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

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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* induced efficient wheat regeneration in the absence of exogenous cytokinins, which facilitates selection of transgenic plants without selectable markers. We also combined *GRF4-GIF1* with CRISPR-Cas9 genome editing and generated 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.

Recent studies have reported improvements in the efficiency of plant regeneration from tissue culture by overexpression of plant developmental regulators, including *LEAFY COTYLEDON1* (refs. [1,2]), *LEAFY COTYLEDON2* (ref. [3]), *WUSCHEL* (*WUS*)[4] and *BABY BOOM* (*BBM*)[5]. These genes promote the generation of somatic embryos or the regeneration of shoots. For example, overexpression of the maize developmental regulators *BBM* and *WUS2* produces high transformation frequencies in previously non-transformable maize inbred lines and other monocot species[6–8]. Another strategy uses different combinations of developmental regulators to induce de novo meristems in dicotyledonous species without tissue culture[9]. However, 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 factor genes 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 *GRF* genes carry a target site for microRNA miR396, which reduces the function of GRFs 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 functional GRF–GIF complex assembly 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 (refs. [11–13,18,19]), while overexpression of *GIF* genes 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* promote the development of larger leaf sizes than are observed when the expression of these genes is increased individually[15]. Based on the observation that GRFs and GIFs interact to form a protein complex[15], we evaluated the effect of a GRF–GIF chimeric protein. Here we show that expression of a sequence encoding a chimera composed of a 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 ten *GRF* genes in the wheat genome (Supplementary Fig. 1a) and selected *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 homolog of *Arabidopsis* and rice *GIF1* (Supplementary Fig. 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 (Fig. 1a) using the primers described in Supplementary Table 1 (Methods). Transgenic plants overexpressing the *GRF4–GIF1* chimera under the control of the maize *UBIQUITIN* promoter (Ubi::*GRF4–GIF1*; Methods) were fertile and showed normal phenotypes (Fig. 1b). However, they exhibited a 23.9% reduction in the number of grains per spike and a 13.7% increase in grain weight (Supplementary Table 2).

We performed 18 transformation experiments in the tetraploid wheat Kronos (Methods) and estimated regeneration frequencies as the number of calli showing at least one regenerating shoot per the total number of inoculated embryos (Supplementary Table 3 summarizes the regeneration frequencies and the number of inoculated embryos). These regeneration efficiencies were assessed for five different comparisons using experiments as blocks (Fig. 1d–h). Across 15 experiments (Supplementary Table 3), the average regeneration efficiency with the *GRF4–GIF1* chimera (65.1% ± 5.0%) was 7.8-fold higher than for the empty vector control (8.3% ± 1.9%, $P < 0.001$; Fig. 1c,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 and transit-amplifying cells[28] and its ability to promote cell proliferation

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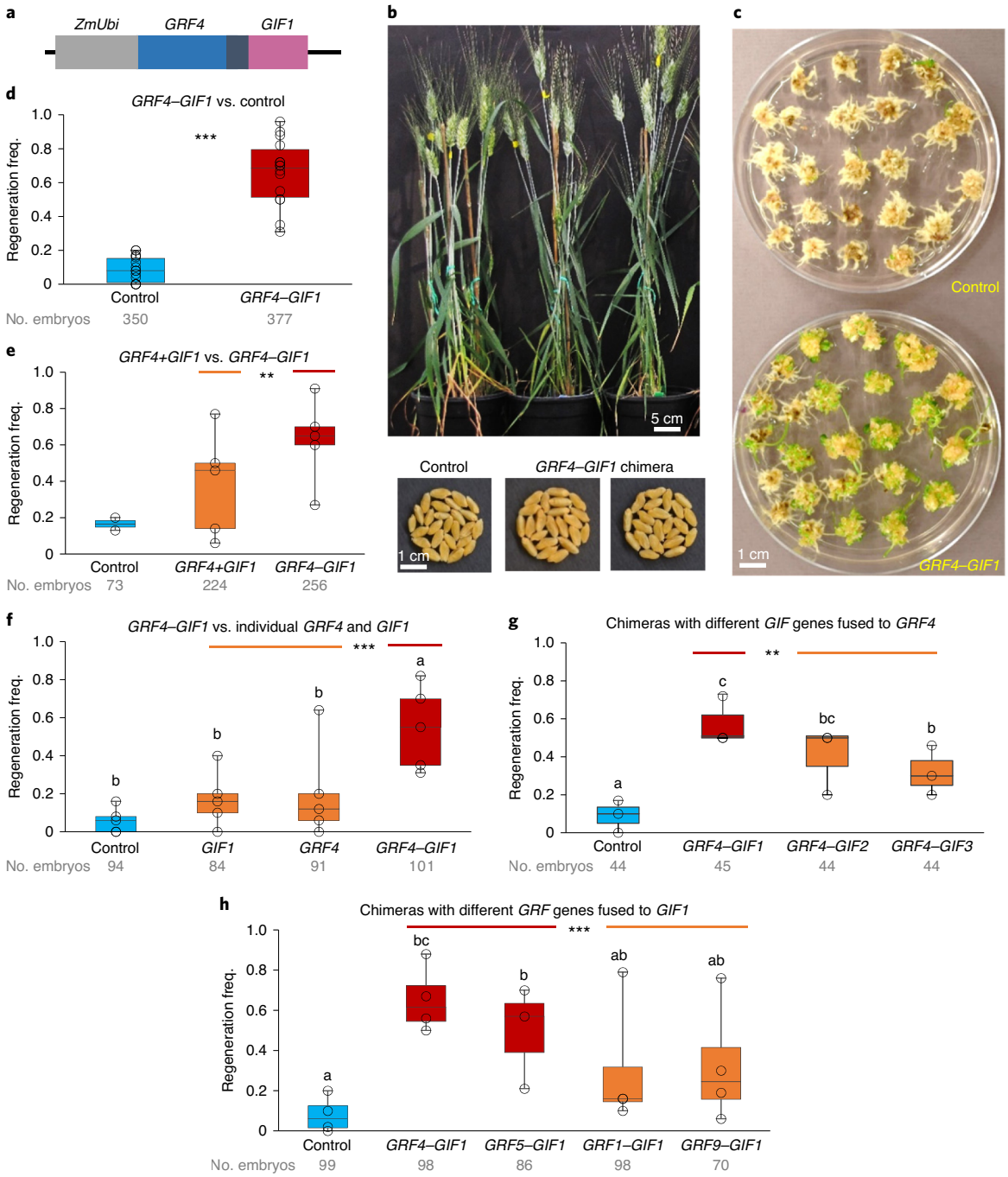

