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# A GRF-GIF chimeric protein improves the regeneration efficiency of transgenic plants

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## Abstract

The potential of genome editing to improve the agronomic performance of crops is often limited by low plant regeneration efficiencies and few transformable genotypes. Here, we show that expression of a fusion protein combining wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) substantially increases the efficiency and speed of regeneration in wheat, triticale and rice and increases the number of transformable wheat genotypes. *GRF4-GIF1* transgenic plants were fertile and without obvious developmental defects. Moreover, *GRF4-GIF1* induces efficient wheat regeneration in the absence of exogenous cytokinins, which facilitates selection of transgenic plants without selectable markers. We also



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Author Contributions

**Juan M. Debernardi**: Investigation, Methodology, Formal analysis, Writing - Original Draft -Review & Editing. **David M. Tricoli**: Investigation, Supervision, Methodology, Project administration and funding acquisition, Writing - Review & Editing. **Javier F. Palatnik**: Conceptualization, Writing - Review & Editing. **Maria F. Ercoli**: Investigation (rice section). Writing - Review & Editing. **Sadiye Hayta**: Investigation (JIC wheat transformation). **Pam Ronald**: Supervision (rice section), Writing - Review & Editing. **Jorge Dubcovsky**: Conceptualization, Formal analysis, Supervision, Project administration and funding acquisition, Writing - Original Draft –Review & Editing.

Competing interest statement

JFP and JMD are co-inventors in patent US2017/0362601A1 that describes the use of chimeric GRF-GIF proteins with enhanced effects on plant growth (Universidad Nacional de Rosario Consejo Nacional de Investigaciones Científicas y Técnicas). JFP, JD, DMT and JMD are co-inventors in UC Davis provisional patent application 62/873,123 that describes the use of GRF-GIF chimeras to enhance regeneration efficiency in plants. Vectors are freely available for research, but commercial applications may require a paid non-exclusive license. There is a patent application from KWS/BASF (WO 2019 / 134884 A1) for improved plant regeneration using Arabidopsis *GRF5* and grass *GRF1* homologs. None of the authors of this manuscript is part of the KWS/BASF patent or is related to these companies. The KWS/BASF patent focuses on a different cluster of *GRF* genes than the one described in our study and does not incorporate the *GIF1* cofactor or the generation of *GRF-GIF* chimeras.

combined *GRF4-GIF1* with CRISPR-Cas9 genome editing, generating 30 edited wheat plants with disruptions in the gene *Q* (*AP2L-A5*). Finally, we show that a dicot *GRF-GIF* chimera improves regeneration efficiency in citrus, suggesting that this strategy can be applied to dicot crops.

## Editorial summary

A method that increases plant regeneration efficiency extends gene editing to more species and genotypes

Recent studies have reported improvements in the efficiency of regenerating plants from tissue culture by overexpressing plant developmental regulators, including *LEAFY COTYLEDON1* [1, 2], *LEAFY COTYLEDON2* [3], *WUSCHEL* (*WUS*) [4], and *BABY BOOM* (*BBM*) [5]. These genes promote the generation of embryo-like structures or somatic embryos, or the regeneration of shoots. For example, overexpression of the maize developmental regulators *BBM* and *WUS2* produced high transformation frequencies from previously non-transformable maize inbred lines and other monocots species [6–8]. Another strategy uses different combinations of developmental regulators to induce *de novo* meristems in dicotyledonous species without tissue culture [9]. Still, there remains a need for new methods that provide efficient transformation, increased ease of use, and suitability for a broader range of recalcitrant species and genotypes.

*GRF* transcription factors are highly conserved in angiosperms, gymnosperms and moss [10]. They encode proteins with conserved QLQ and WRC domains that mediate protein-protein and protein-DNA interactions, respectively [11–13]. Many angiosperm and gymnosperm *GRFs* carry a target site for microRNA miR396, which reduces the function of *GRF*s in mature tissues [14]. The GRF proteins form complexes with GIF cofactors that also interact with chromatin remodeling complexes *in vivo* [15, 16]. Multiple levels of regulation control the efficiency of the assembly of functional GRF/GIF complexes *in vivo* [17]. Loss-of-function mutations in *GIF* genes mimic the reduced organ size observed in *GRF* loss-of-function mutants or in plants overexpressing miR396 [11–13, 18, 19] while overexpression of *GIF* promotes organ growth and can boost the activity of GRFs [12, 13, 15, 20–22]. Furthermore, simultaneous increases in the expression of Arabidopsis *GRF3* and *GIF1* promotes larger increases of leaf size relative to the individual genes [15]. Based on the observation that GRFs and GIFs interact to form a protein complex [15], we decided to evaluate the effect of a GRF-GIF chimeric protein. Here we show that expression of a sequence encoding a chimera of GRF transcription factor and its GIF cofactor substantially increases regeneration efficiency in both monocotyledonous and dicotyledonous species, increases the number of transformable cultivars and results in fertile transgenic plants.

