Ta*GRF4-GIF3*
- [00284] 47: Full vector pLC41 marker free with wheat *GRF4-GIF1* chimera
- [00285] 48: Citrus *GRF4-GIF1*
- [00286] 49: Full pGWB14 construct with Citrus *GRF4-GIF1*
- [00287] 50: Vitis *rGRF4-GIF1* miR396 resistant chimera
- [00288] 51 Ta*GRF4* nucleotide sequence -- From SEQ ID NO: 1 vector
- [00289] 52 Ta*GIF1* nucleotide sequence -- From SEQ ID NO: 2 vector
- [00290] 53 wild-type miR396 target site
- [00291] 54: miR396 target site with silent mutations
- [00292] 55: Mature miR396 sequence

- [00293] 56: Protein sequence is Figure 8
- [00294] 57: LB of pLC41
- [00295] 58: RB of pLC41
- [00296] 59: Zea maize UBIQUITIN promoter
- [00297] 60: HA tag
- [00298] 61: nos terminator
- [00299] 62: 35s promoter
- [00300] 63: HPT
- [00301] 64: TaCas9 from SEQ ID NO: 35
- [00302] 65: TaU6 promoter from SEQ ID NO: 35
- [00303] 66: Guide RNA gene Q from SEQ ID NO: 35
- [00304] 67: Guide RNA scaffold from SEQ ID NO: 35
- [00305] 68: Sequence in Figure 9 B
- [00306] 69: QLQ domain from SEQ ID NO: 9
- [00307] 70: QLQ domain from SEQ ID NO: 10
- [00308] 71: QLQ domain from SEQ ID NO: 11
- [00309] 72: QLQ domain from SEQ ID NO: 12
- [00310] 73: QLQ domain from SEQ ID NO: 13
- [00311] 74: QLQ domain from SEQ ID NO: 14
- [00312] 75: QLQ domain from SEQ ID NO: 15
- [00313] 76: QLQ domain from SEQ ID NO: 16
- [00314] 77: QLQ domain from SEQ ID NO: 17
- [00315] 78: QLQ domain from SEQ ID NO: 18

- [00316]** 79: QLQ domain from SEQ ID NO: 19
- [00317]** 80: QLQ domain from SEQ ID NO: 20
- [00318]** 81: QLQ domain from SEQ ID NO: 21
- [00319]** 82: QLQ domain from SEQ ID NO: 22
- [00320]** 83: QLQ domain from SEQ ID NO: 23
- [00321]** 84: WRC domain from SEQ ID NO: 9
- [00322]** 85: WRC domain from SEQ ID NO: 10
- [00323]** 86: WRC domain from SEQ ID NO: 11
- [00324]** 87: WRC domain from SEQ ID NO: 12
- [00325]** 88: WRC domain from SEQ ID NO: 13
- [00326]** 89: WRC domain from SEQ ID NO: 15
- [00327]** 90: WRC domain from SEQ ID NO: 16
- [00328]** 91: WRC domain from SEQ ID NO: 19
- [00329]** 92: WRC domain from SEQ ID NO: 20
- [00330]** 93: WRC domain from SEQ ID NO: 21
- [00331]** 94: WRC domain from SEQ ID NO: 22
- [00332]** 95: WRC domain from SEQ ID NO: 23
- [00333]** 96: A concatenated QLQ-WRC domain for wheat *TaGRF4* (for which the complete protein sequence is SEQ ID NO:12):
- [00334]** 97: SNH domain from SEQ ID NO: 24
- [00335]** 98: SNH domain from SEQ ID NO: 25
- [00336]** 99: SNH domain from SEQ ID NO: 26
- [00337]** 100: SNH domain from SEQ ID NO: 27

- [00338] 101: SNH domain from SEQ ID NO: 28
- [00339] 102: SNH domain from SEQ ID NO: 29
- [00340] 103: SNH domain from SEQ ID NO: 30
- [00341] 104: Primer Fw-*GRF4b*
- [00342] 105: Primer Rev-*GIF1c*
- [00343] 106 – 118: Table 6 primers
- [00344] 119: Primer Fw_HindIII
- [00345] 120: Primer Rev-HindIII
- [00346] 121: Primer Fw-*GRF*
- [00347] 122: Primer r*GRF*-Rev
- [00348] 123: Primer r*GRF*-Fw
- [00349] 124: Primer Rev-*GIF*
- [00350] 125: QLQ-WRC from TraesCS6A01G335900 Ta*GRFGRF1*
- [00351] 126: QLQ-WRC from TraesCS7A01G165600 Ta*GRF2GRF2*
- [00352] 127: QLQ-WRC from TraesCS6A01G269600 Ta*GRF4*
- [00353] 128: QLQ-WRC from TraesCS2A01G435100 Ta*GRF3*
- [00354] 129: QLQ-WRC from TraesCS7A01G049100 Ta*GRF5*
- [00355] 130: QLQ-WRC from TraesCS4A01G291500 Ta*GRF9*
- [00356] 131: QLQ-WRC from TraesCS4A01G255000 Ta*GRF6*
- [00357] 132: QLQ-WRC from TraesCS6A01G257600 Ta*GRFGRF12*
- [00358] 133: Os11g40100 (*GIF2*)
- [00359] 134: Os12g31350 (*GIF3*)
- [00360] 135: Os03g52320 (*GIF1*)

- [00361] 136: *OsGIF3* SNH Domain
- [00362] 137: *OsGIF1* SNH Domain
- [00363] 138: *Capsicum GRF4* (LOC107869915)
- [00364] 139 *Capsicum GIF1* (LOC107870303)
- [00365] 140 *Capsicum GRF4-GIF1* chimera.

[00366] REFERENCES

- [00367] All references cited in this disclosure are incorporated by reference in their entirety.
- Beltramino, M., Ercoli, M. F., et al. (2018). "Robust increase of leaf size by *Arabidopsis thaliana* *GRF3*-like transcription factors under different growth conditions". *Scientific Reports* **8**:13447.
- Che, R., Tong, H., et al. (2015). "Control of grain size and rice yield by GL2-mediated brassinosteroid responses." *Nat Plants* **2**:15195.
- Debernardi, J. M., Mecchia, M. A., et al. (2014). "Post-transcriptional control of *GRF* transcription factors by microRNA miR396 and *GIF* co-activator affects leaf size and longevity". *Plant J.* **74**:413-426.
- Debernardi, J. M., Rodriguez, R. E., et al. (2012). "Functional specialization of the plant miR396 regulatory network through distinct microRNA-target interactions". *PLoS Genet* **8**: e1002419.
- Duan, P., Ni, S., et al. (2015). "Regulation of *OsGRF4* by *OsmiR396* controls grain size and yield in rice". *Nat Plants* **2**:15203.
- He, Z., Zeng, J. et al., (2017). "*OsGIF1* positively regulates the sizes of stems, leaves, and grains in rice". *Front Plant Sci* **8**:1730.
- Horiguchi, G., Kim, G. T., et al. (2005). "The transcription factor *AtGRF5* and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis thaliana*". *Plant J* **43**: 68-78.
- Hu, J., Wang, Y., et al. (2015). "A rare allele of *GS2* enhances grain size and grain yield in rice". *Mol Plant* **8**:1455-1465.

- Kim, H., Kim, S. T., Ryu, J., Kang, B. C., Kim, J. S., Kim, S. G. (2017). "CRISPR/Cpf1-mediated DNA-free plant genome editing". *Nat Commun* **8**:14406.
- Kim, J. H. and B. H. Lee (2006). "GROWTH-REGULATING FACTOR4 of *Arabidopsis thaliana* is required for development of leaves, cotyledons, and shoot apical meristem." *Journal of Plant Biology* **49**: 463-468.
- Kim, J. H., Choi, D., et al. (2003). "The *AtGRF* family of putative transcription factors is involved in leaf and cotyledon growth in *Arabidopsis*". *Plant J* **36**: 94-104.
- Kim, J. H. and Kende, H. (2004). "A transcriptional coactivator, *AtGIF1*, is involved in regulating leaf growth and morphology in *Arabidopsis*". *Proc Natl Acad Sci U S A* **101**: 13374-13379.
- Lee, B. H., J. H. Ko, et al. (2009). "The *Arabidopsis GRF-INTERACTING FACTOR* gene family performs an overlapping function in determining organ size as well as multiple developmental properties." *Plant Physiol* **151**:655-668.
- Li, S., Gao, F., et al. (2016). "The *OsmiR396c-OsGRF4-OsGIF1* regulatory module determines grain size and yield in rice". *Plant Biotechnol J* **14**:2134-2146.
- Li, S., Tian, Y., et al. (2018). "Modulating plant growth-metabolism coordination for sustainable agriculture". *Nature* **560**:595-600.
- Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., Liu, J., Zhang, H., Liu, C., Ran, Y., Gao, C. (2017). "Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes". *Nat Commun* **8**:14261.
- Liu, D., Song, Y., et al. (2009). "Ectopic expression of *miR396* suppresses *GRF* target gene expression and alters leaf growth in *Arabidopsis*". *Physiol Plant* **136**: 223-236.
- Malnoy, M., Viola, R., et al. (2016). "DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins". *Front Plant Sci* **7**:1904
- Omidbakhshfard, M. A., Proost, S., et al. (2015). "Growth-Regulating Factors (*GRFs*): A Small Transcription Factor Family with Important Functions in Plant Biology". *Mol Plant* **8**: 998-1010.

- Rodriguez, R. E., Mecchia, M. A., et al. (2010). "Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396". *Development* **137**: 103-112.
- Shimano, S., Hibara, K. I., et al. (2018). "Conserved functional control, but distinct regulation, of cell proliferation in rice and *Arabidopsis* leaves revealed by comparative analysis of *GRF*-INTERACTING FACTOR 1 orthologs". *Development* **145**: dev159624.
- Subburaj, S., Chung, S. J., et al. (2016). "Site-directed mutagenesis in *Petunia* x hybrid protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins". *Plant Cell Rep* **35**:1535–1544.
- Sun, P., Zhang, W., et al. (2016). "Os*GRF4* controls grain shape, panicle length and seed shattering in rice". *J Integr Plant Biol* **58**:836-847.
- Svitashev, S., Schwartz, C., Lenderts, B., Young, J. K., Mark Cigan, A. (2016). "Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes". *Nat Commun* **7**:13274.
- van der Knaap, E., Kim, J. H., et al. (2000). "A novel gibberellin-induced gene from rice and its potential regulatory role in stem growth". *Plant Physiol* **122**: 695-704.
- Vercruyssen, L., Verkest, A., et al. (2014). "ANGUSTIFOLIA3 binds to SWI/SNF chromatin remodeling complexes to regulate transcription during *Arabidopsis* leaf development". *Plant Cell* **26**: 210-229.
- Wang, L., Gu, X., et al. (2011). "miR396-targeted *AtGRF* transcription factors are required for coordination of cell division and differentiation during leaf development in *Arabidopsis*". *J Exp Bot* **62**: 761-773.
- Wolter, F., Puchta, H. (2017). "Knocking out consumer concerns and regulator's rules: efficient use of CRISPR/Cas ribonucleoprotein complexes for genome editing in cereals". *Genome Biol* **18**:43
- Woo, J. W., Kim, J., et al. (2015). "DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins". *Nat Biotechnol* **33**:1162–1164
- Zhang, D., Sun, W., et al (2018). "*GRF*-INTERACTING FACTOR 1 regulates shoot architecture and meristem determinacy in maize". *Plant Cell*. **30**:360-374.

What is claimed is:

1. A method of increasing regeneration efficiency of one or more transgenic plant cells, the method comprising,
 - a) introducing into said one or more plant cells a nucleic acid molecule encoding or a polypeptide comprising,
 - i) a Growth Regulating Factor (*GRF*) polypeptide and/or a *GRF*-interacting Factor (*GIF*) polypeptide; or
 - ii) a *GRF*- *GIF* polypeptide chimera; and
 - b) said one or more plant cells having increased regeneration efficiency compared to a plant not comprising said introduced nucleic acid molecule encoding or a polypeptide comprising a *GRF* polypeptide and/or a, *GIF* polypeptide or a *GRF*- *GIF* polypeptide chimera.
2. The method of claim 1, further comprising producing a plant from said one or more plant cells.
3. The method of claim 1 or 2, wherein said *GRF* polypeptide is selected from a wheat *GRFGRF1*, *GRF2GRF2*, *GRF3*, *GRF4*, *GRF5*, *GRF6* or *GRF9* polypeptide or a homolog from other plant species or a combination thereof.
4. The method of claim 1 or 2, wherein said *GIF* polypeptide is selected from a wheat *GIF1*, *GIF2*, *GIF3* polypeptide or a homolog from other plant species thereof or a combination thereof.
5. The method of claim 1 or 2, wherein said *GIF* polypeptide is selected from SEQ ID NO: 24-30, 43-44, 52, 133-135, 139 or a polypeptide sequence having at least 70% identity in the SNH domain thereto, or a combination thereof.
6. The method of claim 1 or 2, wherein said *GRF* polypeptide is selected from SEQ ID NO: 9-23, 37-39, 51, 125-132 or 138 or a polypeptide sequence having at least 70% identity in the QLQ and WRC domains thereto or a combination thereof.