Fig. 1 | The *GRF4–GIF1* chimera and its effects on regeneration frequency. a, Schematic representation of the *GRF4* (blue) and *GIF1* (pink) chimera. The black region represents a sequence encoding a four-amino-acid spacer. **b**, The *GRF4–GIF1* transgenic wheat plants were normal and fertile. **c**, Representative transformation showing a 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 the regeneration frequencies of transgenic Kronos plants and their respective controls. The box shows the range from the first to third quartile 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 included the empty pLC41 vector as a control and the wheat *GRF4–GIF1* chimera. Numbers below the genotypes represent the total number of inoculated embryos, and different letters above the bars indicate significant differences ($P < 0.05$, Tukey test). **d**, Control versus *GRF4–GIF1*. $n = 15$ experiments (***$P < 0.001$, square root transformation). **e**, Comparison of control, *GRF4–GIF1* and a vector including *GRF4* and *GIF1* with expression driven by separate maize *UBIQUITIN* promoters (*GRF4+GIF1*). $n = 5$ experiments (*GRF4–GIF1* versus *GRF4+GIF1*, **$P = 0.0064$; the empty vector control was only included in two experiments). **f**, Comparison of control, *GRF4–GIF1* and vectors including only *GIF1* or only *GRF4*. $n = 5$ experiments (*GRF4–GIF1* versus *GRF4* only and *GIF1* only, ***$P = 0.0007$). **g**, Comparison of control and *GRF4* chimeras fused to *GIF1*, *GIF2* or *GIF3*. $n = 3$ experiments (chimeras with *GIF1* versus chimeras with *GIF2* or *GIF3*, **$P = 0.0046$). **h**, Control versus chimeras combining different wheat *GRF* genes fused with *GIF1*. $n = 4$ experiments except for *GRF5*, where $n = 3$ (*GRF4–GIF1* and *GRF5–GIF1* chimeras versus *GRF1–GIF1* and *GRF9–GIF1* chimeras, **$P = 0.0064$). *GRF4* and *GRF5* are evolutionary related whereas *GRF1* and *GRF9* are more distantly related. In all tests, the normality of the residuals was confirmed by Shapiro–Wilk's test and the homogeneity of the variances was confirmed by Levene's test (raw data are available in Supplementary Table 3).

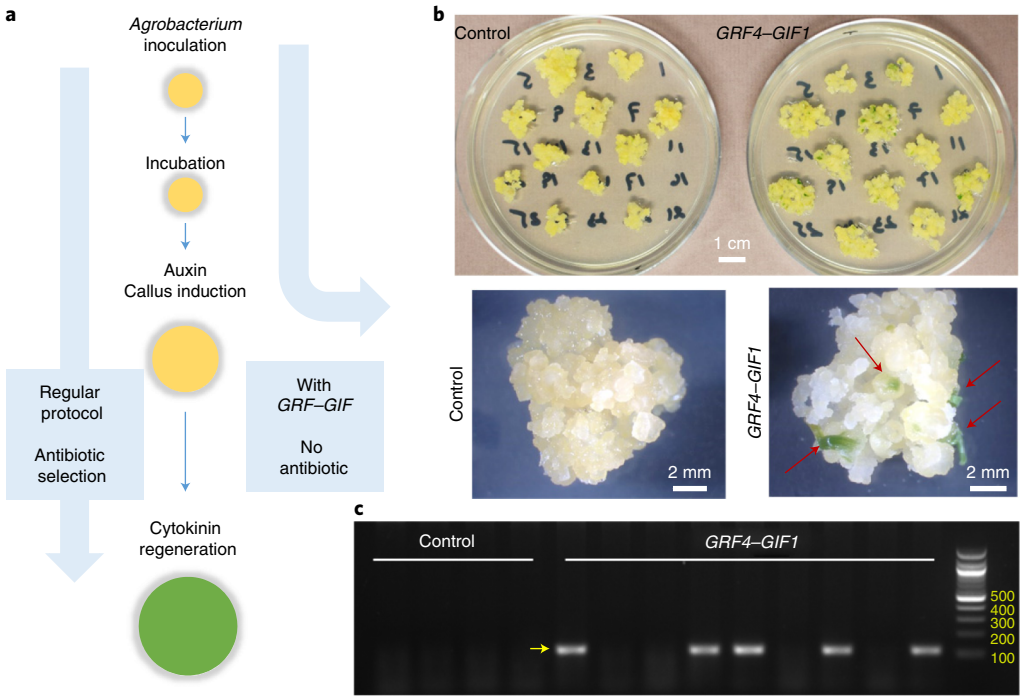

Fig. 2 | The *GRF4–GIF1* chimera induces embryogenesis in the absence of cytokinins. a, Schematic representation of the different steps of wheat transformation. **b**, Representative calli in auxin medium with no hygromycin. Note the growing green shoots in the callus transformed with the wheat *GRF4–GIF1* chimera in the absence of cytokinins (red arrows). Control, pLC41. **c**, Transgenic-specific PCR product (yellow arrowhead) amplified with primers pLC41-1064 and pLC41-1061 (Supplementary Table 1). In the first experiment (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.

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 Fig. 2).

We then compared the effect of having *GRF4* and *GIF1* fused in a chimera versus having each gene expressed separately within the same construct controlled by individual *UBIQUITIN* promoters (not fused) (Supplementary Table 3) on regeneration efficiency. 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 of the *GRF4–GIF1* chimera (62.6% ± 10.3%; Fig. 1e). This result demonstrates that the forced proximity of the two proteins in the chimera increases 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%) than with the *GRF4–GIF1* chimera (54.6% ± 9.8%, $P = 0.0007$; Fig. 1f). The regeneration efficiency of the calli transformed with the individual genes was approximately threefold higher than in the control (6.0% ± 3.0%), but the differences were not significant in the Tukey test (Fig. 1f).

We generated chimeras in which *GIF1* was replaced by other *GIF* genes or *GRF4* was replaced by other *GRF* genes 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* combinations ($P = 0.0046$), and all three chimeras showed higher regeneration efficiency than the control (Tukey test, $P < 0.05$; Fig. 1g). Similarly, the regeneration efficiency of chimeras including the closely related *GRF4* and *GRF5* genes fused with *GIF1* was higher than the regeneration efficiency observed for chimeras including the more distantly related *GRF1* and *GRF9* genes fused with *GIF1* ($P = 0.0064$; Fig. 1h). Only the chimeras including the *GRF4* and *GRF5* genes were significantly different from the control (Tukey test, $P < 0.05$; Fig. 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 all either recalcitrant to *Agrobacterium*-mediated transformation or had low regeneration efficiency in previous experiments performed at the University of California Davis Plant Transformation Facility. With the *GRF4–GIF1* chimera, we observed high increases in regeneration frequencies in the tetraploid wheat Desert King (63.0% ± 17.0% versus 2.5% ± 2.5%; two experiments) and the hexaploid wheat Fielder (61.8% ± 8.2% versus 12.7% ± 10.3%; three experiments) compared 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 9–19% in plants with the *GRF4–GIF1* chimera versus 0% in plants with the control vector (Supplementary Fig. 3 and Supplementary Table 4a,b).