We began by identifying 10 *GRFs* in the wheat genome (Supplementary Figure 1A) and selected wheat *GRF4* based on its homology to *OsGRF4*, a rice gene that promotes grain and plant growth in rice and wheat [23–27]. Among the three wheat GIF cofactors, we selected the closest homologue of Arabidopsis and rice GIF1 (Supplementary Figure 1B), because members of this clade have been shown to control growth in Arabidopsis, rice and maize [12, 13, 21, 22]. We then combined *GIF1* and *GRF4* to generate a *GRF4-GIF1* chimera including a short intergenic spacer (Figure 1A) using primers described in Supplementary Table 1

(Online Methods). Transgenic plants overexpressing the *GRF4-GIF1* chimera under the maize *UBIQUITIN* promoter (*Ubi::GRF4-GIF1*, Supplementary Method 1) were fertile and showed normal phenotypes (Figure 1B). However, they exhibited a 23.9 % reduction in number of grains per spike and 13.7 % increase in grain weight (Supplementary Table 2).

We performed 18 transformation experiments in the tetraploid wheat Kronos (Online Methods) and estimated regeneration frequencies as the number of calli showing at least one regenerating shoot / total number of inoculated embryos (Supplementary Table 3 summarizes regeneration frequencies and number of inoculated embryos). These regeneration efficiencies were used for five different comparisons using experiments as blocks (Figure 1D–H). Across 15 experiments (Supplementary Table 3), the average regeneration efficiency of the *GRF4-GIF1* chimera (65.1 ± 5.0 %) was 7.8-fold higher than the empty vector control (8.3 ± 1.9 %, $P < 0.001$, Figure 1C and D).

We hypothesize that the increased regeneration efficiency of the *GRF4-GIF1* chimera is associated with the ability of the GRF-GIF complex to regulate the transition between stem cells to transit-amplifying cells [28] and their capacity to promote cell proliferation in a broad range of organs [19]. The wheat *GRF4-GIF1* chimera also accelerates the regeneration process, which allowed us to develop a faster wheat transformation protocol that takes 56 d instead of the 91 d required for all the wheat experiments presented in this manuscript (Supplementary Figure 2).

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) (Supplementary Table 3). In five different experiments, the average regeneration efficiency of the separate *GRF4* and *GIF1* genes (38.6 ± 12.9 %) was significantly lower ($P < 0.0064$) than the regeneration efficiency with the *GRF4-GIF1* chimera (62.6 ± 10.3 %, Figure 1E). This result demonstrated that the forced proximity of the two proteins in the chimera increased its ability to induce regeneration.

In another five separate transformation experiments (Supplementary Table 3), we observed significantly lower regeneration efficiencies in embryos transformed with the *GRF4* gene alone (20.4 ± 11.4 %) or the *GIF1* gene alone (17.2 ± 6.6 %) relative to the *GRF4-GIF1* chimera (54.6 ± 9.8 %, contrast $P = 0.0007$, Figure 1F). The regeneration efficiency of the calli transformed with the individual genes was approximately 3-fold higher than the control (6.0 ± 3.0 %) but the differences were not significant in the Tukey test (Figure 1F).

We generated chimeras in which *GIF1* was replaced by other *GIFs* or *GRF4* was replaced by other *GRFs*, and tested their regeneration efficiency in three and four separate experiments, respectively (Supplementary Table 3). The *GRF4-GIF1* combination resulted in higher regeneration efficiency than the *GRF4-GIF2* and *GRF4-GIF3* combination (contrast $P = 0.0046$), and all three chimeras showed higher regeneration efficiency than the control (Tukey test $P < 0.05$, Figure 1G). Similarly, the regeneration efficiency induced by chimeras including the closely related *GRF4* and *GRF5* genes fused with *GIF1*, was higher than the regeneration observed for chimeras including the more distantly related *GRF1* and *GRF9* genes fused with *GIF1* (contrast $P = 0.0064$, Figure 1H). Only the chimeras including the

*GRF4* and *GRF5* genes were significantly different from the control (Tukey $P < 0.05$, Figure 1H).

We then tested the potential of the *GRF4-GIF1* chimera to generate transgenic plants from commercial durum, bread wheat and a Triticale line that were recalcitrant to *Agrobacterium*-mediated or had low regeneration efficiency in previous experiments at the UCD Plant Transformation Facility. With the *GRF4-GIF1* chimera we observed high increases in regeneration frequencies in tetraploid wheat Desert King (63.0 ± 17.0 % *vs.* 2.5 ± 2.5 %, 2 experiments) and hexaploid wheat Fielder (61.8 ± 8.2 % *vs.* 12.7 ± 10.3 %, three experiments) relative to the control. For the hexaploid wheat varieties Hahn and Cadenza and the Triticale breeding line UC3190, for which we were not able to generate transgenic plants using the Japan Tobacco protocol, we observed regeneration frequencies of 9 to 19 % with the *GRF4-GIF1* chimera (versus 0 % with the control, Supplementary Figure 3 and Supplementary Table 4A and B).