7. The method of any of claims 1-6, wherein said *GRF* polypeptide comprises a QLQ selected from SEQ ID NO: 69-83 and WRC domain that selected from SEQ ID NO: 84-95 or a sequence having at least 70% identity thereto.
8. The method of any of claims 1-7, wherein said *GIF* polypeptide comprises a SNH domain selected from SEQ ID NO: 97-103, 136, 137 or a sequence having at least 70% identity thereto.
9. The method of any of claims 1-8, wherein said *GRF* polypeptide comprises one or more mutations in the miR396 target site and lowering repression of said *GRF* polypeptide by miR396 in said plant.
10. The method of claim 9, wherein said mutations comprise silent mutations of said miR396 target site.
11. The method of claim 9, wherein said miRNA target site comprises SEQ ID NO: 53
12. The method of any of claims 1-11, further comprising an inducible nucleic acid molecule operably linked to said *GRF*, *GIF* or *GRF-GIF* chimera.
13. The method of any of claims 1-12, wherein said one more plant cells are regenerated on media comprising cytokinin that is at a concentration not sufficient to regenerate a plant cell that has not had said *GRF*, *GIF* or *GRF-GIF* chimera introduced.
14. The method of claim 13, wherein said one or more transformed plant cells comprising said heterologous nucleic acid molecule or polypeptide is selected by its ability to grow on said media.
15. The method of any of claims 1-14, wherein said plant has at least 10% increased regeneration efficiency compared to a plant cell not comprising said introduced *GRF*, *GIF* or *GRF-GIF* chimeras.

16. The method of any of claims 1-15, wherein said plant is selected from *Triticum*, *Oryza*, *Vitis*, *Citrus*, avocado, walnut, pistachio, peach, apple, cherry, strawberry, blueberry, raspberry, bean, broccoli, cauliflower, cowpea, leek, melon, onion, pepper, spinach, squash or watermelon.
17. The method of any of claims 1-16 wherein said plant comprises an elite plant.
18. The method of any of claims 1-17, wherein said one or more plant cells comprises one or more leaf explant cells, or cell from any other tissue.
19. The method of any of claims 1-18, wherein said nucleic acid molecule or polypeptide of said *GRF*, *GIF* or *GRF* and *GIF* chimera is combined with CRISPR-CAS9 or any other gene editing system.
20. The method of claim 19, wherein said construct combining *GRF*, *GIF* or *GRF* and *GIF* chimera with gene editing is removed from said plant by segregation in the progeny of the edited plant.
21. The method of any of claims 1-20, wherein said nucleic acid molecule encoding or polypeptide comprising a *GRF*, *GIF* or *GRF-GIF* chimera polypeptide are introduced into said plant prior to introducing said heterologous nucleic acid molecule or polypeptide.
22. A method of increasing regeneration efficiency of a plant, wherein said plant contains miR396, the method comprising
- a) introducing into said plant a nucleic acid molecule encoding or a polypeptide comprising,
 - i) a Growth Regulating Factor (*GRF*) polypeptide and/or a *GRF*-interacting Factor (*GIF*) polypeptide; or
 - ii) a *GRF-GIF* polypeptide chimera; and
 - b) said *GRF* polypeptide comprising a miR396 target site having one or more mutations and lowering repression of said *GRF* polypeptide by said miRNA in said plant.
23. The method of any of claims 1-22, wherein said plant is a transgenic plant.

24. A plant produced by any of the methods of claims 1-23.
25. The method of any of the previous claims where *GRF*, *GIF* or *GRF-GIF* chimeras are used to accelerate the time required to regenerate plants.

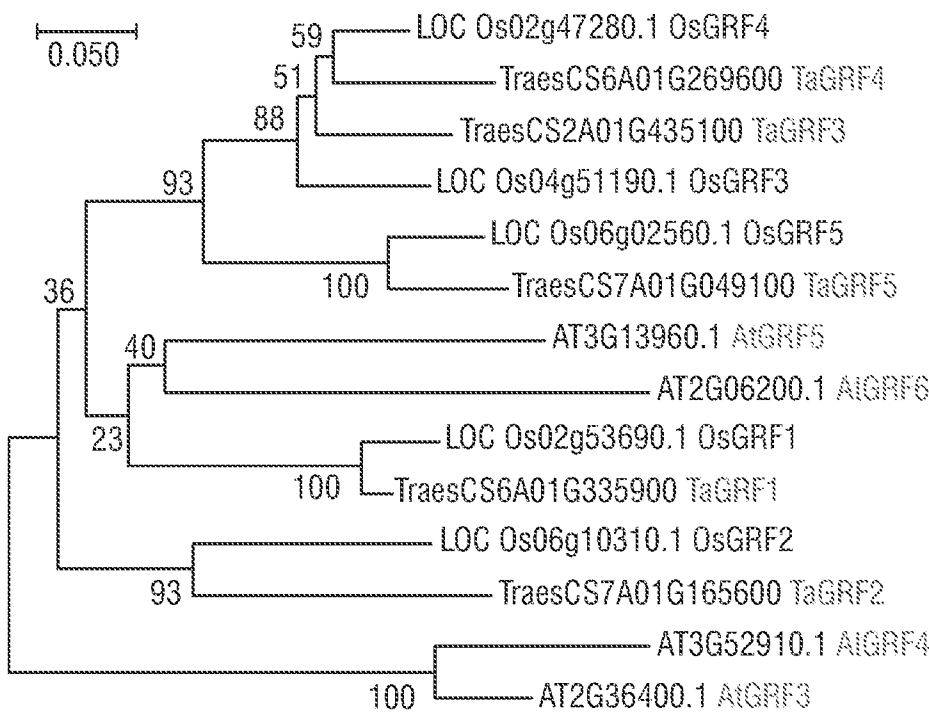


FIG. 1A

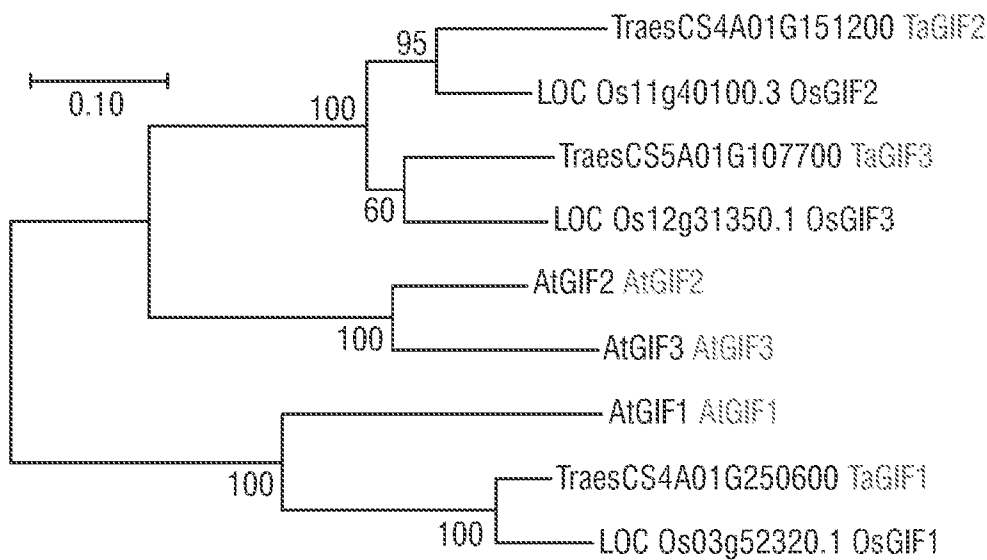


FIG. 1B

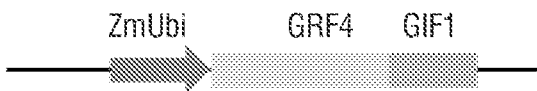


FIG. 1C

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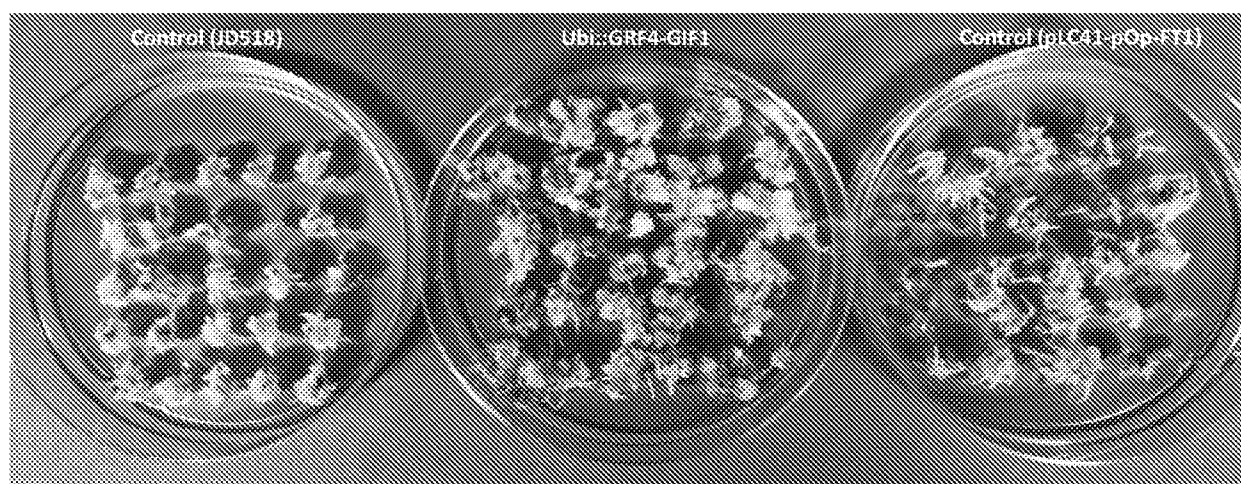


FIG. 2

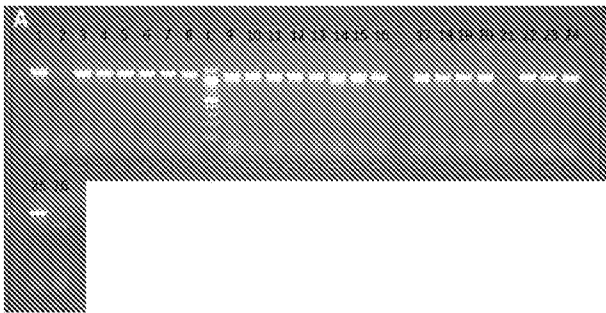


FIG. 3A

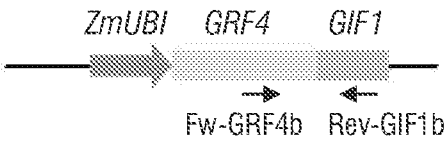
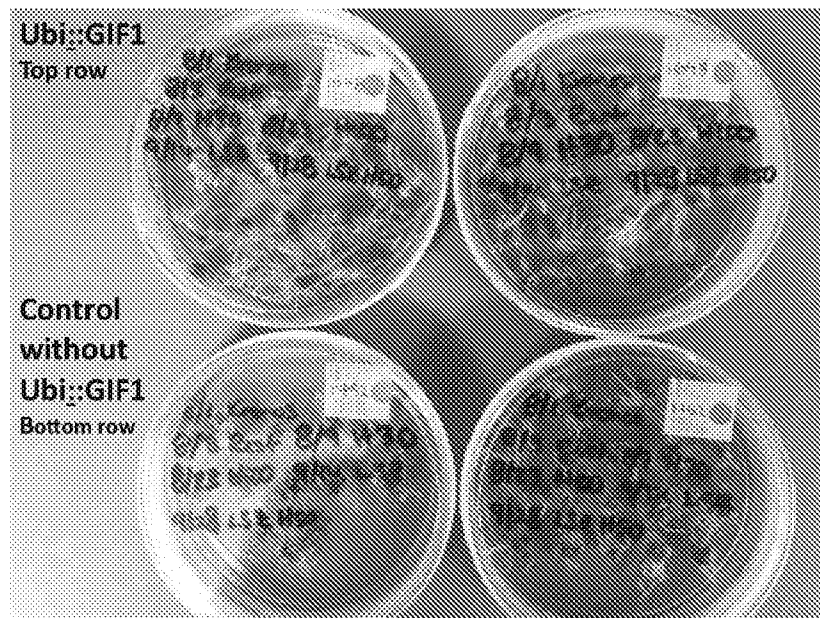
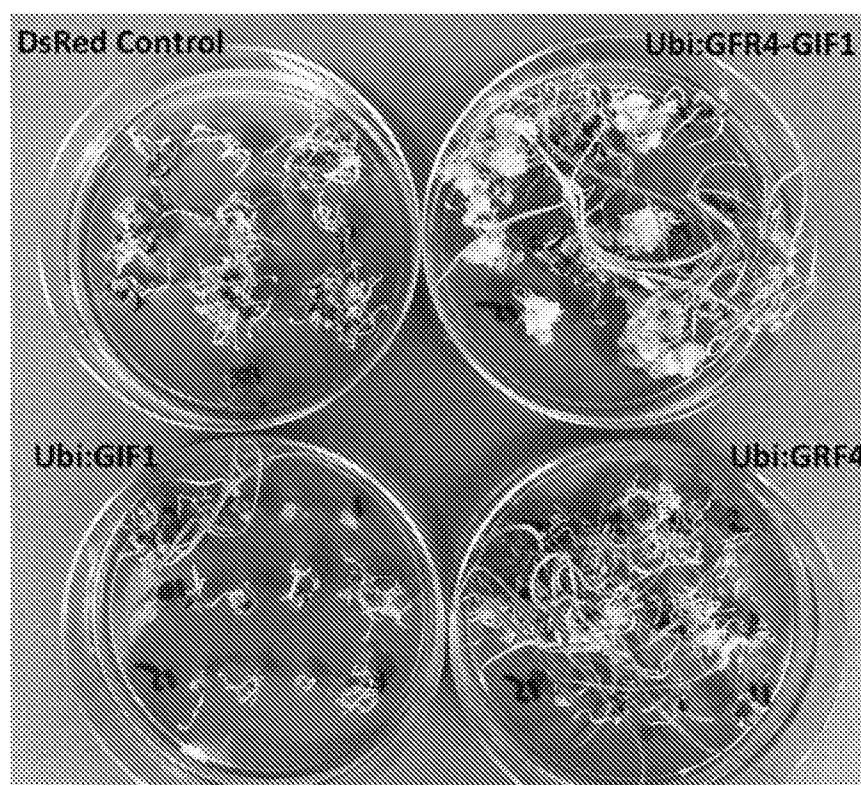