High wheat regeneration efficiencies have been reported using the proprietary Japan Tobacco method in the variety Fielder[29–31]. 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 (ref. [29]; Supplementary Table 5). The addition of the *GRF4–GIF1* chimera overcame some of the constraints 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 (1.5–3.0 mm) obtained from plants grown in diverse environmental conditions. High regeneration efficiencies were observed even when we used different vectors and genotypes

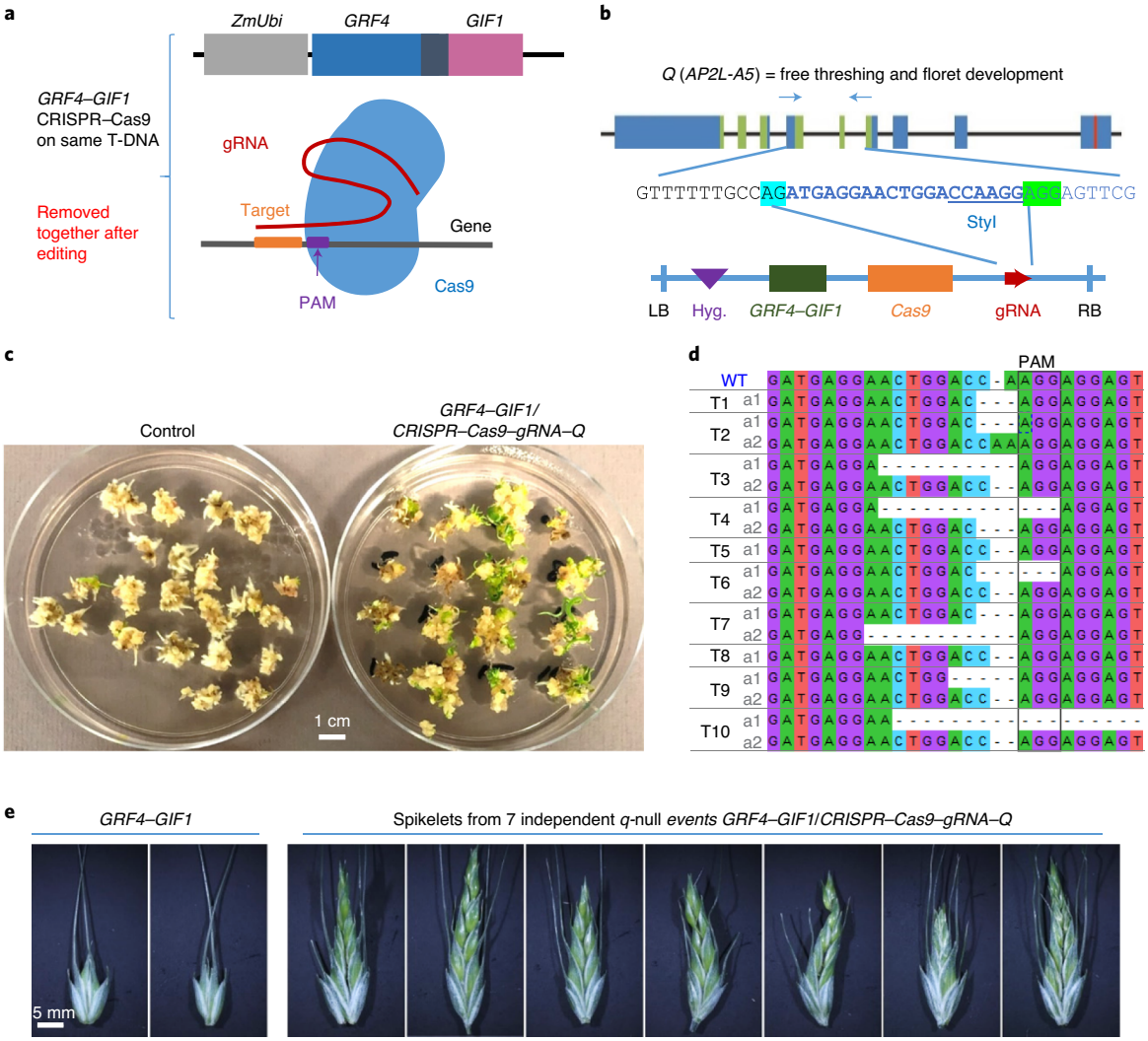

Fig. 3 | High frequency of genome-edited plants using combined *GRF4–GIF1* and CRISPR–Cas9 technology. a, Technologies combined in a single vector. PAM, protospacer-adjacent motif. **b**, Region of the gene *Q* (*AP2L-A5*) targeted with the gRNA and schematic representation of the vector combining both 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–Q* construct (93.7% regeneration efficiency). **d**, All ten sequenced transgenic T_0 plants showed *Q* editing. Seven of the ten plants (T1–T10) carried two different mutations (a1 and a2), demonstrating high editing efficiency. WT, wild type. **e**, Edited T_0 plants showing an increased number of florets per spikelet (characteristic of *q*-null plants).

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 to a 33.3% regeneration efficiency 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 (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 to 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 (Fig. 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 when cultured in auxin medium without cytokinin (Fig. 2b). We then tested the regeneration efficiency of immature embryos from stable *GRF4–GIF1* transgenic ($n = 27$) 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 that of the non-transgenic sister lines (11.5%; Supplementary Fig. 4). These results indicate that the *GRF4–GIF1* chimera can promote 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 medium without using antibiotic-based markers. We recovered 40 shoots using a *GRF4–GIF1* marker-free vector and 15 shoots using the empty vector across 3 experiments. Genotyping revealed that 10 of the 40 (25%) *GRF4–GIF1* shoots were transgenic, while none of the shoots from the control were

positive (Fig. 2c presents results from the first experiment). These highly 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* chimera and a second transgene, facilitating segregation of the *GRF4–GIF1* insertion in the next generation.

This strategy is not necessary for genome editing, as 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 guide RNA (gRNA) targeting the wheat gene *Q* (also known as *AP2L-A5*)[33] in the same T-DNA (Fig. 3a,b). We recovered 30 independent transgenic events for 32 infected calli (93.7% efficiency; Fig. 3c). Disruption of a StyI restriction site showed Cas9-induced editing in all 30 transgenic calli (Supplementary Fig. 5). We sequenced the PCR products obtained from ten independent lines and confirmed editing (Fig. 3d). Of the ten edited T_0 plants transferred to soil, seven showed clear mutant *q*-null phenotypes (Fig. 3e), and the other three died before heading. These T_0 transgenic plants were fertile, and the edited *Q* gene and the *GRF4–GIF1/CRISPR–Cas9–gRNA–Q* 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 chimera and a heterologous grape *GRF–GIF* chimera using the closest homologs to wheat *GRF4* and *GIF1* in both species (Supplementary Fig. 1a,b). In three independent transformation experiments in the citron rootstock Carrizo (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 compared to those transformed with the empty vector control (Supplementary Fig. 6a). The heterologous grape *GRF–GIF* chimera produced similar increases in citrus regeneration efficiency as the citrus chimera (Supplementary Fig. 6b).

We also tested the effect of a grape *GRF–GIF* chimera that was resistant to miR396 (henceforth referred to as *rGRF–GIF*) in which we introduced silent mutations in the binding site for miR396 in *GRF* to avoid cleavage (Supplementary Fig. 6b,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 and the three *GRF–GIF* constructs in combination was also significant ($P=0.0136$; Supplementary Fig. 6d and Supplementary Table 7). In spite of its higher regeneration frequency, the *rGRF–GIF* construct would require additional optimization (for example, an inducible system) because some of the transgenic events produced large calli that were unable to generate shoots (Supplementary Fig. 6b).

In summary, expression of a *GRF4–GIF1* chimera significantly increased the efficiency and speed of wheat regeneration and the ability to generate large numbers of fertile edited plants. Expression of the chimera also extended the range of transformable genotypes and eliminated the requirement of cytokinin for regeneration, thereby eliminating the need for antibiotic-based selectable markers. The *GRF4–GIF1* technology results in fertile and normal transgenic plants without the need for 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* (refs. [6–8]), there is the potential to combine these technologies and have synergistic effects in the regeneration efficiency of recalcitrant genotypes. A concurrent and independent study showed that overexpression of *Arabidopsis AtGRF5* and *AtGRF5* homologs positively enhances 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.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41587-020-0703-0.