High wheat regeneration efficiencies have been reported before using the proprietary Japan Tobacco method in the variety Fielder [29, 30, 31]. However, the company warns that these high values require the optimization of multiple factors with narrow optimal windows and that "those values can drop drastically when one of the factors become suboptimal" [29] (Supplementary Table 5). The addition of the *GRF4-GIF1* chimera overcame some of the constrains imposed by these narrow optimal windows and allowed us to obtain high transformation efficiencies using a shorter protocol and embryos of a wider range of sizes (1.5 to 3.0 mm) obtained from plants grown in diverse environmental conditions. High regeneration efficiencies were observed even when we used different vectors and genotypes and without embryo excision, a critical step in the Japan Tobacco technology[29].

To test the robustness of our method, we transferred our *GRF4-GIF1* vector to the John Innes Centre Transformation facility for testing with their recently published wheat transformation method [32]. Fielder plants transformed with the *GRF4-GIF1* chimera showed a 77.5% regeneration efficiency, compared with 33.3% in the control (Supplementary Table 4A). Taken together, these results indicate that the addition of the *GRF4-GIF1* chimera increases the robustness of wheat transformation under different conditions and protocols.

We also tested the wheat *GRF4-GIF1* chimera in the rice variety Kitaake (Online Methods). In four independent transformation experiments, we observed a 2.1-fold increase in rice regeneration efficiency ($P < 0.00001$) in the calli transformed with the wheat *GRF4-GIF1* chimera (average 42.8 ± 2.6 %) compared with those transformed with the control vectors (20.3 ± 2.9 %, Supplementary Table 6). These results suggest that the wheat *GRF4-GIF1* chimera is effective in enhancing regeneration in another agronomically important monocotyledonous species.

In many plant transformation systems cytokinins are required to regenerate shoots (Figure 2A). Notably, in both laboratories we observed that Kronos and Fielder embryos inoculated with *Agrobacterium* transformed with the *GRF4-GIF1* chimera were able to rapidly regenerate green shoots in auxin media without cytokinin (Figure 2B). We then tested the regeneration efficiency of immature embryos from stable *GRF4-GIF1* transgenics (n=27)

and non-transgenic (n=26) $T_1$ 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 %, Supplementary Figure 4). These results indicated that the *GRF4-GIF1* chimera can promote either embryogenesis, shoot proliferation, or both, in wheat without the addition of exogenous cytokinin.

Based on the previous result, we developed a protocol to select transgenic shoots in auxin media without using antibiotic-based markers. We recovered 40 shoots using a *GRF4-GIF1* marker-free vector and 15 for the empty vector across three experiments. Genotyping revealed that 10 out of the 40 (25 %) *GRF4-GIF1* shoots were transgenic, while none of the shoots from the control was positive (Figure 2C presents results from the first experiment). These high-regenerating transgenic plants overexpressing the *GRF4-GIF1* chimera without selection markers could potentially be used for future transformation experiments to incorporate other genes using selectable markers. This approach could generate separate insertion sites for the *GRF4-GIF1* and the second transgene, facilitating the segregation of the *GRF4-GIF1* insertion in the next generation.

This strategy is not necessary for genome editing, since both the CRISPR-Cas9 and *GRF4-GIF1* sequences can be segregated out together after editing the desired region of the genome. Therefore, the GRF-GIF system is ideal to extend genome editing technology to crops with low regeneration efficiencies. As a proof of concept, we generated a binary vector for *Agrobacterium* transformation that contained a cassette including the *GRF4-GIF1* chimera, Cas9 and a gRNA targeting the wheat gene *Q* (= *AP2L-A5*) [33] in the same T-DNA (Figure 3A and B). We recovered 30 independent transgenic events out of 32 infected calli (93.7% efficiency, Figure 3C). Disruption of a *Sty*I restriction sites showed Cas9-induced editing in all 30 transgenics (Supplementary Figure 5). We sequenced the PCR products obtained from 10 independent lines and confirmed editing (Figure 3D). Of the ten edited $T_0$ plants transferred to soil, seven showed clear mutant *q*-null phenotypes (Figure 3E) and the other three died before heading. These $T_0$ transgenic plants were fertile, and the edited *Q* gene and the CRISPR-Cas9 / *GRF4-GIF1* construct are expected to segregate in the $T_1$ progeny, facilitating the selection of edited plants without the transgene.