FIG. 3B

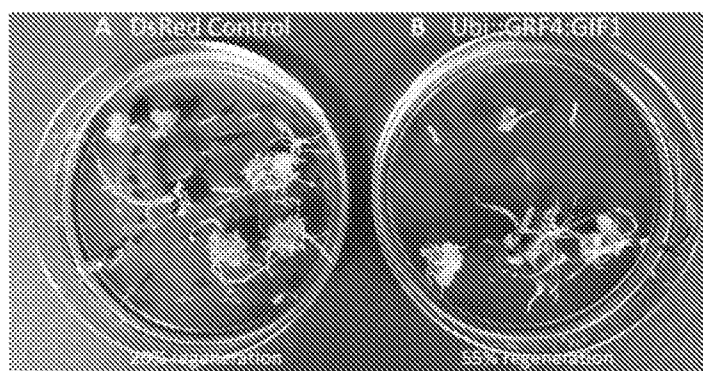
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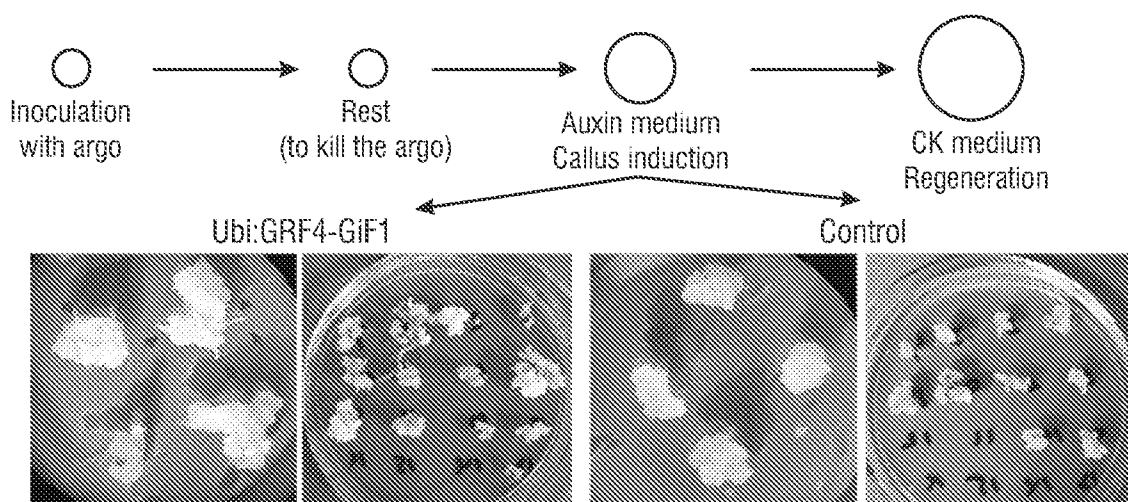
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**FIG. 5**

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**FIG. 6A****FIG. 6B**

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**FIG. 7A****FIG. 7B**

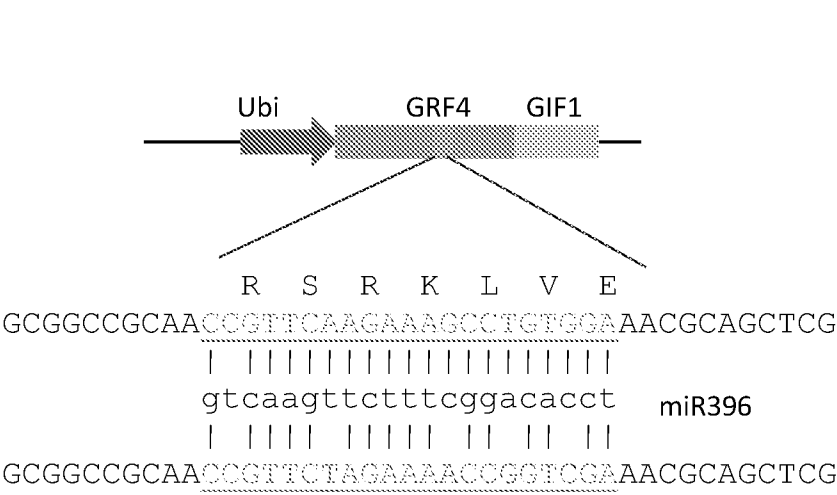


FIG. 8

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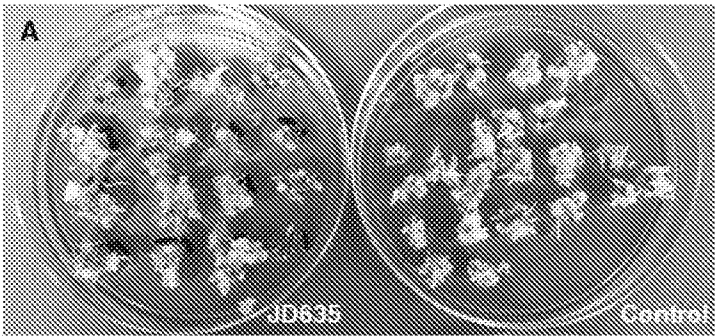


FIG. 9A

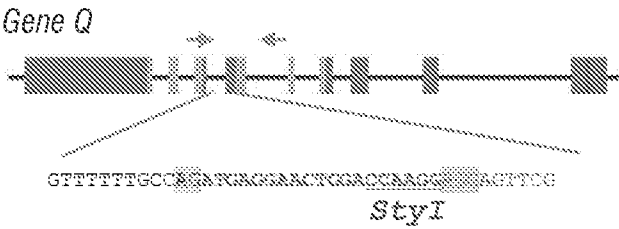


FIG. 9B

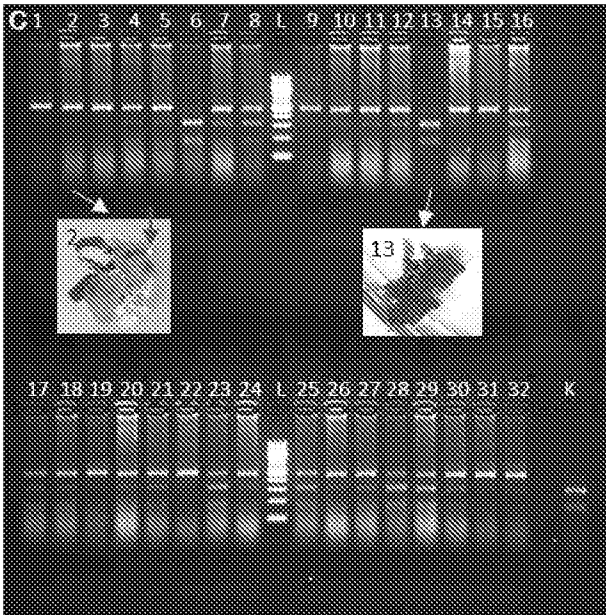


FIG. 9C

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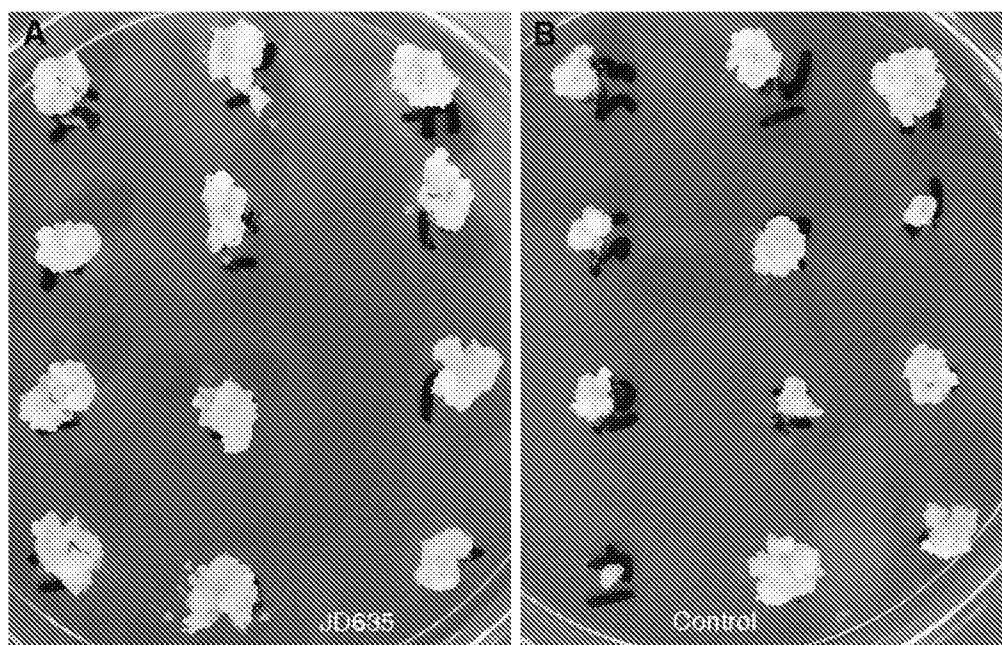


FIG. 10A

FIG. 10B

[illegible]

FIG. 11A

[illegible]