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Methods

Wheat vectors. We performed all PCR cloning with Phusion High-Fidelity DNA Polymerase (New England BioLabs). We extracted RNA from spikes using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), treated the extracted RNA with RQ1 RNase-free DNase (Promega) and then synthesized the cDNA using SuperScript II Reverse Transcriptase (Invitrogen). To clone the coding regions of wheat *GRF4* and *GIF1*, we performed PCR using cDNA generated from Kronos spikes. The sequences 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 Gateway BP reaction and generated the *GRF4–GIF1* chimera by overlapping PCR.

In the first step, we amplified the *GRF4* and *GIF1* coding sequences with the FW-GRF4a/Rev-GRF4b and Fw-GIF1b/Rev-GIF1b primers from the pDONR-GRF4 and pDONR-GIF1 clones. The Rev-GRF4b primer generates a 3′ end that overlaps with 12 nucleotides on the 5′ end of Fw-GIF1b. Those 12 nucleotides generate a bridge of four alanine residues 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 into pDONR. Next, we cloned *GRF4*, *GIF1* and the *GRF4–GIF1* chimera into the binary vector pLC41 by a Gateway LR reaction under the control of 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 into *Agrobacterium* strain EHA105 and in a few experiments into strain AGL1 (Supplementary Table 4). Both strains were handled in the same way.

To develop the vector expressing both *GRF4* and *GRF1* under the control of their own *UBIQUITIN* 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_HindIII and Rev-HindIII (Supplementary Table 1). We cloned the PCR fragment into pGEMT-easy and then subcloned the Ubi::*GRF4*-term fragment into the HindIII 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. We then 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 into a pLC41 vector by a Gateway LR reaction. We verified all the vectors by restriction digestion and transformed them by electroporation into *Agrobacterium* strain EHA105.

To develop the JD635-*GRF4–GIF1/CRISPR–Cas9–gRNA–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) into the AscI site of the pYP25F binary vector, which contains a wheat codon-optimized Cas9 (TaCas9) with two nuclear localization signals and is a modified version of pDIRECT_25F (Addgene, 91143) from the laboratory of D. Voytas (University of Minnesota). We validated the vector sequence by Sanger sequencing. Next, we cloned a gRNA construct targeting the coding region of gene *Q*[35] by GoldenGate reaction into two AarI sites of the vector and transformed it into chemically competent *Escherichia coli* DH5α. We validated the JD635-*GRF4–GIF1/CRISPR–Cas9–gRNA–Q* vector by Sanger sequencing and transformed it 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 Fig. 1. We cloned the DNA fragments into pDONR by a Gateway BP reaction. We cloned the *GRF–GIF* chimeras into the pGWB14 binary vector by a Gateway LR reaction under the control of a viral *35S* promoter and transformed them by electroporation into *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 the pGBW14-*Vitis GRF–GIF* clone as template. The primers rGRF-Fw and rGRF-Rev overlap by 17 nucleotides and introduce silent mutations in the miR396 target site (Supplementary Fig. 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 a Gateway BP reaction. Next, we cloned the chimera *rGRF–GIF* in the binary vector pGWB14 by a Gateway LR reaction under the control of the viral *35S* promoter and transformed them by electroporation into *Agrobacterium* strain EHA105.

Wheat transformation. Wheat transformation followed previously published protocols[29]. Briefly, we grew the different wheat and triticale cultivars in a greenhouse or a growth chamber under a long-day photoperiod (16 h of 380 μM m^{-2} s^{-1} light, 26 °C during the day and 18 °C at night). We harvested immature grains from spikes approximately 2 weeks after anthesis and surface sterilized the grains for 1 min in 70% ethanol followed by 10 min in a solution of 1.2% (vol/vol) sodium hypochlorite and 5 μl Tween-20. After surface sterilization, we washed the seeds three times with sterilized water and isolated immature embryos using a stereoscopic microscope (embryo sizes, 1.5–3.0 mm).

We centrifuged the isolated immature embryos in liquid medium and then inoculated them with *Agrobacterium*. We transferred the embryos to co-cultivation medium with the scutellum side up and incubated them at 23 °C in the dark. After 2–3 d, we excised the embryo axis, transferred them to callus induction medium without selection and incubated them at 25 °C in the dark. After 5 d, we transferred the embryos to selection medium with 30 mg $liter^{-1}$ hygromycin and incubated them at 25 °C in the dark.

After 3 weeks, we transferred calli to selection medium that contained 100 mg $liter^{-1}$ hygromycin. After an additional 3 weeks, we transferred the proliferating tissue to regeneration medium containing 50 mg $liter^{-1}$ 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 that contained 50 mg $liter^{-1}$ hygromycin. Rooted plants were acclimated to soil by transferring them to a 1020 tray containing 36-cell inserts filled with Sunshine Potting Mix, covering them with a 28 cm × 53 cm × 5 cm clear plastic dome and maintaining them for 10 d under 16 h of 100 μM light at 26 °C. More recently, we developed a shorter transformation protocol to generate *GRF4–GIF1* transgenic wheat plants that is summarized in Supplementary Fig. 2. Transformation at the John Innes Centre was performed as published before[32].

Rice transformation. Rice transformation was performed following previously published protocols[36]. Briefly, we selected fresh rice seeds, dehusked them and surface sterilized them in a rotating flask containing 20% (vol/vol) bleach for 30 min. We then rinsed the seeds three times with sterile water. We placed about 25–50 seeds per plate onto callus induction medium (MSD, 1× Murashige and Skoog with vitamins medium containing 30 g $liter^{-1}$ sucrose, 2 mg $liter^{-1}$ 2,4-dichlorophenoxyacetic acid and 1.2% (wt/vol) agar, pH 5.6–5.8) without letting the embryo touch the medium, wrapped the plates with surgical tape and incubated them under a 16-h light/8-h dark cycle at 28 °C. After 10–14 d, we separated the callus from the rest of the germinating seed and transferred it 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 that was isolated from a plate. We then inoculated 1 ml LB containing the appropriate antibiotics to maintain the *Agrobacterium* and the plasmid, and we incubated the culture overnight at 28 °C in a shaking incubator set at 250 r.p.m. The following day, we added 300 μl of the *Agrobacterium* culture to 20 ml of TY (pH 5.5) containing the appropriate antibiotics and 200 μM acetosyringone. We incubated the culture at 28 °C in a shaking incubator set at 250 r.p.m. until the culture reached an OD_{600} of between 0.1 and 0.2 (approximately 2–4 h).

Transformation and co-cultivation. We placed the calli in an *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, 1× Murashige and Skoog with vitamins medium containing 30 g $liter^{-1}$ sucrose, 5% sorbitol, 2 mg $liter^{-1}$ 2,4-dichlorophenoxyacetic acid, 200 μM acetosyringone and 1.6% (wt/vol) agar, pH 5.6–5.8) and incubated them for 3 d in the dark at 22 °C.