Lastly, 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 and organogenic-based transformation protocols. We generated a citrus and a heterologous grape *GRF-GIF* chimera using the closest homologs to wheat *GRF4* and *GIF1* in both species (Supplementary Figure 1A and B). In three independent transformation experiments in the citron rootstock Carrizo (Online Methods), epicotyls were transformed with the citrus and the grape *GRF-GIF* chimeras. Epicotyls transformed with the citrus *GRF-GIF* chimera showed a 4.7-fold increase in regeneration frequency relative to those transformed with the empty vector control (Supplementary Figure 6A). The heterologous grape *GRF-GIF* chimera produced similar increases in citrus regeneration efficiency as the citrus chimera (Supplementary Figure 6B).

We also tested the effect of a miR396-resistant grape *GRF-GIF* version (henceforth, *rGRF-GIF*), in which we introduced silent mutations in the *GRF* binding site for miR396 to avoid

cleavage (Supplementary Figure 6B and C). In three independent experiments, we observed that the grape *rGRF-GIF* chimera produced the highest frequency of transgenic citrus events (7.4-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 ($P = 0.0136$, Supplementary Figure 6D and Supplementary Table 7). 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 (Supplementary Figure 6B).

In summary, expression of a *GRF4-GIF1* chimera increased significantly the efficiency and speed of wheat regeneration and the ability to generate large numbers of fertile edited plants, extended the range of transformable genotypes and eliminated the requirement of cytokinin for regeneration, thereby eliminating the need of antibiotic-based selectable markers. The *GRF4-GIF1* technology results in fertile and normal transgenic plants without the need of specialized promoters or transgene excision, overcoming some of the limitations of transformation technologies with other morphogenic genes (Supplementary Table 8). Because *GRF4-GIF1* likely operates at a later stage of meristem differentiation and stem cell proliferation [28] than *Bbm-Wus2* [6–8], there is potential to combine both technologies and have synergistic effects in the regeneration efficiency of recalcitrant genotypes. A concurrent and independent work showed that overexpression of Arabidopsis *AtGRF5* and *AtGRF5* homologs positively enhance regeneration and transformation in monocot and dicot species not tested here [34]. We hypothesize that the benefits of the *GRF4-GIF1* technology can be rapidly extended to other crops with low regeneration efficiencies by incorporating the *GRF4-GIF1* chimera into current protocols. This hypothesis is supported by the high conservation of the GRF and GIF proteins across the plant kingdom and by the higher regeneration frequencies observed for rice and citrus in this study.

## Methods

### Wheat vectors

We performed all PCRs cloning with Phusion High-Fidelity DNA Polymerase (NEB). We extracted RNA extracted from spikes using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), treated with RQ1 RNase-free DNase (Promega), and then synthesized the cDNA using SuperScript II Reverse Transcriptase (Invitrogen). To clone the coding region of wheat *GRF4* and *GIF1*, we performed PCRs using 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 Supplementary Table 1. We first cloned the PCR fragments in pDONR by a B/P gateway reaction and generated the *GRF4-GIF1* chimera by overlapping PCR.

In the first step, we amplified the *GRF4* and *GIF1* coding sequences with primers FW-GRF4a/Rev-GRF4b and Fw-GIF1b/Rev-GIF1b from the pDONR-GRF4 and pDONR-GIF1 clones. 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. We gel-purified both PCR fragments and used them as template in a second PCR with the primers Fw-GRF4/Rev-GIF1b (Supplementary Table 1). We cloned

the resulting product in pDONR. Next, we cloned the *GRF4*, the *GIF1* and the chimera *GRF4-GIF1* chimera the binary vector pLC41 by a L/R gateway reaction under the maize *UBIQUITIN* promoter. We verified the resulting vectors for the individual genes pLC41:*GRF4* and pLC41:*GIF1*, and for the chimera pLC41:*GRF4-GIF1* by restriction digestion, and transformed them by electroporation in *Agrobacterium* strain EHA105 and in a few experiments in strain AGL1 (Supplementary Table 4). Both strains were handled in the same way.

To develop the vector expressing both *GRF4* and *GRF1* under their own promoters (not fused, Ubi::*GRF4*-term and Ubi::*GIF1*-term), we amplified the complete Ubi::*GRF4*-term cassette by PCR using pLC41:*GRF4* as template with primers Fw_*Hind*III and Rev-*Hind*III (Supplementary Table 1). We cloned the PCR fragment in pGEMT-easy and then sub-cloned the Ubi::GRF4-term fragment into the *Hind*III site of pLC41:*GIF1*.