FIG. 11B

FIG. 12A

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CATGTTACCGATGCTATTTCGGAAGAACGGCAACTAAGCTGCCGGGTTTGAAACACGGATGATCTCGCGGAGGGTAGCATGTTGATTGTAACGATGAC
 AGAGCGTTGCTGCCGTGTGATCAAATATCATCTCCCTCGCAGAGATCCGAATTATCAGCCTTCTTATTCATTTCTCGCTTAACCGTGACAGGCTGTCCG
 ATCTTGAGAACTATGCCGACATAATAGGAAATCGCTGGATAAAGCCCGCTGAGGAAGCTGAGTGGCGCTATTTCCTTTAGAAAGTGAACCTTGACGATCG
 TCGACCGTACCCCGATGAATTAATTCGGACGTACGTTCTGAACACAGCTGGATACTTACTTTGGGCGATTGTCTATACATGACATCAACAATGTACCCG
 TTTGTGTAACCGTCTCTTGAGGTTCTGATGACACTAGGTCGCTACCTTAGGACCGTTATAGTTACTAGCGAATTGACATGAGGTTGCCCGTATTC
 AGTGTGCGTGATTGTTGTTCTGAAGTTGTTTTACGTTAAGTTGATGACAGATCAATTAATACGATACCTGCGTCATAATTGATTATTTGACGTGG
 TTTGATGGCCTCCAGCGCAAGTTCGGCGGCTGATTATGTCCTTGCGCAGCTCCGCTGGCGGAAGGTCTGGACCGCAAGCCTGGTGC CGCGCTCGTGGTC
 CGCGGGTTTTTCGCTATTTATGAAAAATTTCCGGTTTAAGGCGTTTCCGTTCTCTTCGTCATAAATTAAATGTTTTTATTTAAAAATACCCCTCTGAAAA
 GAAAGGAAACGACAGGTGCTGAAAGCGAGCTTTTTGGCCTGTGTCGTTTCCCTTCTCTGTTTTGTGTCGTGGAATGAACAATGGAAGGATCTTCTCG
 GCGGCGATCACGACGCCGGCCCTGCGGAGCCTTCCGCGGTGCGCGATTTCATGGCGGCCGTGGAGGCCAAGGATTTCCGCGGAGTGCAAGAGCTGAT
 CGAGGCGCTGGAGCCAAGTCGGCGGCTGATTATGTCCTTGCGCAGCTCCGCTGGCGGAAGGTCTGGACCGCAAGCCTGGTGC CGCGCTCGTGGTC
 GGGAAAGCGCGGGCAGCATGGCAATGCCGCTGCGGCGTGGGTTTTACGCCAAGGGGAGAAGCGGCATACGCCATCGAGCGGTGACGCTATGGTG
 AGCCGAGGTCCAGCATTTGCGAAGCAGTACCAGCAGGAATGGAACCGGAAGGCGGCGACTGGTGGGCGATGGCCGGTGTGGCCGGCATCATCGGCGC
 GATCCTGGCGGCGGCGGCAACCGGCTTTGTTGGGCTGGCAGTGTGATCCGCAACCGAGTGAAGCGCGTGGCGGACCTGTTGGTGTGAGCGCGGGT
 GCAGGCGCTAAGCGCAAGAGCAAGAACCGGCTTTCCGGCTTTGTTTTGTTTCCGCAAGGACAGGTTTAAAGCTTAAAGCTTTGACGTGCG
 AGAAATGTTTAGCTAAACTTCTCTCATGTGCTGGCGGCTGTCAACCGCTATGTTCAACCAAGGCGCGGAGCAAAATATGGGTGTTATCCATGAAGAAA
 CGGCTTACCAGAAAGCAGTTCCAGGAGGCGATCCAGGGGCTGGAAGTGGGGCAGCAGACCATCGAGATAGCGCGGGGCGCTTCTAGTCGATGGGAAGC
 CACAGGCGACGTTCCCAACGTGCTGGGACTGACAGGGGCGCAGTGTGCAAGCGGTGATCGCGTGTGGGCGCGCTTCGAGGACAAGAACTTGCC
 CGAGGCGTACGCGGAGTAAAGCGGCTTCTGCCGGAACATCAGGCTCAAGCGTTCGCGGCCGGAAGGTTCGAGCGTTCGAGCTGAGCGGCGGCTGAC
 CGATGAAAACTTTGGTCACGGCCAACAGAAAGGCGGCGTGGCAAGACTTCGACCTTGTGTCATCTTGCTTTCGACTTTTTTCGAGCGCGGCTTGCG
 GGTTCGCGTGTGACCTGGACCCCGAGGGCAATGCGTCTACACGCTCAAGGACTTTGCTACCGGCGTGCATGCAAGCAAGCTGTTCCGCGCTGTG
 CCTGCCGCGGCTGGACCGAAACCGCACCCGAGCGCGGCGAGGCCAGGCGCGCGCTCGCCCTCATCGAGTCCAACCCGCTACTGGCGAACCGCGC
 AACCGCTGTGCTGGACTGAGCAAGCGGCGGAGCTGTTCCGGGCAACATCAGGCTCAAGCGTTCGCGGCCGGAAGGTTCGAGCTGAGCGGCTGAGCGGCGC
 GACCTTGGCGTTCGCGCTGGCGCGCGCTCTTCCGCGGCGACTATGTGCTGTCCCCCATCGAGCTTGAGGCGTACAGCATCCAGGGCATCAAGAA
 ATGTTTCACGACCATTTGCGAAGCTGCGCCAGAAAGACGCCAAGCTGCAATTCCTTGGCATGGTGCCAGCAAGGTTCGATGCGCGGAATCCGCGCCACG
 CGCGCCACCAAGCGAGCTGCTGGCGCGTACCCCAAGATGATGATTCGCGGCCACCGTTGGCCTGCGCAGCAGCATGCGCGATGCCCTCGCATCCGG
 TGTGCGGCTCTGGAAGATCAAGAAACCGGCGCGCCAGGCTCAAGAGAGTTTCCGCGCTTGGCTGATTAGTGTTCAGGAAGTGAAGATTTCC
 CAATGACTGCGGCTCAAGCCAGACCAAGAAACACCGCTGCGGCGGCTCAGGAAGCCGAGGCGCGCGCAGCCGTCCGGCCTGGGGTTGGA
 TAGCATCGGCGACCTGTCGAGCCTCTGAGCGCTCTGCGGCGTCTCAGGCGGTTCCGCGCTATCGAGCTGGACCTGGACCTGATGACGAAGAT
 CCGCATCAGCGCGGACGGCGGACAAACCCGGCTTTTCCCGGAGAGCATCGCGGAAATCGGTGCCAGCATCAAAGAGCGCGGGGTGAAGTACACCA
 TTTCCGTCGCGGAGCAACAGGACGCGCGGCGCTATATCATCAATCAGCGGCGGCTACCGTGCTGAGTGGCGGCGGCAAGGAAGTGAAGTCCAT
 CCGCGGCTTCATCGAACACGACTACAACGAAGCCGACCGAGTTTATCGAGAACCTGCAACGCAACGAGCTGACCCCGCGCGAAATTCGCCAGTTTCATT
 GGCGCGAGCTGGCGAAGGGCAAGAAGAAAGGCGATATCGCCAAGGAAATCGGCAAGTTCGCGGCGTTCATCACCCAGCAGCTACGCTGCTGGACC
 TGCCGGAAGAAGATCGCGATGCGTTCAACACCGGCGCGTGC CGCAGCTGACCGTGTGAACGAGCTGGTGACGGCTTCAAGAAAGCGCCCGGAGGA
 AGTCGAGCGTGGCTTGACGACGACACCCAGGAAATCAGCGCGCGCAGGCTCAAGCTGCTGCGCGAGTTCTTGAGCAGAGAAGGCGCGCATCCCAAC
 ACCGTGATGCTTCAACCGGCGAGCTGATGCGGAGCGTGAGCTGAGCGGCGGCGAGCGGCAAGGCGAGGCGGCGGAGGCGGAGGCGGATTAAGGACG
 CCAAGGAAAAGGGCGGAAGGAGCCGACCGGACAAGCTGAAAAAGGCCATCGTCCAGGTCGAGCAGCAGCAGCGCCCTGCCCGCTTATCCTCAA
 CCGTCGCGCGCGCGCGGAAGGCTATGCTGGTTGAAGTACGAGGACGACGCGCAGGAGTTCGAGGCGCAACCTTGCCGAGCTGAAACTGCTCGCGCTC
 ATCGAGGCTGATCCCCAAAGACAGCGCGCGGCGCACCCGCGCGCAGACAACGGTTCCGCTACAAGGAGGACCAAGAATGAATCCGATGCTG
 TTTTCATACGCGGGAGGCGTAGGCGCGGCGTGTGCTGCTGTTTTCCGCGCATGATGCTTTCAAGCTGCGCGAGCGCAAGGAAGGAACACCGACCGCAGC
 GCAAGGCGGCGGCGCCGACGCGCGACCGGTCGATAACGAGCTGCTGCGCACTCTAGTGATATTCACAAAAACAGCAGGGAAGCAGCGCTTTTCCGC
 TGCATAACCTGCTTCGGGGTCATTATAGCGATTTTTTTCGTTATATCATCTCTTTTCGACGATATACAGGATTTTGCCAAAGGGTTCGTGTAGAC
 TTTCTTGGTGTATCAACGGCGTCAGCCGGGCGAGATAGGTGAAGTAGGCCACCCCGCAGCGGGTGTCTCTTCTACTGTCTCTTATTCGACCT
 TGGCGGTGCTCAACCGGCGAGCTGATGCGGAGCTGAGCTGAGCGGCGGCGAGCGGCAACGATGAGGGCAAGCGGATGAGTGAAGCAACCAAC
 CAGGAAGGGCAGCCACCTATCAAGGTGTACTGCTTCCAGACGAACGAAGAGCGATTGAGGAAAAGGCGGCGGCGCGGCGCATGAGCTGTGCGCC
 TACCTGCTGGCGCTCGGCCAGGCTACAAAATCACGGGCGTGTGGAATATGAGCAGCTCCGCGAGCTGGCCCGCATCAATGGCGACCTGGGCGCGC
 TGGGCGGCGTGTGAAACTCTGGCTCACCGACGACCCGCGCAGCGCGGCTGCGTATGATGCCAGGATCTCGCCCTGCTGGCGAAGATCGAAGAGAA
 GAGGACGAGCTTGCGAAGGTGATGATGCGGCAAAACCACTGATGCTGCTGATTCGCGGCGGCGGCGACGCTGCTTTGAAAGGATGACCCGCAATTCGCGGAG
 TGCCCTCGCGTCAAGGTTCGTTGACTCCAGGAGAAGAGGTAGGGGAGTTTCCAGGCGCTCGGCAATGGCCTCCATCACCTTCAACGAGGGGTTGGCC
 TTACCGTTGGTTAAGTCTGATAAAAAACGAAATGAAACCCCTGCCCTCTCCGACAGCTCATGTTTCGTCATGCCCGCTCATCGAGCAGACGAAGGA
 TGTGTTGTAAGAAATATCTGGTTGTACACAGCGGAAGCGCGCCCTCGCACCTTTGGTTCGCGGCGCGCAAAATTTAGCCGCTAAAGTTCTTGACAGCG
 GAACCAATGTTTAGCTAAACTAGAGTCTCTTTCTCAAGGAGACTTTCGATATGAGCCATAATCAGTTCCAGTTTATCGGTAATCTTAGCCGTGACA
 CCGAGGTACGTCATGGCAATTTCAACAGCCGCAAGCAATTTTCGATATAGCGGTTAATGAAGAGTGGCGCAACGATGCCGCGGACAAGCAGGAGCG
 CACCGACTTCTTCCGCATCAAGTGTTTTGGCTCTCAGGCGGAGGCCACGCGAAGTATTTGGCAAGGGGTTCGTGGTATTCGTGCAGGGGAAGATT
 CGGAATACCAAGTACGAGAAGGACGGCCAGACGGTCTACGGGACCGACTTCATTGCCGATAAGGTGGATTATCTGGACACCAAGGCACCGGCGGT
 CAAATCAGGAATAAGGGCACATTTGCCCGGCGTGTGTCGGGCAACATCCGCAAGGAGGCTGAATGAATCGGACGTTTGACCGGAAGGCATACAGGCA
 AGAACTGATCGACGCGGGGTTTTCCGCGGAGGATGCCGAAACCATCGCAAGCCGACCGTCTGCGTGC CGCGCCCGGCAAACTTCCAGTCCGTGCGC
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 CCTGCGCAAAACAGGTGACGAGGCGCAAGGCGGCTGCTGTAACACACGAAGCAGAGATCAAGGAAATCGAGCTTCCCTTGTGTTGTTGCGTGGC
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 ACAAGGTCATTTTCCACGTCAACAAGGACGTGAAGATCACCTACACCGGCGTCGAGCTGCGGCGGACGATGACGAACCTGGTGGGAGCAGGTGTT
 GGAGTACGCGAAGCGCACCCCTATCGGCGAGCGGATCACCTTACGTTCTACGAGCTTTGCCAGGACCTGGGCTGGTTCGATCAATGGCGGGTATTAC
 ACGAAGGCCGAGGAATGCTGTCGCGCTACAGGCGCAGCGGCTGGCTTCCGACCGGCTGGGCGGTTGGGCAACTGGGATCGGTGCTGCTGTCGAC
 GCTTCCGCGTCTGGACCGTGGCAAGAAACGTCCCGTTGCCAGGTCTGATCGACGAGGAAATCGTGTGCTGTTTTGCTGGCGACCACTACACGAA
 ATTCATATGGGAGAAGTACCGCAAGCTGTGCGCGACGGCGGCGGATGTTGCGACTATTTACGCTCGCACCGGAGCGGTACCGGCTCAAGCTGGAA
 ACCTTCCGCTCATGTGCGGATCGGATTCACCCGCGTGAAGAGTGGCGCGAGCAGGTTCGCGAAGCCTGCGAAGAGTTGCGAGGCGAGCGGCTGG

FIG. 12B

FIG. 12C

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ATGAAGCAAAGCTTTGTGGGGTTTCTTCACAAACAGAGGCTGAGACAAGAGCCAGAGAGAGTGAACAC
 AGACACACCCACAAAGGAACCTCAGTCACTGTCCCCAAAAACATCCTCAAAACTTGCAAAGAACCCAAAA
 TGGTGGGTCTGTGGGGGGGCATCAATGGCAACAGGGATGATGGTGAGGTCATCACCATTACAGTGTCT
 CAGTGGCAGGAGCTGGAGCATCAAGCTTTGATCTTTAAGTATCTAATGGCAGGACTACCCGTCACCTG
 ATCTTGTGCTTCCTATTGAGAAGAGCTTTGAATCTATGTCTTCTAGGTTCTTCACCATCCTACCATGGG
 TTAAGTGTCTTCTATGGGAAGAAGGTGGACCGGAGCCAGGTAGATGCCGAGGACTGATGGCAAGAAA
 TGGAGGTGCTCCAAAGACGCCTACCCGAGCTCCAAGTACTGTGAGCGCCACATGCACCGTGGCCGCAACC
 GTTCAAGAAAGCCTGTGGAATCACAACTACTACACAGTCTTCTTCGACTGTGACTTCACTAACTGTAC
 TGGAGGCAGTGGCAGTGGAGCCGGGACCTTCCAGAACCTTCCCTTGCAAACTTCAATAACCCCAAGGC
 GGCCCGGGGTCCGGAACCAACCAATCCCAATTCCATATGGAGCCCATTCCTATGGCATTCCTAATAAAG
 ATTACAGGTATCTTCATGGACTTAAACCCGAGGTAGGCGAACATAGTTTCTTCTCTGAAGCTTCAGGAAG
 CAACAGGGGTCTTCAGATGGACTCGCCTTTGGACAGCACATGGCCTCTGATGCCATCACGGGTCTCATCA
 TTCCCCCATCAAAATCAAGTGAAAACCTCATCTTGCAGAGTGACTACCCCGAGCATTCGTTCTTCAGCA
 GCGAATTTGCGTCGGGAGAGGTTGTGAAACAGGAGGGTCACTCTCTCCGTCTTCTTTTGACGAGTGGCC
 TAAACCAGAGACTCCTSGTCCGGCCTTGAGGAAGAGAGGTCCAACCAGACCTCATTCTCCACAACCCAG
 CTCTCAATTTTCGATCCCAATGGCCTCCTCTGACTTCTCAACAACCAGCTCTCGGTCTCCCCACGATAACg
 cggccgctgccatGCAGCCCATGATGGCAGCCTATTACCCAGCAACGTCACCACTGATCACATTCAGCA
 GTATCTTGATGAAAACAAGTCATTGATTCTGAAGATTGTTGAGAGCCAGAATTCAGGAAAATTGACTGAA
 TGTGCAGAGAACCCAGGCAAGACTACAGAGAAACCTCATGTACCTGGCTGCAATGCTGATCTCAACCCC
 AACCACCCACCATGCATGCTCAGTTCCTCTCTAGTGGCATTGTTGAGCCAGGAGCTCACTACATGCAGCA
 CCAACAAGCTCAACAAATGACACACAGTGGCTCCTGGCTGCACGCTCCTCCATGCTGTACTCCCAACAA
 CCATTTTTCGGCCCTGCAACAACAACAAGCCATCCATAGCCAGCTTGGCATGGGCTCTGGTGGAGTGCAG
 GACTTCACATGCTGCAAAAGCGAGGGGAGTAATCCAGGAGGCAATGGAACACTGGGGACTGATATGGGAAA
 TGCAGAAAGGCGAGGAGGGAACCTCAGGAGGTGAGGTTGGGATGAGGTTGAGACTCTTACTTGAAAGCT
 GCTGAAGATGGGAATTGA