Selection. We transferred the co-cultivated calli to selection medium (MSD + CH + PPM, 1× Murashige and Skoog with vitamins medium containing 30 g $liter^{-1}$ sucrose, 2 mg $liter^{-1}$ 2,4-dichlorophenoxyacetic acid, 400 mg $liter^{-1}$ carbenicillin, 200 mg $liter^{-1}$ timentin, 1 ml $liter^{-1}$ Plant Preservative Mixture, 80 mg $liter^{-1}$ hygromycin and 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 medium every 8–9 d.

Regeneration and rooting. After 4–5 weeks on selection medium, resistant microcalli that were approximately 2–5 mm wide started to appear. We picked these off the original callus and transferred them to Petri dishes with regeneration medium (BN + S + CH, 1× Murashige and Skoog with vitamins medium containing 30 g $liter^{-1}$ sucrose, 5% sorbitol, 3 mg $liter^{-1}$ BAP, 0.5 mg $liter^{-1}$ NAA, 400 mg $liter^{-1}$ carbenicillin, 200 mg $liter^{-1}$ timentin, 1 ml $liter^{-1}$ Plant Preservative Mixture, 50 mg $liter^{-1}$ hygromycin and 1.6% (wt/vol) agar, pH 5.6–5.8) and incubated the dishes under continuous light at 28 °C. We subcultured these calli onto fresh regeneration medium every 8–9 d. After 4–5 weeks, the calli that started to turn green were transferred to regeneration medium with reduced hygromycin (BN + S + CH, 1× Murashige and Skoog with vitamins medium containing 30 g $liter^{-1}$ sucrose, 5% sorbitol, 3 mg $liter^{-1}$ BAP, 0.5 mg $liter^{-1}$ NAA, 400 mg $liter^{-1}$ carbenicillin, 200 mg $liter^{-1}$ timentin, 1 ml $liter^{-1}$ Plant Preservative Mixture, 25 mg $liter^{-1}$ hygromycin and 1.6% (wt/vol) agar, pH 5.6–5.8). When the shoot was properly developed, we transferred the regenerated plants to rooting medium (MS + H, 1× Murashige and Skoog with vitamins medium containing 25 mg $liter^{-1}$ hygromycin and 1.2% (wt/vol) agar, pH 5.6–5.8) and incubated them under a 16-h light/8-h dark cycle at 28 °C. When the roots were well developed, we transferred the plants to soil.

***Citrus* transformation.** We placed seeds of Carrizo citrange rootstock in water to soak and then peeled off the seed coats, making sure not to remove the integument. We surface sterilized seeds in a solution of 0.6% (vol/vol) sodium hypochlorite

and 5 µl of Tween-20, placed them in a 50-ml centrifuge tube and shook them at 100 r.p.m. for 20 min. We rinsed the seeds three times in 150–200 ml of sterile distilled water. We placed seeds on agar (solidified ½× Murashige and Skoog minimal organics medium (½× MSO) containing 15 g liter^{-1} sucrose and 7 g TC agar (pH 5.6–5.8)) and pushed seeds slightly into the medium for more uniform germination. The seeds were then incubated 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 r.p.m. 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 r.p.m. and then diluted the overnight culture grown in TY medium to an OD_{600} of 0.1–0.2.

Co-cultivation. We collected 2- to 5-week-old etiolated epicotyls and placed them into a Petri dish containing 10 ml of the *Agrobacterium* solution prepared as described above (OD_{600} = 0.1–0.2). We cut submerged epicotyls into 0.5-cm sections and soaked them for 10 min. We then transferred the epicotyl sections onto co-cultivation medium consisting of MSO modified with 30 g liter^{-1} sucrose, 3.0 mg liter^{-1} BAP, 0.1 mg liter^{-1} NAA and 200 µM acetosyringone, pH 5.6–5.8. The sections were then incubated at 23 °C in the dark.

Induction. After 2–3 d, we transferred the epicotyl pieces to induction medium consisting of MSO modified with 30 g liter^{-1} sucrose, 3.0 mg liter^{-1} BAP, 0.1 mg liter^{-1} NAA, 400 mg liter^{-1} carbenicillin, 150 mg liter^{-1} timentin and 100 mg liter^{-1} kanamycin sulfate and incubated them in the dark. After 10 d, we subcultured the epicotyl sections in fresh medium of the same formulation and then subcultured them every 21 d. After the second 21-day cycle in the dark, we transferred the cultures to light under a 30 µM light with a photoperiod of 16 h of light and 8 h of darkness. We continued to transfer the cultures every 21 d to fresh medium until organogenic shoot buds developed 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 liter^{-1} sucrose, 0.1 mg liter^{-1} BA, 400 mg liter^{-1} carbenicillin, 150 mg liter^{-1} timentin and 100 mg liter^{-1} kanamycin sulfate. We incubated the cultures as described above and subcultured them 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 liter^{-1} sucrose, 5 mg liter^{-1} NAA, 250 mg liter^{-1} cefotaxime and 100 mg liter^{-1} kanamycin sulfate. After 3–5 d, we transferred shoots to MSO modified with 30 g liter^{-1} sucrose, 400 mg liter^{-1} carbenicillin, 100 mg liter^{-1} kanamycin sulfate and no NAA. Shoots started rooting after 14 d.

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

Data availability

Accession numbers and gene names are available in the phylogenetic tree in Supplementary Fig. 1. All wheat gene names are based on genome release RefSeq v1.0. The raw data for the different experiments are available in Supplementary Tables 3, 4, 6 and 7. The steps for the generation of the different vectors and the transformation protocols are described in the Methods. The following vectors will be available through Addgene (http://www.addgene.org/): JD553-wheat *GRF4–GIF1* in pDONR, JD633-wheat *GRF4–GIF1* in the 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.

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36. Chern, M. S. et al. Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in *Arabidopsis*. *Plant J.* **27**, 101–113 (2001).

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Author contributions

J.M.D. contributed to the investigation, methodology, formal analysis, writing and editing. D.M.T. contributed to the investigation, supervision, methodology, project administration, funding acquisition, writing and editing. J.F.P. contributed to study conceptualization, writing and editing. M.F.E. contributed to the experiments involving rice, writing and editing. S.H. performed wheat transformation experiments at the John Innes Centre. P.R. supervised the experiments involving rice and contributed to writing and editing. J.D. contributed to study conceptualization, formal analysis, supervision, project administration, funding acquisition, writing and editing.

Competing interests

J.F.P. and J.M.D. 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). J.F.P., J.D., D.M.T. and J.M.D. 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 nonexclusive 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.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41587-020-0703-0.