To generate the different wheat GRF-GIF chimeras, we obtained the coding sequences of *GRF1*, *GRF5*, *GRF9*, *GIF2* and *GIF3* by gene synthesis. Then, we generated the different chimeras (*GRF1-GIF1*, *GRF5-GIF1*, *GRF9-GIF1*, *GRF4-GIF2*, and *GRF4-GIF3*) by overlapping PCR following the same strategy described to generate *GRF4-GIF1*. All the chimeras were cloned in pLC41 vector by L/R reaction. We verified all the vectors by restriction digestion and transformed by electroporation in *Agrobacterium* strain EHA105.

To develop the JD635-*GRF4-GIF1*-Cas9- gRNA-Gene *Q* vector, we amplified by PCR a cassette including the maize *UBIQUITIN* promoter, the *GRF4-GIF1* chimera and the Nos terminator (primers Fw_ZmUbi-AscI and Rev_NosTerm-AscI). The PCR product was gel-purified and cloned by In-fusion (Takara Bio USA, Inc.) into the *Asc*I site of the pYP25F binary vector, which contains a wheat codon optimized Cas9 (TaCas9) with two nuclear localization signals (NLS), and is a modified version of pDIRECT_25F (https://www.addgene.org/91143/) from Dr. Daniel Voytas group. We validated the vector sequence by Sanger sequencing. Next, we cloned a guide RNA construct targeting the coding region of Gene *Q*[35] by Golden Gate reaction into two *Aar*I sites of the vector and transformed it into chemical competent *E. coli* DH5α. We validated the JD635-*GRF4-GIF1*-Cas9-gRNA-Gene *Q* vector by Sanger sequencing and transformed by electroporation into *Agrobacterium* strain EHA105.

## Citrus and Vitis vectors

We generated the *Citrus* and *Vitis GRF-GIF* chimeras by gene synthesis using the *GRF* and *GIF* homologs highlighted in Supplementary Figure 1. We cloned the DNA fragments into pDONR by B/P gateway reaction. We cloned the *GRF-GIF* chimeras in the binary vector pGWB14 binary vector (L/R gateway reaction under viral 35S promoter) and transformed them by electroporation in *Agrobacterium* strain EHA105.

We generated a miR396-resistant version of *Vitis GRF-GIF* (*rGRF-GIF*) by overlapping PCR. Two PCR reactions were performed with primers Fw-GRF/rGRF-Rev and rGRF-Fw/Rev-GIF (Supplementary Table 1) using pGBW14-*vitis GRF-GIF* clone as template. The primers rGRF-Fw and rGRF-Rev overlap in 17 nucleotides, and introduce silent mutations in the miR396 target site (Supplementary Figure 6). We gel-purified both PCR fragments

and used them as template in a second PCR with the primers Fw-GRF/Rev-GIF (Supplementary Table 1). We cloned the resulting product in pDONR by B/P gateway reaction. Next, we cloned the chimera *rGRF-GIF* in the binary vector pGWB14 by a L/R gateway reaction under the viral 35S promoter and transformed them by electroporation in *Agrobacterium* strain EHA105.

## Wheat transformation

Wheat transformation followed previously published protocols [29]. Briefly, we grew the different wheat and triticale cultivars in a green house or a growth chamber under long-day photoperiod (16 h of 380 μM $m^{-2}$ $s^{-1}$ 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 (embryo sizes 1.5 to 3.0 mm).

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.

After 3 weeks, we transferred 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 μM $m^{-2}$ $s^{-1}$) 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 × 21 × 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 Supplementary Figure 2. Transformation at the John Innes Centre was performed as published before [32].

## Rice transformation

Rice transformation followed previously published protocols [36]. 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 end 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.

**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 $OD_{600}$ between 0.1 – 0.2 (approximately 2–4 h).

**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.

**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.

**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, the calli that started to turn green, were transferred to regeneration media with reduced hygromycin (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, 25 mg/l hygromycin, 1.6 % (w/v) agar, pH 5.6–5.8). When the shoot was properly developed, we transferred the regenerated plants to rooting media (MS + H, 1x Murashige and Skoog with vitamins medium containing 25 mg/l hygromycin, 1.2 % (w/v) agar, pH 5.6–5.) and incubated in 16 h light/ 8 h dark 28 °C. When roots were well developed, we transferred the plants to soil.

### *Citrus* Transformation

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.

**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.

**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 OD $_{600}$). 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.