FIG. 13A

MKQSEVGVSSQTEAETFAFESDTQTHFQRNLSHCPQKHQPONLQRTQNGGSSVGGASMATGMMVRESPTTVS
 QWQELEHQALIFKYLMAGLFVFPDLVLPPIQKSEFESMSSRFFHHPTMGYCSFYGKKVDPEPGRCPRTDGGK
 WRCSKDAYPDEKYCERHMHRRNRNRKPVESQTTTQSSSTVTSLSLTVTGGSGSGAGTFQNLPLQNFNNPQG
 GPGSGTNQSQFHMEPIPYGIPNKDYRYLHGLKPEVGEHSFFSEASGSNFGQLQMDSPLDSTWPLMPSRVSS
 FPFSSKSSSENSILQSDYPQHSFFSSEFASGEVVVKQEGQSLRPFDFEWPKTRDSWSGLEEERSNQTSFSTTQ
 LSISIPMASSDFSTTSRSPHDNAAAAAQPMMAAYYPSNVTTDHIQQLDENKSLILKIVESQNSGKLTE
 CAENQARLQRNLMYLAALADSQFPPTMBAQFPFSGIVQPGAHYMQBQQAQOMTPQSLLAARSSMLYSQQ
 PFSALQQQQAHSQQLGMSSGGSAGLHMLQSEGSNFGNGTLGTDMGNAEGPGGNSGGQGGGSGGETLYLKA
 AEDGN

FIG. 13B

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>TraesCS6A01G269600 TaGRF4

PFTAAQYEELEHQALIYKYL VAGVSVPPDLVLP I R D P E P G R C R R T D G K K W R C A K E A A S D S K Y C E R H M H R G
R N R S R K P V E

FIG. 14A

Triticum aestivum cultivar Chinese Spring

>TraesCS6A01G335900 TaGRF1

M M M M G G R A G A G G V G A G G G R C P F T A T Q W Q E L H Q A L I Y K Y M A S G V P I P S D L L L P L R R S F L L D S A L A T S P S L
A F P P Q A A L G W G C F G M G F G R K A E D P E P G R C R R T D G K K W R C S K E A Y P D S K Y C E K H M H R G K N R S R K P V E M S L A
T P P P P S S S A S S S S S N V H S A V N V A T T T T S P A P S Y H R H A A A T H D T T P Y H A L Y G G P Y S S A G R Q Q H A S A Y H H A
A Q V S P F H L H L D T T H P H P P S Y Y S T M D H S K D S Y A Y G H S V K E V H G G G E H A F F S S D V S T D R D H H H H Q H Q H H A S
A G G N G Q W Q F K Q L G G M E P K Q H N P T S L F P G Y G N N A A Y A I D L S S K E E D E E K E R R Q Q Q Q H C F L L G A D L R L D K P S
S G H G D S A D Q K P L R P F F D E W P H E K T G S K G S W M G L E G E T Q L S I S I A N E L P I T T T S R Y H H G E

>TraesCS7A01G165600 TaGRF2

M M L G G H G G G G R C L F T A S Q W R E L H Q A L I Y K Y M A A G S Q V P H E L V L P L R H R D A A A F A G I D T A P S V A C Y P P P
Q P S L G W G L Y G A G A Q Y A R K P E D P E P G R C R R T D G K K W R C S R E A Y G E S K Y C D R H M H R G K N R S R K P V E P M S S S S
V S S P A A S Y R Q T T L S M S P P T P A D T P S Y G H G L R A A A S Q S Q I N P L Q L H L D T P S P P S Y H R Y A P A Q Q Y G G S F F
P S R Q Q V Q E E A E A E A R R R Q H F L A L G A D L S L D K P D A T T A A S S T T E E K P L R R F F D E W P R D G N A V E V R P W N M G H
R D E T L L S M S I P T T T A S H P D L A A Y R H H N G T F A

>TraesCS2A01G435100 TaGRF3

M A M P F A S L S P A A D H R S S P I F P F C R S S P L Y S A G E E A A Q Q Q Q Q Q H A M S G A R W A A A R P A T F T A A Q Y E E L E Q
Q A L I Y K Y L V A G V P V P P D L L L P I R R G F D S L A S R F Y H H H A L G Y G S Y F G K K L D P E P G R C R R T D G K K W R C S K E A
A Q D S K Y C E R H M H R G R N R S R K P V E T Q L V S H S Q Q L Q Q Q A P A A F H G H S P Y P A I A T G A G A P G S F A L G S T A Q L H
M D N A A P Y A T A G A A G N K D F R Y S A Y G F R T S A M E D H N Q F I S A A M D T A M D N Y S W R L L P A Q N S S F S L S S Y P M L S
T L S D L D Q S A I C S L A K T E R E P L S F F G V G G G F D D D E S A V K Q E N Q T L R P F F D E W P K D R D S W P E L Q D H D S N H N N
E A F S A T K L S I S I P V T S S D F S T T A G S R S P H G I Y S R

>TraesCS6A01G269600 TaGRF4 (used in presented transgenic events)

M A M P Y A S L S P A G D R R S S P A A T A T A S L L P F C R S S P F S A G G N G M G E E A R M D G R W M A R P V P F T A A Q Y E E L E H
Q A L I Y K Y L V A G V S V P P D L V L P I R R G I E S L A A R F Y H N P L A I G Y G S Y L G K K V D P E P G R C R R T D G K K W R C A K E
A A S D S K Y C E R H M H R G R N R S R K P V E T Q L V P H S Q P P A A S A V P P L A T G F H S H S L Y P A I G G G T N G G G G G N N G M
S M P G T F S S A L G P P Q Q H M G N N A A S P Y A A L G G A G T C K D F R Y T A Y G I R S L A D E Q S Q L M T E A M N T S V E N P W R L P
P S S Q T T T F L S S Y S P Q L G A T S D L G Q N N S S N N N S G V K A E G Q Q Q Q P L S F P G C G D F G S G D S A K Q E N Q T L R P F
F D E W P K T R D S W S D L T D D N S N V A S F S A T Q L S I S I P M T S S D F S A A S S Q S P N G M L F A G E M Y

>TraesCS7A01G049100 TaGRF5

M L S S S A A M G M L G G Y G Q Q Q Q Q Q M Q M Q R G A G P V F T P A Q W A E L E Q Q A L I Y K Y L M A G V P V P P D L L L P I R P
H P A G A G A T F S F A N P A A S P F Y H H H H P S M S Y Y A Y Y G K K L D P E P W R C R R T D G K K W R C S K E A H P D S K Y C E R H M H
R G R N R S R K P V E S K S A S P A H Q S Q Q P P L S A V T S A T R D A E P L P S L P A G A K T H G L S L G G A G S S Q M H V D A S S Y G N
K Y S L G A K S D V G E L S F F S G A S G N N N R G F T I D S P T D S S W H S M G S S L P P Y Q L S K P R D S G L M Q G G F S Y S H F E P S
Q E L G Q V T I A S L S H S Q E Q D R R S F G G G G G G G G G A G L M G N V K Q E N Q P L R P F F D E W P G R R D S W S E M D D E R S N G
T S F S T T Q L S I S I P M P R C D

FIG. 14B

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Oryza sativa subsp. japonica

>LOC_Os02g53690.1 OsGRF1

MMMSGRPSGGAGGGGRYPFTASQWQELEHQALIYKYMASGTPIPSDLILPLRRSFLLD
 SALATSPSLAFP
 PQPSLGWGCFGMGFGRKAEDPEPGRRCRRTDGKKWRCSKEAYPDSKYCEKHMHRGKNRSRKP
 VEMSLATPP
 PPSSSATSAASNTSAGVAPTTTTTSSPAPSYSRPAPHDAAPYQALYGGPYAAATARTP
 AAAAYHAQVSPF
 HLQLDTTHPHPPPSYYSMDHKEYAYGHATKEVHGEHAFFSDGTEREHHHAAAGHGQWQFQ
 LGMPEPKQST
 TPLFPGAGYGHTAASPYAIDLKEDDDEKERRQQQQQQQQHCFLLGADLRLEKPA
 GHDHAAAAQKPLRH
 FFDEWPHEKNSKGSWMGLEGETQLSMSIPMAANDLPITTTSTRYHNDD

>LOC_Os06g10310.1 OsGRF2

MMAGGGSGRCLFTATQWQELEHQALIYKYMAAGAPVPPDLLLHLRHRAAAAAAADVDT
 VPSLAFPPHHLG
 WGCYGA AAAAQYGRRVEDPEPGRRCRRTDGKKWRCSREAYGESKYCEKHMHRGKNRSRKP
 VEMPPPA AAVY
 RPSALSISPPPHDADAPSYGAGAGAPLQLHLD SFHASTSPPPSYHRYAHTSSAPLFPSSA
 AGYGGGWSLS
 KEHCLTLGGAAADLSLDKPADHHHDATSATTEKPLRRFFDEWPRSDDGRTPWDGTQLS
 ISIPTAAAASPD
 LAIAGAASRYHSNGDHLRTSE

>LOC_Os04g51190.1 OsGRF3

MAMPFASLSPAADHRPSFIFPFCRSSPLSAVGEEAQQHMMGARWAAAVARPPPFTAAQYEE
 LEQQALIYK
 YLVAGVPVPADLLLPIRRGLDSLASFYHHPVLGYGSYFGKKLDPEPGRRCRRTDGKKWRCS
 KEAAPDSKY
 CERHMHGRGNRSRKPVEAQLVAPHSQPPATAPAAAVTSTAFQNHSLYPAIANGGGANGG
 GGGGGGGSAP
 GSFALGSNTQLHMDNAASYSTVAAGAGNKDFRYSAYGVRPLADEHSPLITGAMDTSIDNS
 WCLLPSQTST
 FSVSSYPMLGNLSELDQNTICSLPKVEREPLSFSGSDYVTVDSGKQENQTLRPFFDEWP
 KARDSWPD
 LAD
 DNSLATFSATQLSISIPMATSDFTSSSRSHNGIYSR

>LOC_Os02g47280.1 OsGRF4

MPPCLRRWPTTARPRQPRPPPSSPSAAPPRS PRKQREPAATTHFLGSSGACDNTVRRCVW
 VGGCRGGGGV
 AMGEDAPMTARWPPAAAARLPPFTAAQYEELEQQALIYKYL VAGVPVPPDLVLP
 IRRGLDSLAA RFYNHP
 ALGYGPYFGKKLDPEPGRRCRRTDGKKWRCSKEAAPDSKYCERHMHGRGNRSRKP
 VETQLVAQSQPPSSV
 V
 GSAAAPLAAASNGSSFQNHSLYPAIAGSNGGGGGRNMPSSFGSALGSQLHMDNAAPYAA
 VGGGTGKDLRY
 TAYGTRSLADEQSQLITEAINTSIENPWRLPSQNSPFPLSSYSQLGALSDLGQNT
 PSSLSKVQRQPLSF
 FGNDYAAVDSVKQENQTLRPFFDEWPKGRDSWSDLADENANLSSFGTQLSISIPMASSD
 FSAASSRSTN
 GD

>LOC_Os06g02560.1 OsGRF5

MLSSSPSAAAPGIGGYQPQRGA AVFTAAQWAELEQQALIYKYL VAGVPVPGDLLLPIRPH
 SSAAATYSFA
 NPAAAPFYHHHHHPSLSYYAYYGKKLDPEPWRRCRRTDGKKWRCSKEAHPDSKYCERH
 MHGRGNRSRKPVE
 SKTAAPAPQSQPQLSNVTTATHDTDAPLPSLTVGAKTHGLSLGGAGSSQFHV
 DAPSYGSKYSLGAKADV
 G
 ELSFFSGASGNTRGFTIDSPDSSWHSPLSSVPPYPMSKPRDSGLLP
 GAYSYSHLEPSQELGQVTIASLS
 QEQRERSFGGGAGGMLGNVKHENQPLRPFFDEWPGRRDSWSEMDEERSNQTSFSTTQLS
 ISIPMPRCGSP
 IGPRLP

FIG. 15

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Arabidopsis thaliana

>AT2G36400.1 AtGRF3

MDLQLKQWRSQQQQQHQTSEEEQPSAAKIPKHVFDQIHSHSTATSTALPLFTPEPTSSKLSSLSPDSSSRF
 PKMGSSFFSWAQWQELELQALIIYRYMLAGAAVPQELLPIKKSLLHLSPSYFLHHPLQHLPHYQPAWYLGR
 AAMDPEPGRCRRTDGKKWRCSRDVFAGHKYCERHMHGRNRSRKPVETPTTVNATATSMASVAAAATTT
 TATTTSTFAFGGGGGSEEVVGQGGSSFFSGSSNSSSELLHLSQSCSEMKQESNNMNNKRPYESHIGFSNN
 RSDGGHILRPFFDDWPRSSLQEADNSSSPMSSATCLSI SMPGNSSSDVSLKLSTGNEEGARSNNNGRDQQ
 NMSWWSGGGSNNHHHNMGGPLAEALRSSSSSSSPTSVLHQLGVSTQAFH