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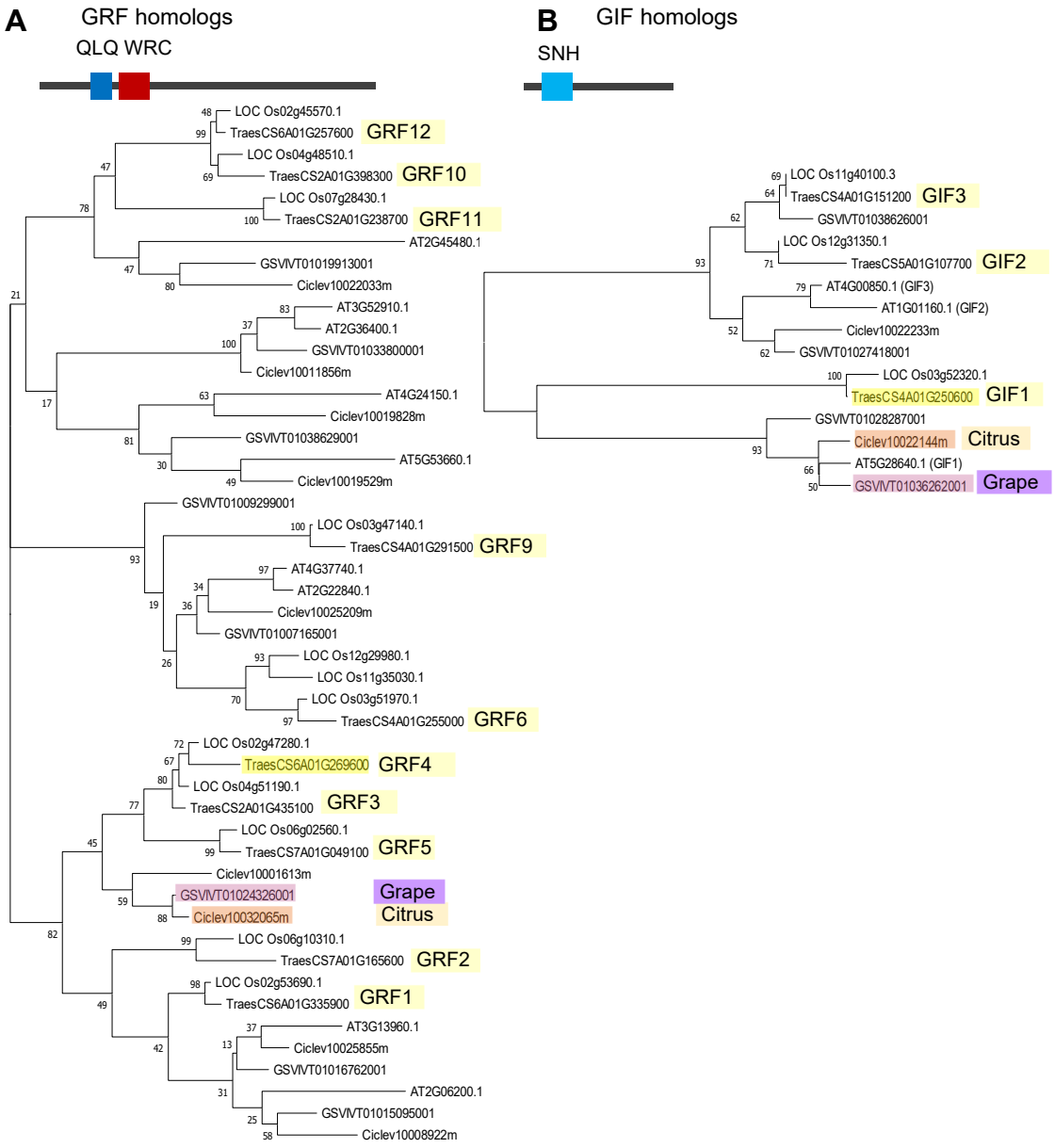
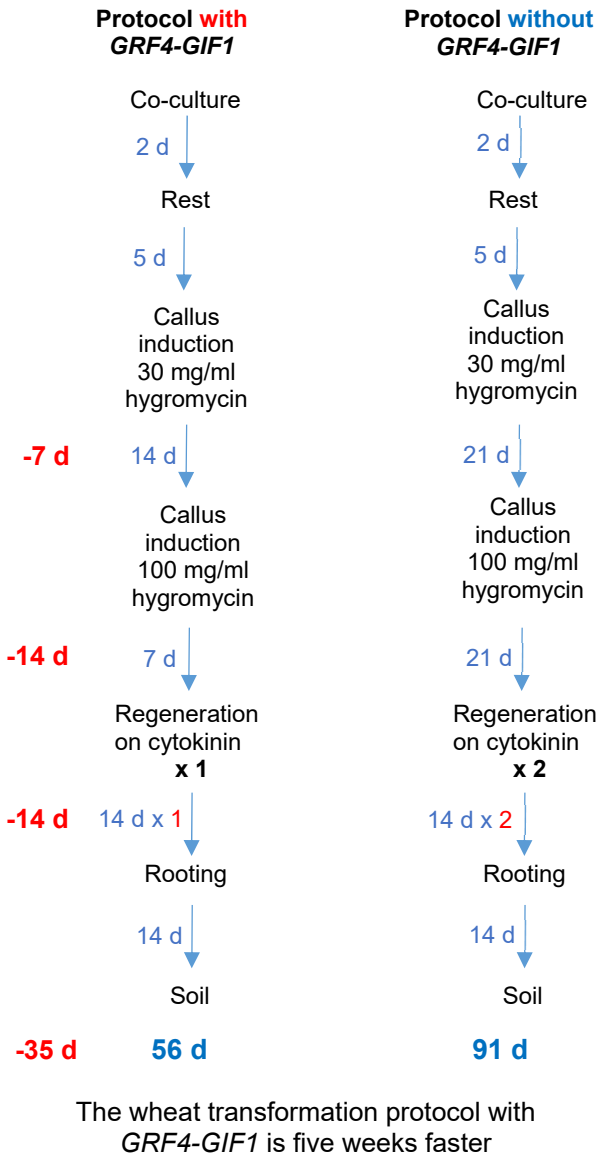
Supplementary Figures