**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.

**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.

**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.

## Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

### Data availability statement

Accession numbers and gene names are available in the phylogenetic tree in Supplementary Figure 1. All wheat gene names are based on genome release RefSeq v1.0. The raw data for the different experiments is available in Supplementary Tables 3–4 and 6–7. The methods for the generation of the different vectors and the transformation protocols are described in Online Methods. The following vectors will be available through Addgene (http://www.addgene.org): JD553 - wheat GRF4-GIF1 in pDONR, JD633 - wheat GRF4-GIF1 in CRISPR vector, JD630 - Vitis GRF4-GIF1 in pDONR, JD638 - Vitis miR396-resistant GRF4-GIF1 in pDONR, JD689 - citrus GRF4-GIF1 in pDONR, JD690 - citrus GRF4-GIF1 in pGWB14, JD631 - Vitis GRF4-GIF1 in pGWB14, and JD639 - Vitis miR396-resistant GRF4-GIF1 in pGWB14.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Methods-only References

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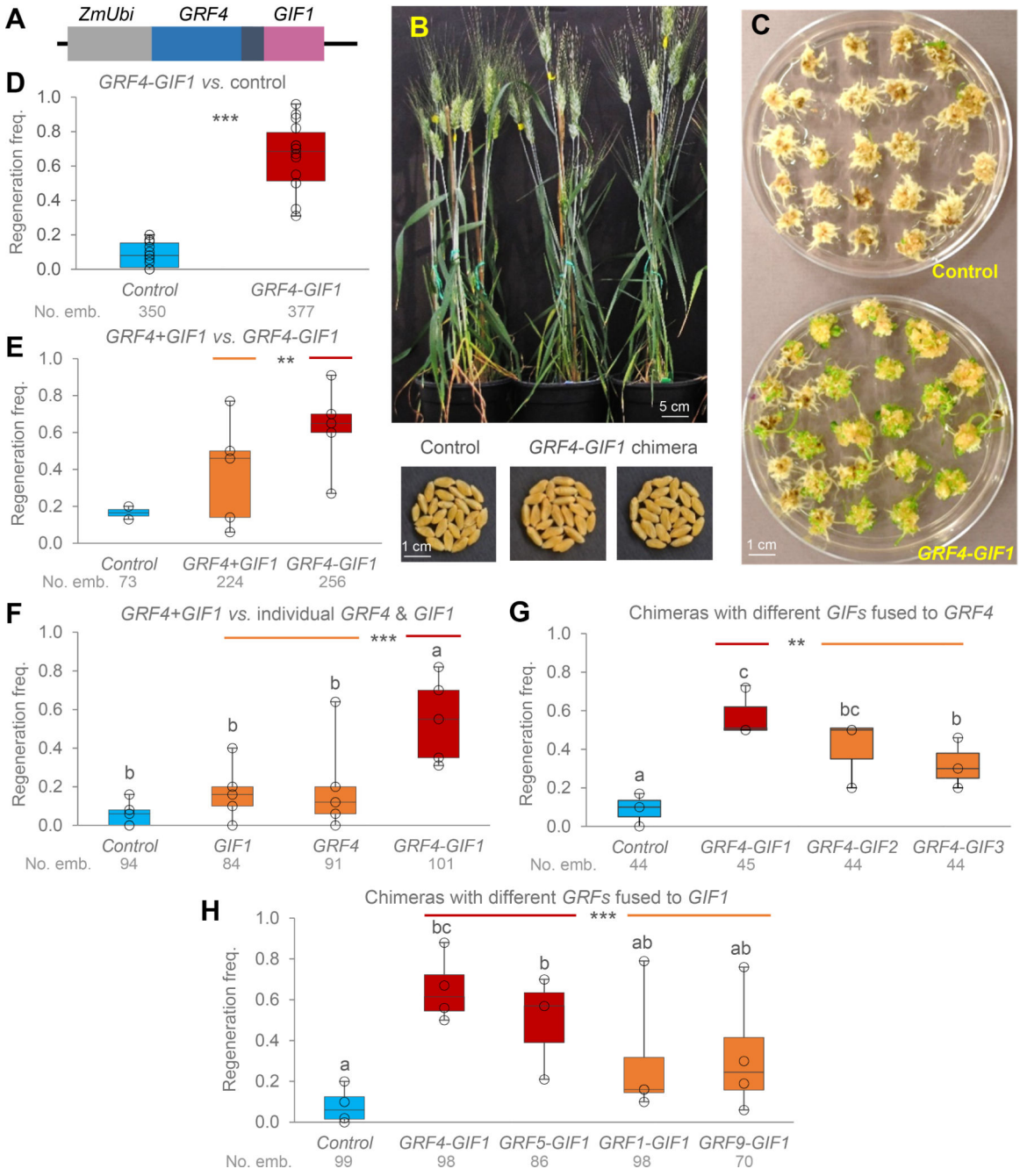