>AT3G52910.1 AtGRF4

MDLQLKQWRSQQQNESEEQGSAAATKISNFFFDQIQSQTATSAAAAPLPLFVPEPTSSSSSFSCFSPDSSNS
 SSSSRFLKMGNFFSWAQWQELELQALIIYRYMLAGASVPQELLPIKKSLLHQSPMHFLHHPLQHSFPHHQ
 PSWYWGREGAMDPEPGRCRRTDGKKWRCSRDVVAGHKYCDRHIIHRGNRSRKPVETATTTITTTATTTASS
 FVLGEELGHGPNNNHFFSSGSSQPLHLHQSCSSEMKQESNNKRPYEANSFGSNGRSDDGHLRHFFD
 DWPRSSDSTSSPMSSSTCHLSISMPGNNTSSDVSLKLSTGNEEEENMRNNNNEREQMNWWSNGGNHNN
 MGGPLAEALRSASSTSSVLHQMISTQVFH

>AT3G13960.1 AtGRF5

MMSLSGSSGRTIGRPPFTPTQWEELEHQALIIKYMVSGVPVPPELIFSIRRLDTSLSVSRLLPHQSLGWG
 CYQMGFGRKPDPEPGRCRRTDGKKWRCSREAYPDSKYCEKMHHRGNRARKSLDQNTTTTPTLSPSLSF
 TNNNNPSPTLSSSSSSNSSSTTYSASSSSMDAYSNSNRFLGGSSSNTRGYFNHSLDYPPSTSPKQQQ
 QTLHHASALS LHQNTNSTSQFNVLASATDHKDFRYFQGIGERVGGVGERTFFPEASRSFQDSPYHHHQQP
 LATVMNDPYHHCSTDHNNKIDHHHTYSSSSSQHLHHDHHRQQQCFVLGADMFNKPTRSVLANSSRDQDQ
 QEEDEKDSSESSKSLHHFFGEDWAQNKNSSDSWLDLSSHSRLDTGS

>AT2G06200.1 AtGRF6

MATRIPFTESQWEELENQALVFKYLAANMPVPPHLLFLIKRPFLEFSSSSSSSSSSSSSFFSPTLSPHFGWNV
 YEMGMGRKIDAEPPGRCRRTDGKKWRCSKEAYPDSKYCERHMHGRNRSRKPPPTQFTPNLFLDSSSR
 RRGYMDFFSIEPSGSIKSCSGSAMEDNDGSCRGINNEEKQPDHRCFILGTDLRTREPLMLEEKLKQ
 RDHDNEEEQGSKRFRFLDEWPSSKSSVSTSLFI

FIG. 16A***Vitis vinifera* GRF**

Vitis vinifera GRF GSVIVT01024326001

MKQSFVGVSSQTEAETRARESDTQTHPQRNLSHCPQKHPQNLQRTQNGGSVGGASMATGMMVRSSPFTVS
 QWQELEHQALIFKYL MAGLPVPPDLVLPIQKSFEISMSSRFFHHPTMGYCSFYGKKVDPEPGRCRRTDGKK
 WRCSKDAYPDSKYCERHMHGRNRSRKPVESQTTTQSSSTVTSLTVTGGSGSGAGTFQNLPLQNFNNPQG
 GPGSGTNQSQFHMEPIPYGIPNKDYRYLHGLKPEVGEHSFFSEASGSNRGLQMDSPLDSTWPLMPSRVSS
 FPPSKSSENSILQSDYPQHSFFSSEFASGEVVKQEGQSLRPFFDEWPKTRDSWSGLEEERSNQTSFSTTQ
 LSISIPMASSDFSTTSSRSPHDN

FIG. 16B

19/32***Arabidopsis thaliana* SNH conserved domain highlighted**

>AtGIF1

MQQHLMQMMPMAGYPSNVTSDHIQQYLDENKSLILKIVESQNSGKLSECAENQARLQRNLMYLAADIAD
 SQPQPPSVHSQYGSAGGGMIQGEGGSHYLQQQQATQQQQMTQQSLMAARSSMLYAQQQQQQQPYATLQHQ
 QLHHSQLGMSSSSGGGSSGLHILQGEAGGFHDFGRGKPEMGSGGGGEGRGSSGDGGETLYLKSSDDGN

>AtGIF2

MQQQQSPQMFPMPVPSIPPANNITTEQIQKYLDENKKLIMAIMENQNLGKLAECAQYQALLQKNLMYLAADI
 ADAQPPPPTPGPSPSTAVAAQMATPHSGMQPPSYFMQHPQASPAGIFAPRGPLQFGSPLQFQDPQQQQQI
 HQQAMQGHMGIRPMGMTNNGMQHAMQQPETGLGGNVGLRGKQDGADGQGKDDGK

>AtGIF3

MQQSPQMI PMVLPSFPPTNNITTEQIQKYLDENKKLIMAILLENQNLGKLAECAQYQALLQKNLMYLAADIA
 DAQPQPPAATLTSGAMTPQAMAPNPSSMQPPSYFMQQHQAVGMAQQIPPGIFPFRGPLQFGSPHQFLDP
 QQQLHQQAMQGHMGIRPMGLNNNGLQHQMHHHETALAANNAGPNDASGGGKPDGTNMSQSGADGGGSA
 ARHGGGDAKTEGK

FIG. 17A**Wheat SNH conserved domain highlighted**

>TraesCS4A01G250600 TaGIF1

MQQQHLMQMNQSMGGYASSTTVTTDLIQYLDENKQLILAILDNQNNGKVEECARNQAKLQQNLMYLAADI
 ADSQPPQTASLSQYPSNLMMQSGPRYMQQQSAQMMSQSLMAARSSMMYAQQAMSPLQQQQQQQQHQQA
 ASHGQLGMSSGATTGFNLLHGEASMGGGGGATGNSMMNASVFSQDYGRGGSGAKEGSTLSADARGANSQA
 HSGDGEYLLKGTEEEGS

>TraesCS4A01G151200 TaGIF2

MQQAMSLPPGAVGAVSPPAGITTEQIQKYLDENKQLILAILLENQNLGKLAECAQYQAQLQKNLLYLAADIA
 DAQPPQNPASRPQMVMVQQPGSMQGAGHYMSQVPMFPPTPLTPQQMQEQQHQQQLQQQQAQALSFPQVVMR
 PGTVNGMQQPMQAAGDLQPAAPGGSKQDAVAGASSEPSGTSKSHKNAGAEVGVADVAEQS

>TraesCS5A01G107700 TaGIF3 Re-annotated 1st two exons to match
 Brachypodium and rice orthologs

MQQAMPMPAAAAPGMPPSAGLSTEQIQKYLDENKQLILAILLENQNLGKLAECAQYQAQLQKNLLYLAADI
 ADTQPQTTSRPMAPPSASPGAGHYMSQVPMFPPTPLTPQQMQEQQLQQQQAQMLPFAGQMVARPGAV
 NGMPQAPQVEPAYAAGGASSEPSGTESHRSTGADNDGGSGGLADQS*

FIG. 17B

20/32***Vitis vinifera* GIF**

>SeqID#30 Vitis vinifera GIF GSVIVT01036262001
MQPMMAAYYPSNVTTDHIQQYLDENKSLILKIVESQNSGKLTECAENQARLQRNLMYLAAIADSQPQPPT
MHAQFPFSGIVQPGAHYMQHQQAAQQMTPQSLAARSSMLYSQQPFSAQQQQAIHSQLGMGSGGSAGLHM
LQSEGSNPGNGTLGTDMGNAEGRGGNSGGQGGDGGETLYLKAAEDGN