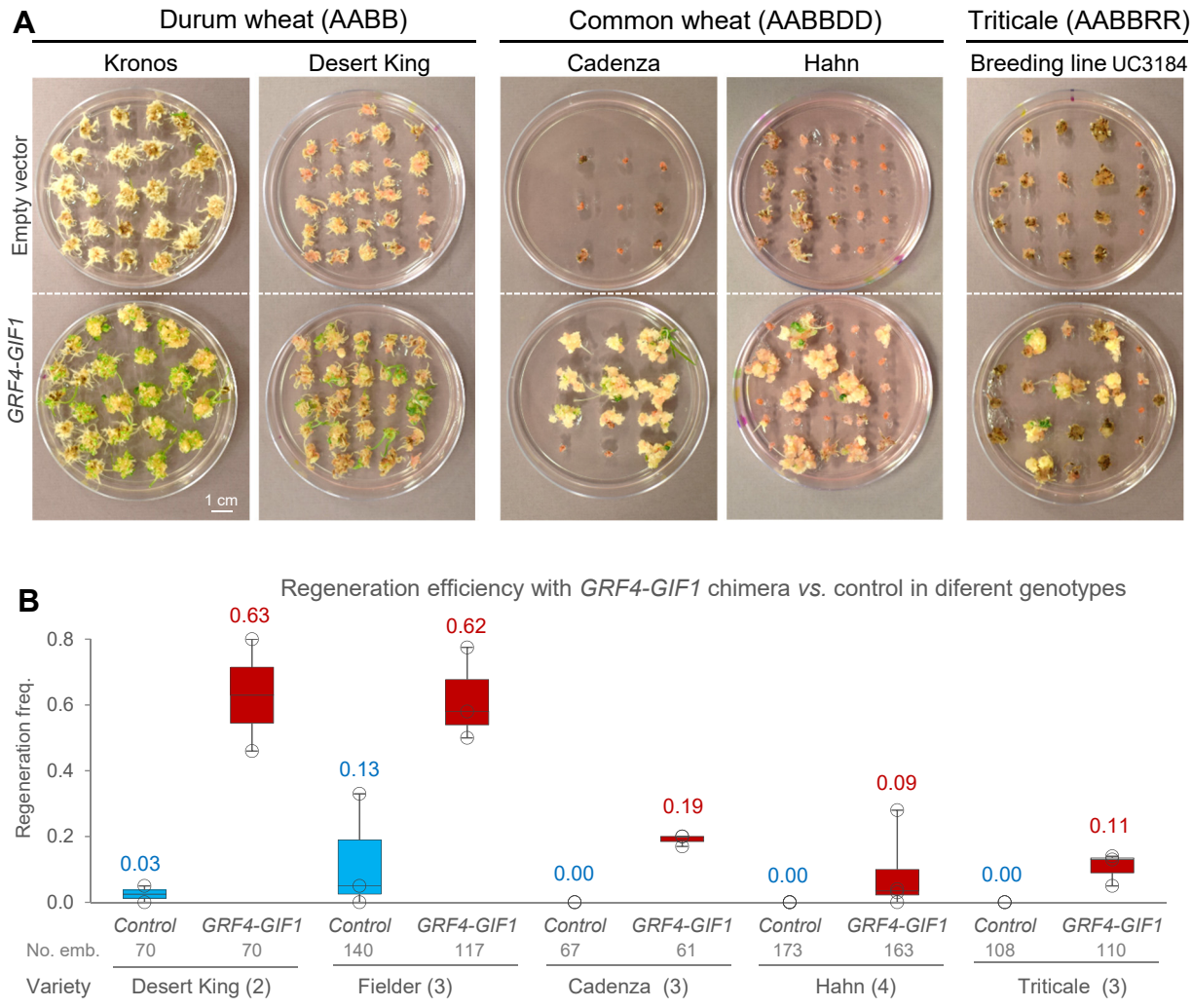
Supplementary Figure 1. Phylogenetic trees of GRF and GIF families for wheat (yellow highlight and corresponding RefSeq v1.0 names), rice, Arabidopsis, citrus and grape. The closest homologs to wheat GRF4 and GIF1 are highlighted in orange for citrus and in violet for grape. **A**) We used the QLQ and 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 MEGA X [1]. Yellow highlight: wheat. Orange highlight: selected *Citrus* homolog. Violet highlight: selected *Vitis* homolog. Note that the cluster including wheat and rice GRF3, GRF4, and GRF5 proteins does not include any Arabidopsis protein.



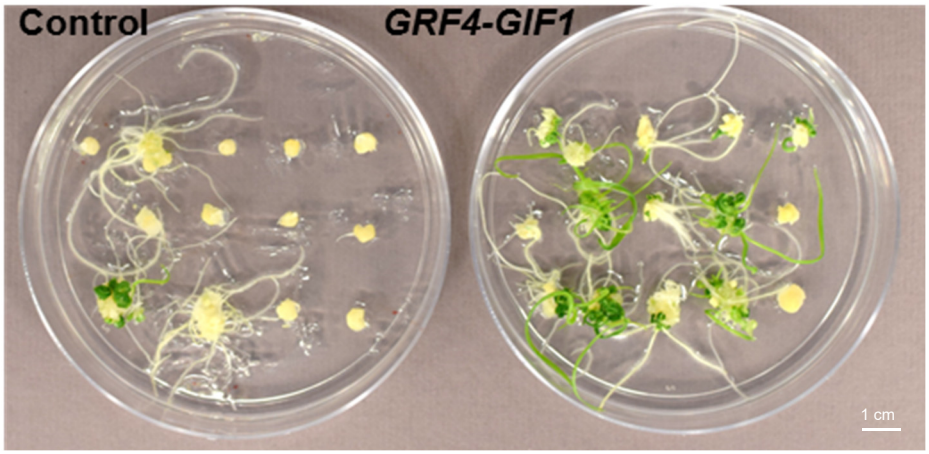
Supplementary Figure 2. Accelerated wheat transformation protocol using the *GRF4-GIF1* chimera relative to normal protocol of wheat transformation at the UC Davis transformation facility. The protocol with the *GRF4-GIF1* chimera is faster, reducing the overall process by five weeks.



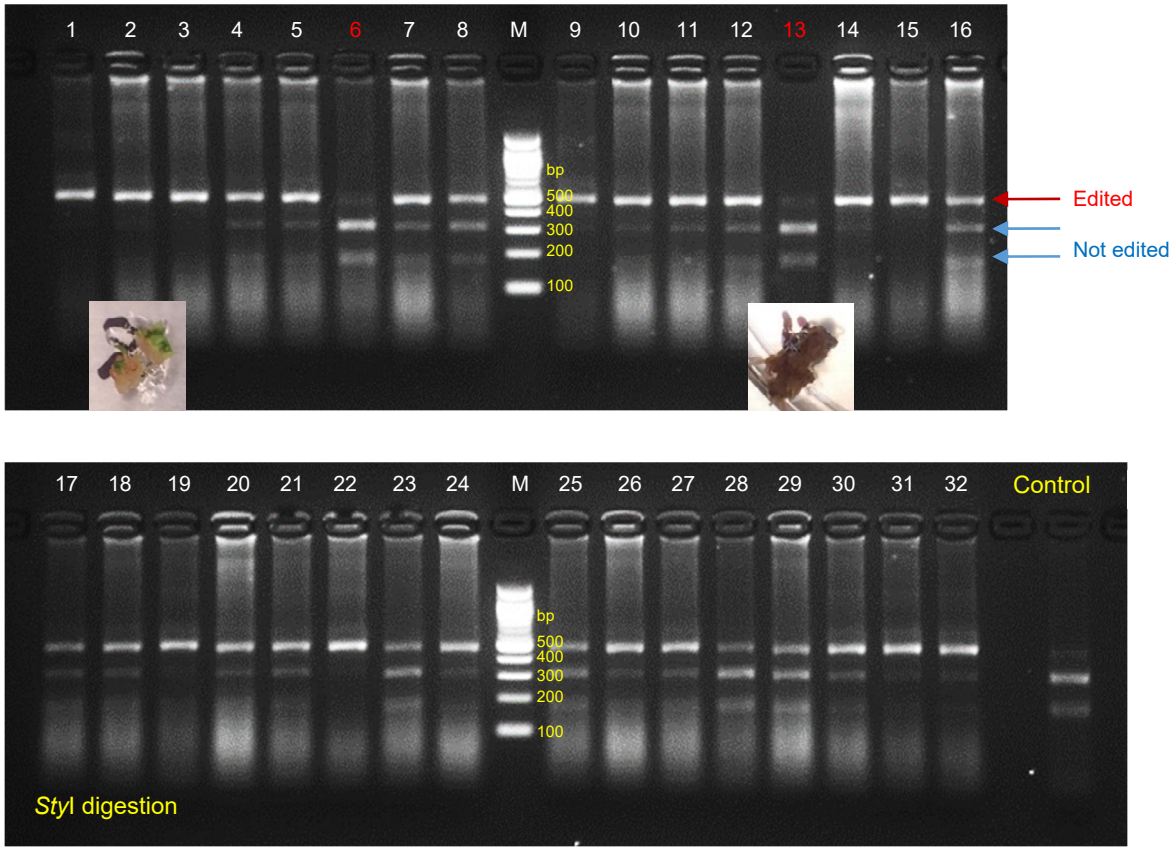
Supplementary Figure 3. Effect of the *GRF4-GIF1* chimera in regeneration efficiency in different genotypes. **A**) Representative transformations showing higher frequency of regenerated shoots in the presence of the *GRF4-GIF1* chimera than in the control (empty vector) in different wheat and Triticale genotypes. **B**) Box-plots showing regeneration efficiencies of *GRF4-GIF1 vs.* control in the same cultivars as in A. The raw data is available in Supplementary Table 4A and B. The number of independent experiments is indicated in parenthesis after the genotype name and the total number of inoculated embryos is indicated below. 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. Empty black circles are regeneration results from individual experiments. No statistical analysis is presented for these experiments because transformations of many of these cultivars without the *GRF4-GIF1* chimera showed 0 or close to 0 regeneration frequencies. Averages are presented above the box-plots.