**Figure 1. *GRF4-GIF1* chimera**

**A)** Schematic representation of the *GRF4* (blue)-*GIF1* (pink) chimera. The black region represents a four amino acid spacer. **B**) The *GRF4-GIF1* transgenic wheat plants were normal and fertile. **C)** Representative transformation showing higher frequency of regenerated shoots during Kronos transformation in the presence of the *GRF4-GIF1* chimera than in the control. **D-H**) Box-plots showing regeneration frequencies of transgenic Kronos plants and their respective controls. The box shows the range from first to third quartiles, and is divided by the median. The whiskers span down to the minimum, and up to the maximum observation. Results from individual experiments are indicated by empty black

circles. All experiments include the empty pLC41 vector as control and the wheat *GRF4-GIF1* chimera. Numbers below the genotypes are total number of inoculated embryos and different letters above bars indicate significant differences ($P < 0.05$, Tukey test). **D**) Control *vs. GRF4-GIF1*, n= 15 experiments (*** $P < 0.001$, square root transformation). **E**) Control, *GRF4-GIF1* and vector including *GRF4* and *GIF1* driven by separate maize *UBIQUITIN* promoters (*GRF4+GIF1*), n = 5 experiments (contrast *GRF4-GIF1 vs. GRF4+GIF1*, ** $P = 0.0064$, the empty-vector control was included only in two experiments). **F**) Control, *GRF4-GIF1* and vectors including only *GIF1* or only *GRF4*, n = 5 experiments (contrast *GRF4-GIF1 vs.* combined *GRF4* & *GIF1* $P = 0.0007$). **G**) Control and *GRF4* chimeras fused to either *GIF1*, *GIF2* or *GIF3*, n = 3 experiments (contrast chimeras with *GIF1* vs. combined *GIF2* and *GIF3* ** $P = 0.0046$). **H**) Control and chimeras combining different wheat *GRF* genes fused with *GIF1* (n= 4 experiments, except for *GRF5* n=3). ** $P = 0.0064$ in contrast comparing combined *GRF4-GIF1* and *GRF5-GIF1* chimeras (evolutionary related) with combined *GRF1-GIF1* and *GRF9-GIF1* chimeras (more distantly related). In all tests, normality of residuals was confirmed by Shapiro-Wilk's test and homogeneity of variances by Levene's test (raw-data is available in Supplementary Table 3).

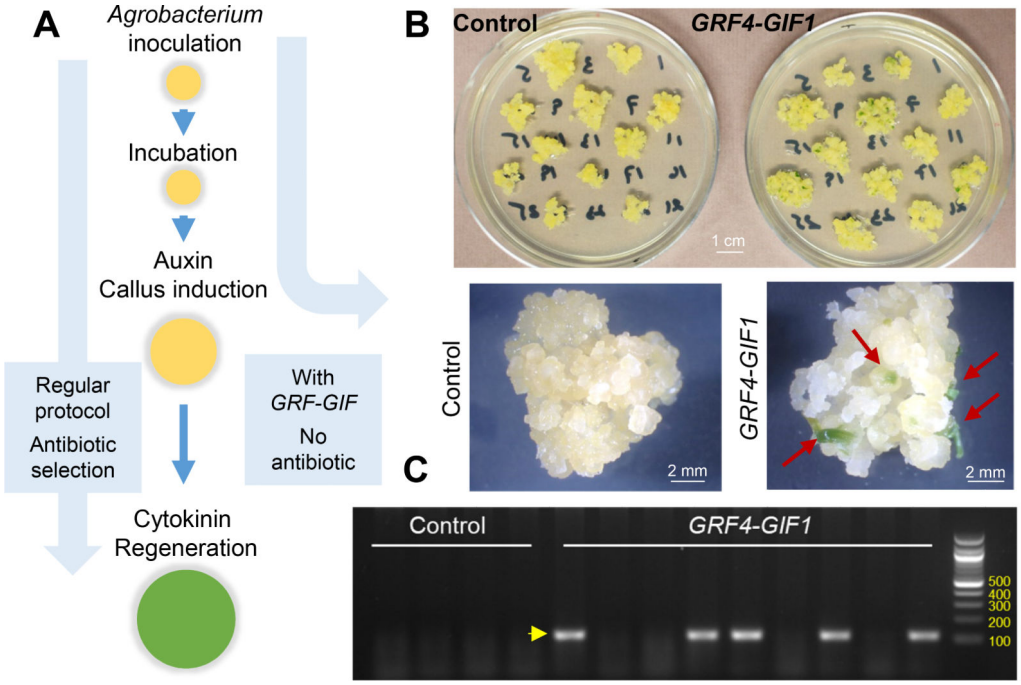


**Figure 2. The *GRF4-GIF1* chimera induces embryogenesis in the absence of cytokinins.**

**A)** Schematic representation of the different steps of wheat transformation. **B).** Representative calli 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 (yellow arrow) amplified with primers pLC41–1064 and pLC41–1061 (Supplementary Table S1). In this first experiment (out of three), we identified five transgenic plants among nine regenerated from the *GRF4-GIF1* marker-free vector and no transgenic plants among four regenerated from the control.