FIG. 17C

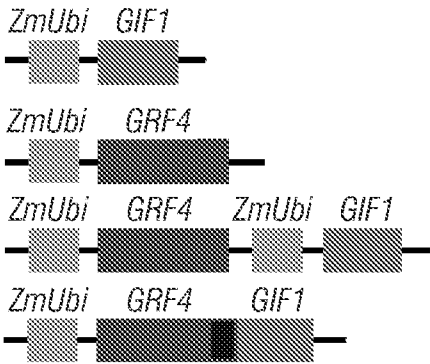


FIG. 18A

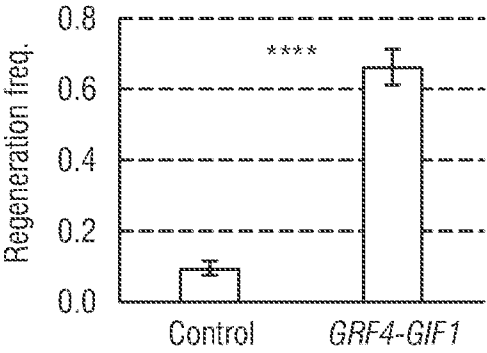


FIG. 18B

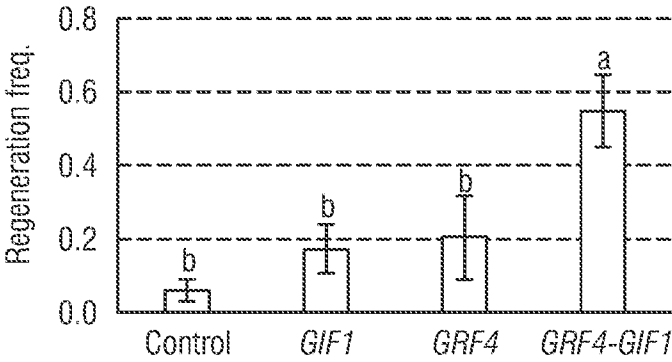


FIG. 18C

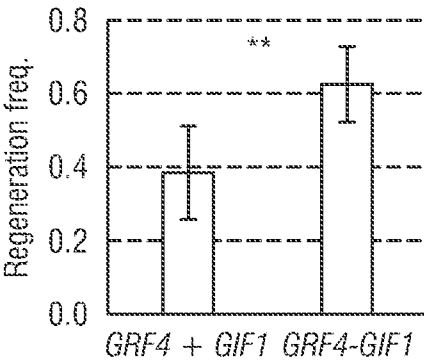


FIG. 18D

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GRF homologs
QLQ WRC

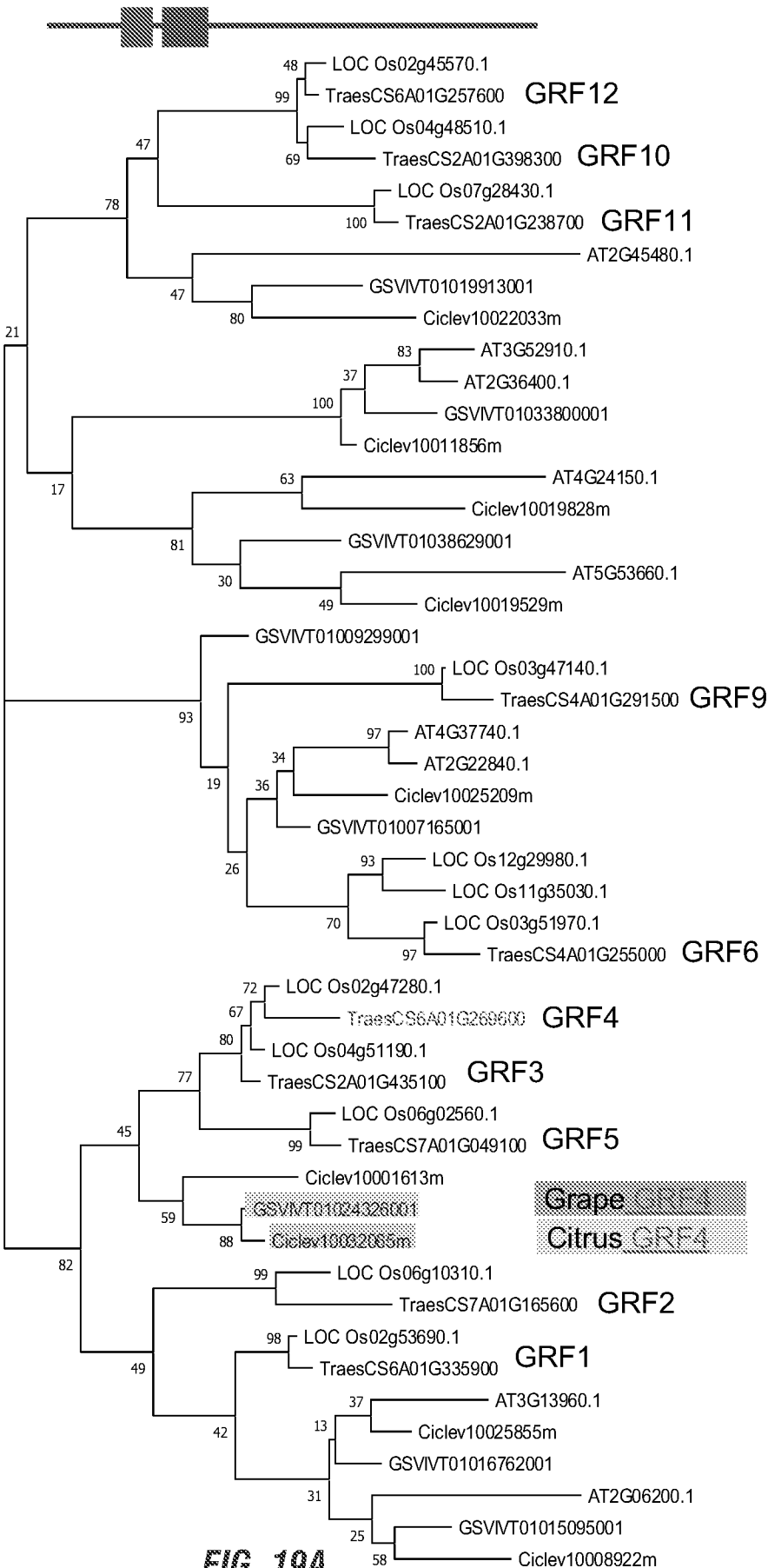


FIG. 19A

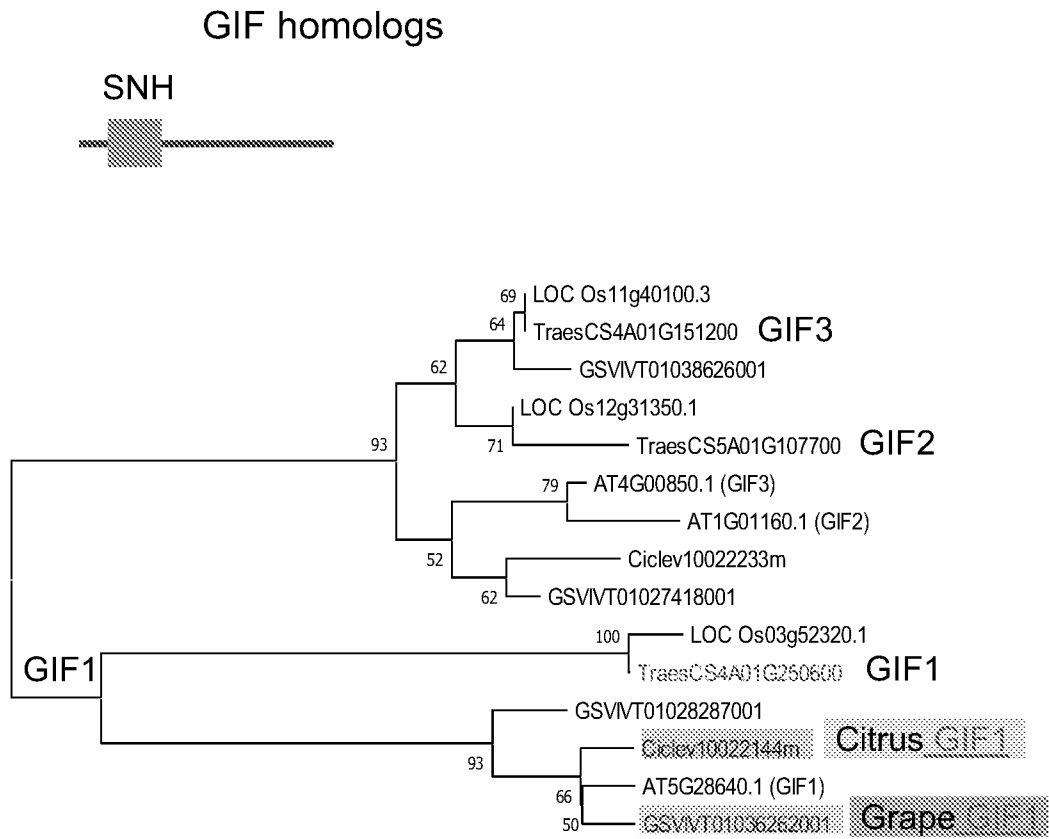


FIG. 19B

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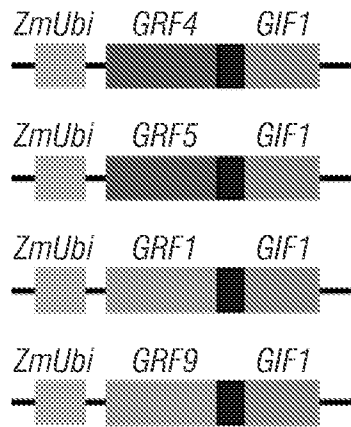


FIG. 20A

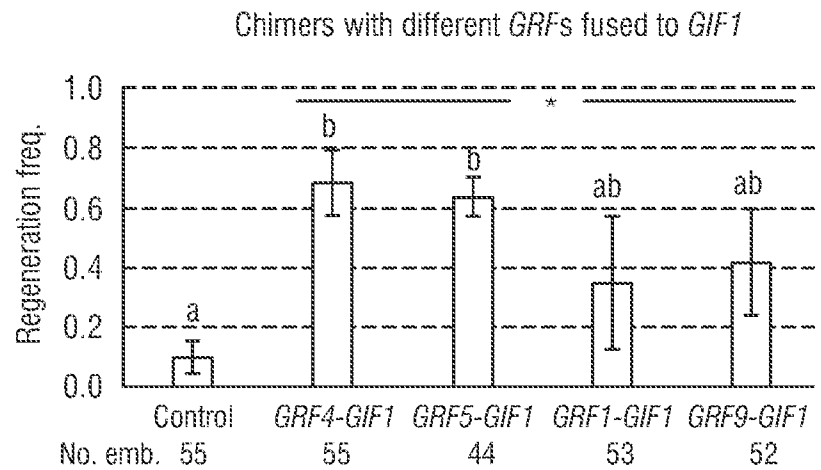


FIG. 20B

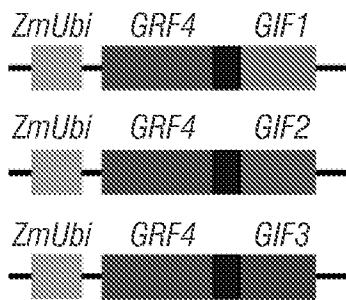


FIG. 20C

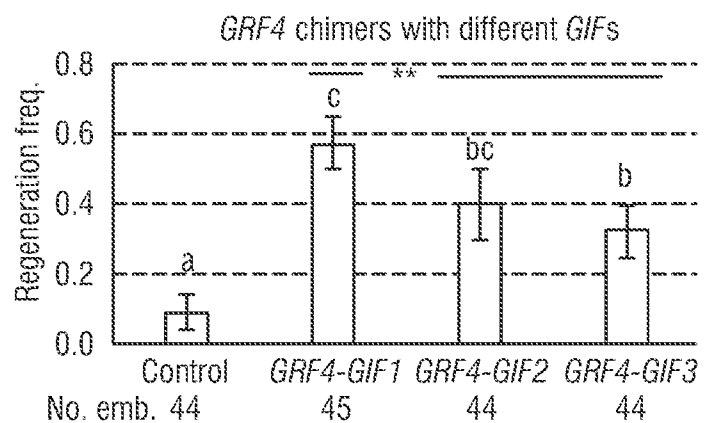


FIG. 20D

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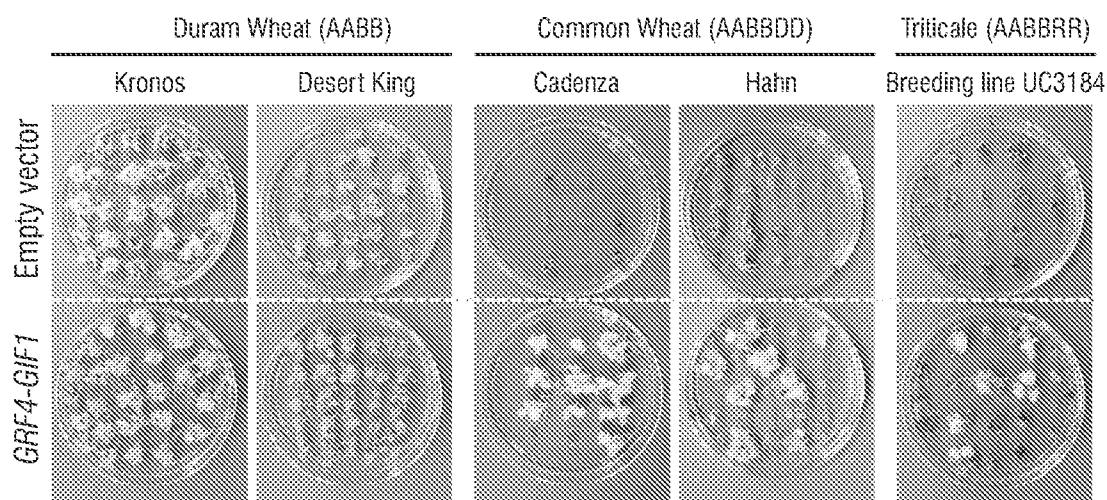


FIG. 21A

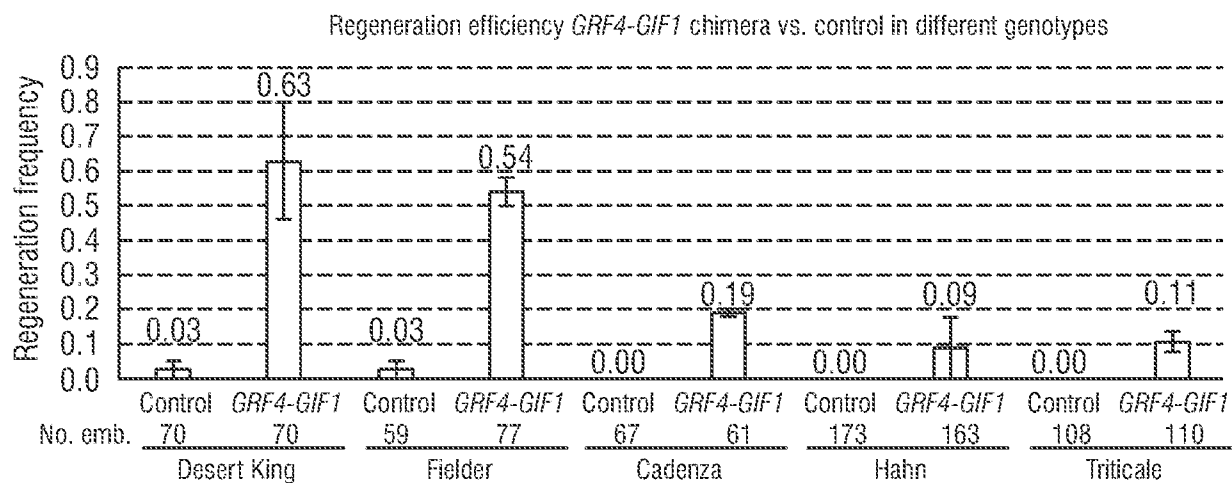
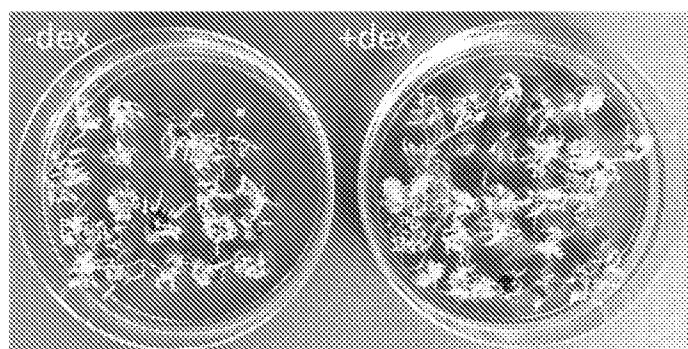
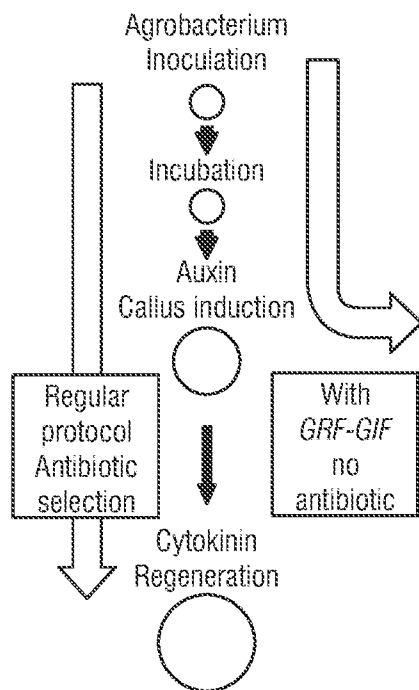
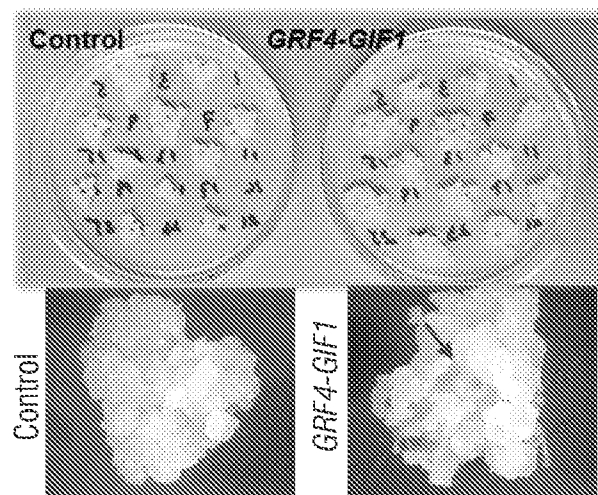


FIG. 21B

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**FIG. 22A****FIG. 22B**

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**FIG. 23A****FIG. 23B****FIG. 23C**

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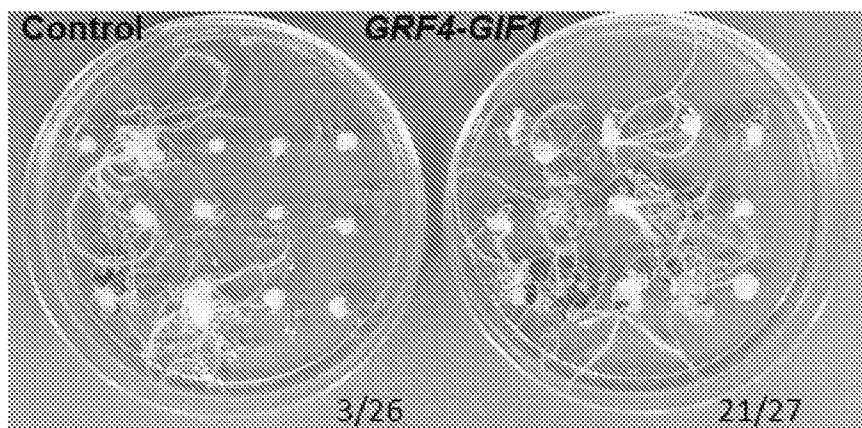