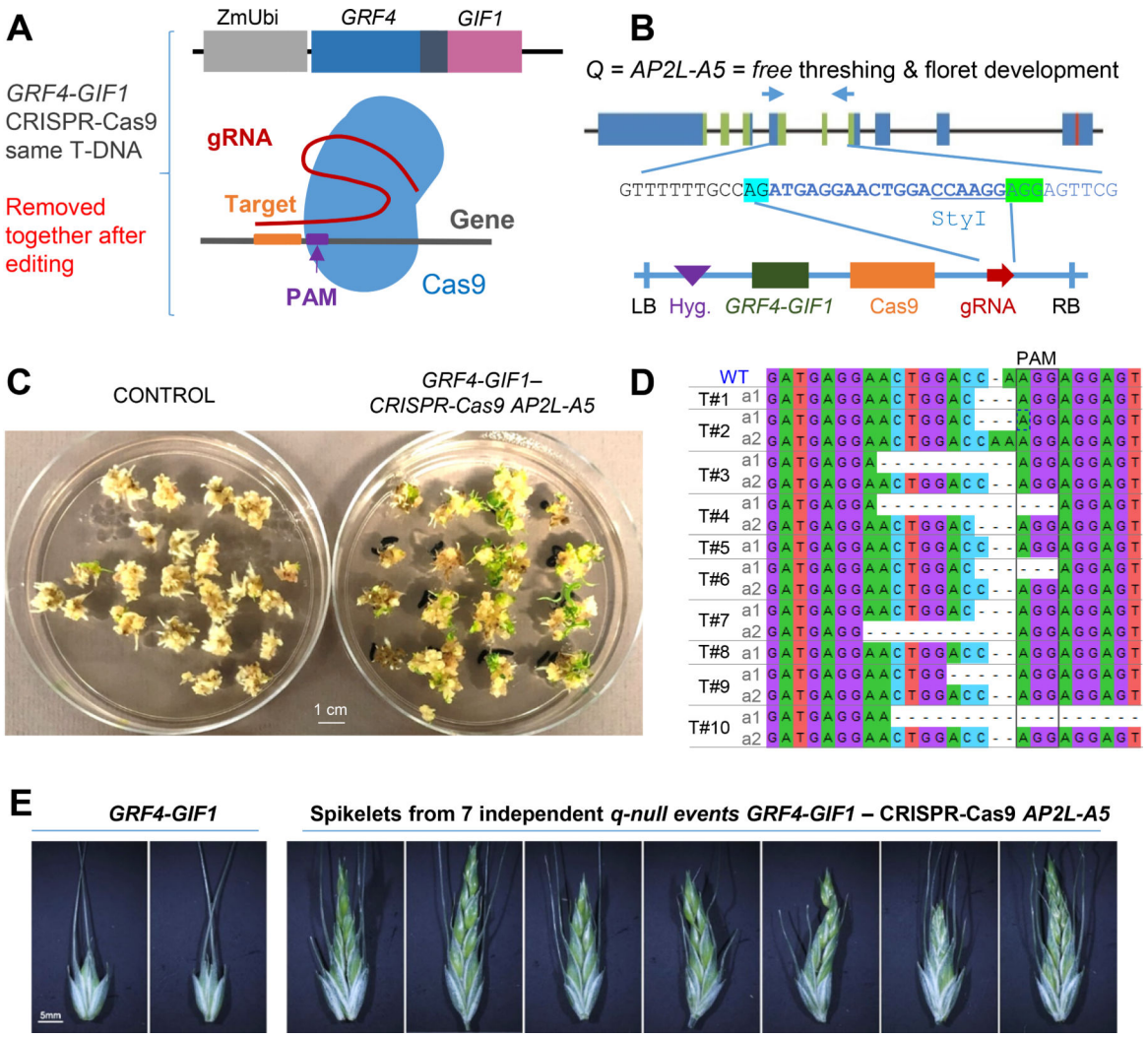


**Figure 3. High frequency of genome edited plants using combined GRF4-GIF1 – CRISPR-Cas9 technology.**

**A)** Technologies combined in a single vector. **B)** Region of the gene *Q* (*AP2L-A5*) targeted with the guide RNA (gRNA) and schematic representation of the vector combining both technologies (LB = left border, Hyg. = hygromycin resistance, RB = right border). **C)** Kronos shoot regeneration of embryos transformed with an empty vector and with the combined GRF4-GIF1 - CRISPR-Cas9-gRNA-*AP2L-A5* construct (93.7 % regeneration efficiency). **D)** All 10 sequenced transgenic $T_0$ plants showed *AP2L-A5* editing. Seven of the 10 plants (T#1 to T#10) carried two different mutations (a1 and a2), documenting high editing efficiency. **E**) Edited $T_0$ plants showed increased number of florets per spikelet (characteristic of *q*-null plants).