FIG. 24

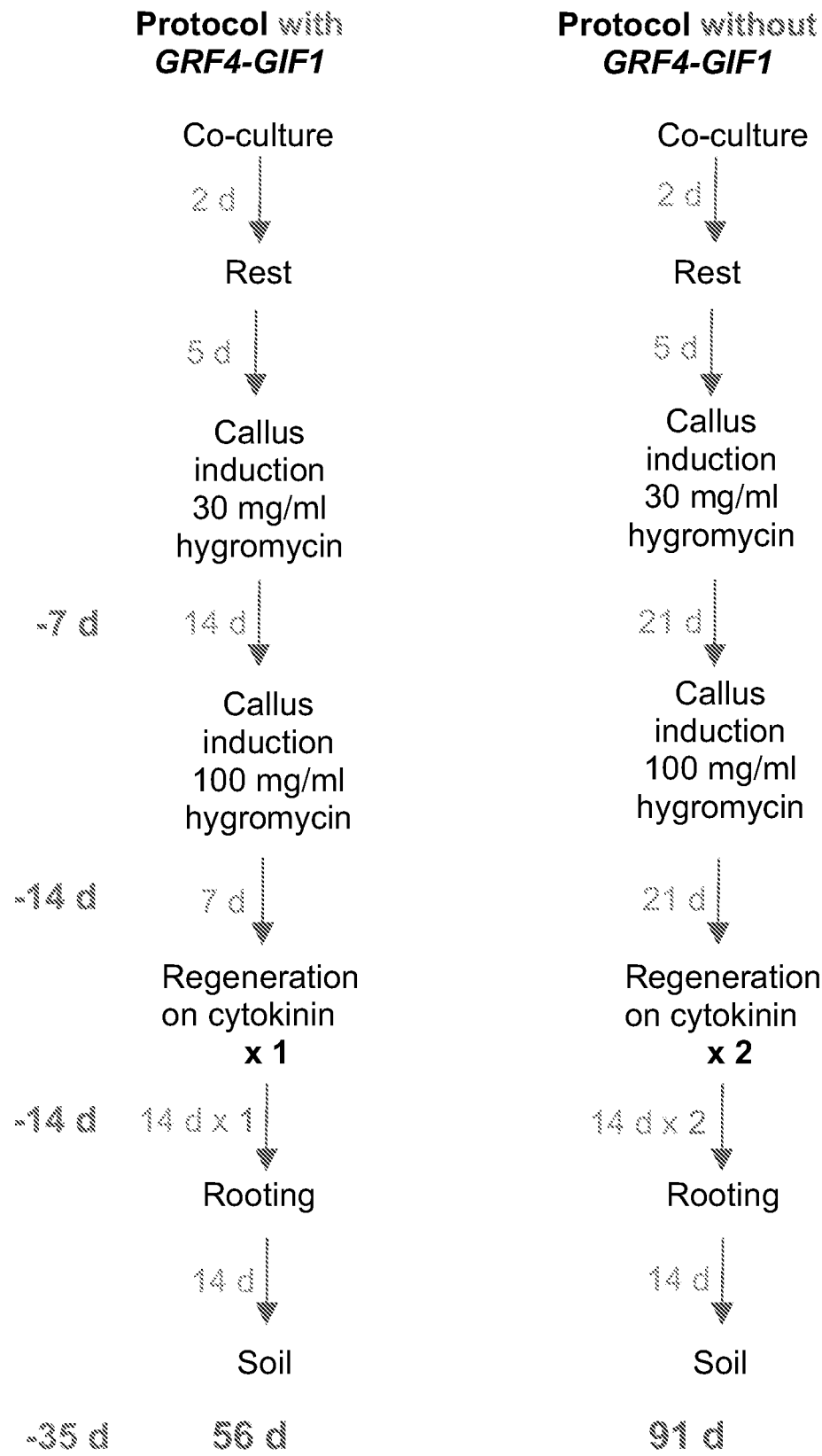


FIG. 25

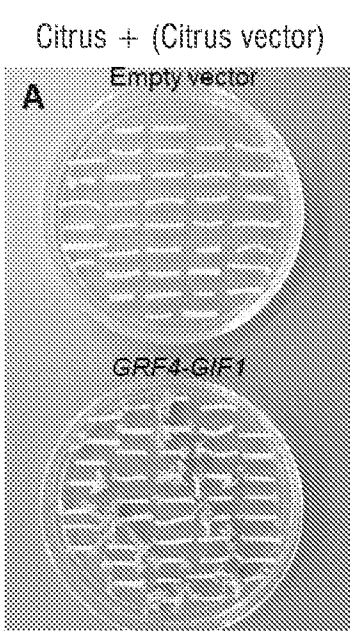


FIG. 26A

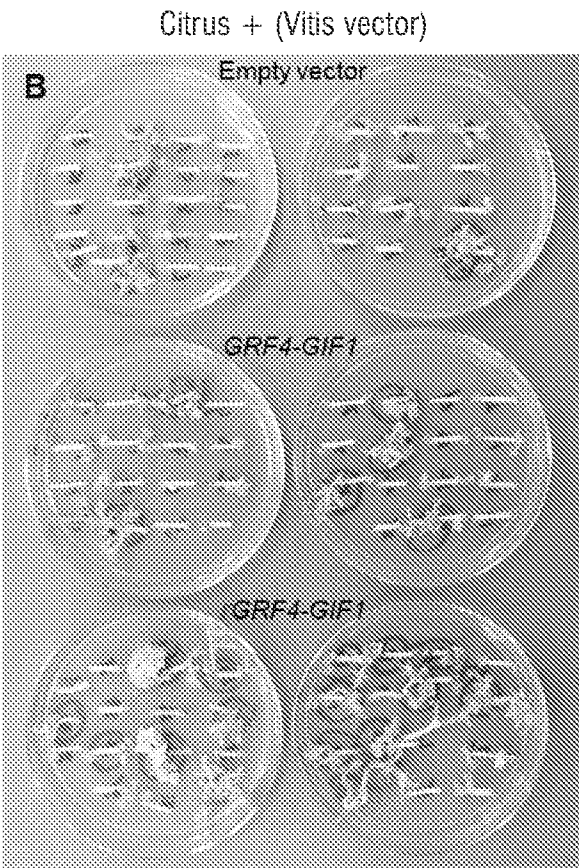


FIG. 26B

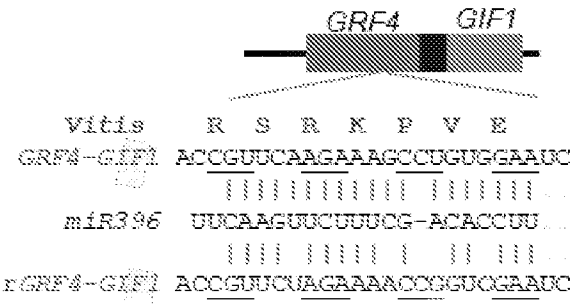


FIG. 26C

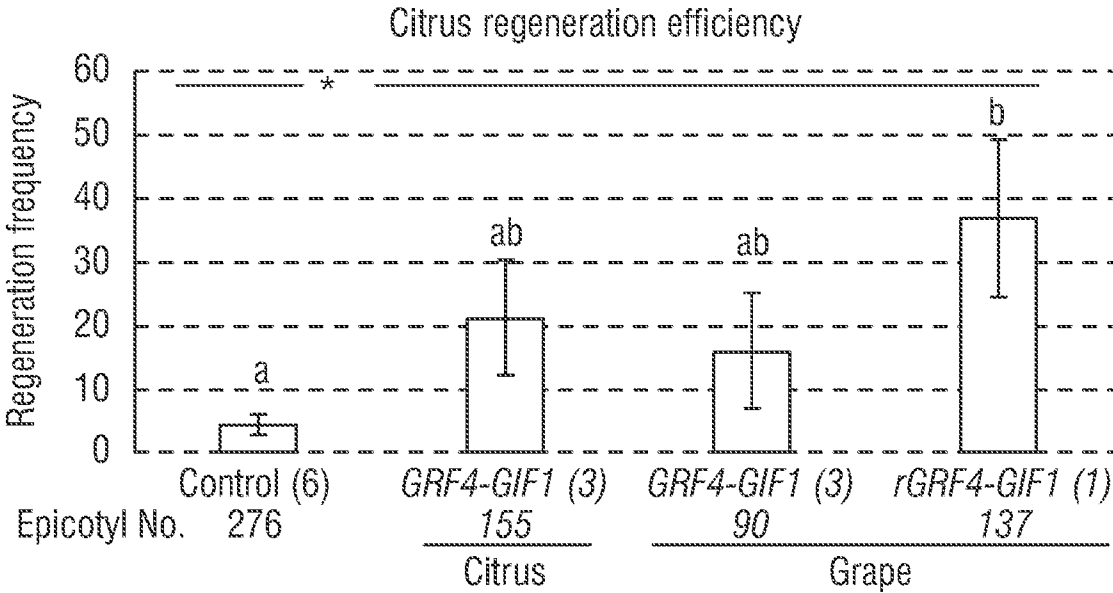


FIG. 26D

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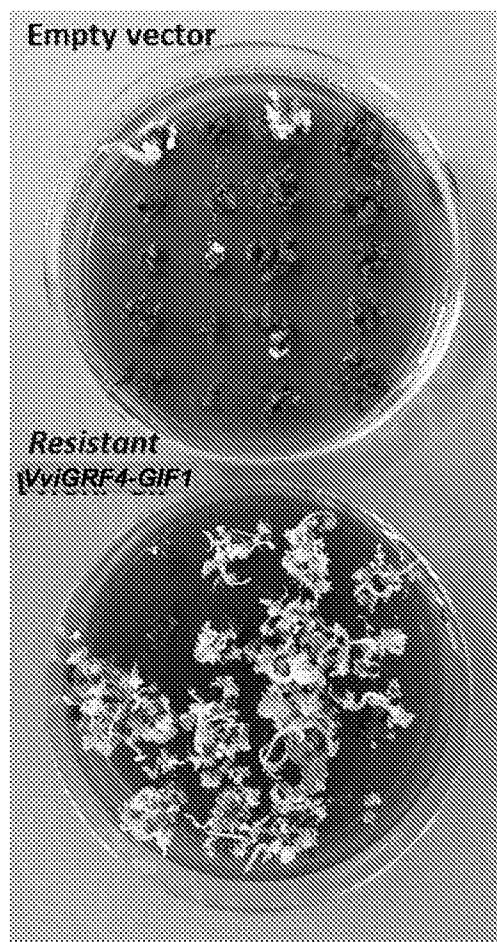


FIG. 27

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Capsicum annuum (pepper cultivar R&C Cayenne). Exp # 201027/28

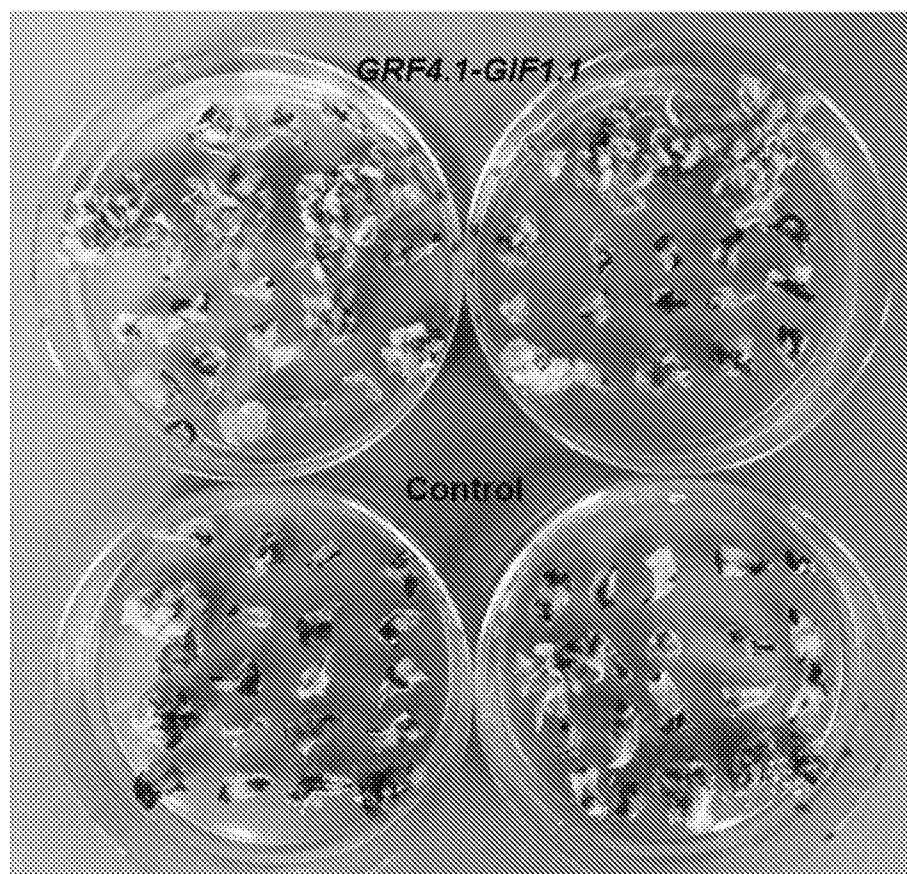


FIG. 28