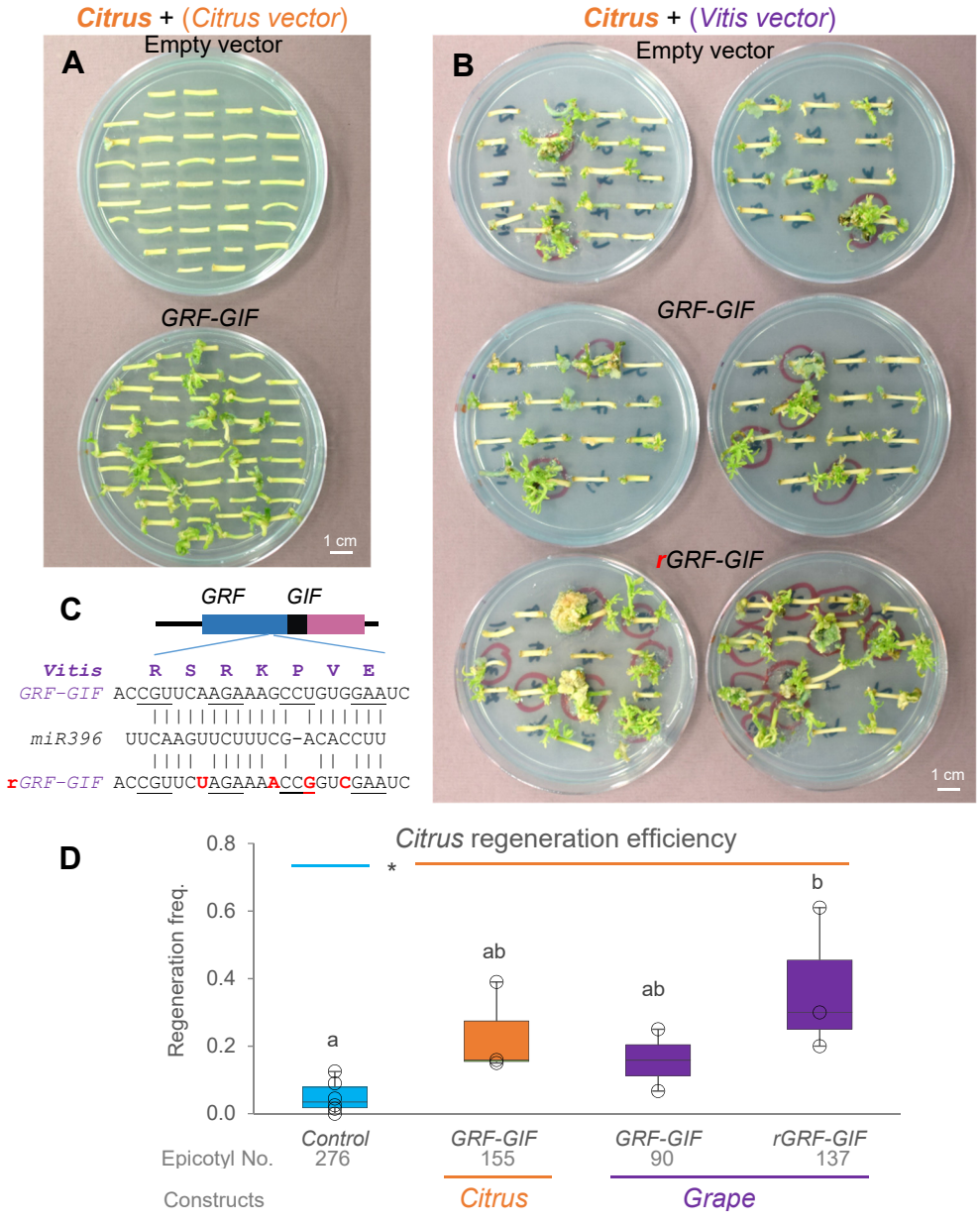
Supplementary Figure 4. Effect of the *GRF4-GIF1* chimera in regeneration efficiency in the absence of exogenous cytokinin. Immature wheat embryos from a *GRF4-GIF1* transgenic Kronos T_1 plant and a segregating non-transgenic T_1 sister line where treated following the standard transformation protocol, excluding the *Agrobacterium* inoculation and the addition of hygromycin to the plates. In the last step, the calli where transferred to regeneration media in the absence of cytokinin. The number of calli regenerating green shoots was significantly higher in the *GRF4-GIF1* transgenic plant (21 out of 27) than in the non-transgenic sister control (3 out of 26). The picture shows representative plates with calli in regeneration media without cytokinin.



Supplementary Figure 5. Genome edited wheat plants using combined *GRF4-GIF1* – CRISPR-Cas9 technology. We recovered 30 independent transgenic events (white numbers) out of 32 infected callus. Calli 6 and 13 (red numbers) were not transgenic. Figures are examples of a transgenic (2) and non-transgenic (13) callus. Editing disrupts a *Sty*I restriction site in the target region resulting in an undigested band (red arrow). Not edited sequences are digested (blue arrows). Ten of the edited events were sequenced and the detected mutations are presented in Figure 3D. M = 100 bp ladder. Control = non-transgenic Kronos.



Supplementary Figure 6. Transformation of dicot species with GRF-GIF chimers. A) *Citrus* epicotyls transformed with an empty vector and the *Citrus GRF-GIF* chimera (60 d after *Agrobacterium* inoculation). **B**) *Citrus* epicotyls transformed with an empty vector and the *Vitis GRF-GIF* and miR396-resistant *Vitis GRF-GIF* (*rGRF-GIF*) (120 d after inoculation). **C**) Scheme of a *Vitis GRF-GIF* chimera showing the miR396 target site and its interaction with miR396 below. In the miR396-resistant *rGRF-GIF* version, we introduced silent mutations (in red) to reduce interactions with miR396. **D**) Box-plots comparing three *Citrus* experiments. Regeneration results from individual experiments are indicated by empty black circles. Box-plot definition is the same as in Supplementary Figure 3. Different letters above the box-plots indicate significant Tukey test ($P < 0.05$). Horizontal lines on top indicate a significant contrast between the control and combined *GRF-GIF* constructs ($P = 0.0136$). Normality of residuals was confirmed by Shapiro-Wilk's test and homogeneity of variances by Levene's test.



SUPPLEMEN

SUPPLEMENTARY TABLES

Supplementary Table 1. Primers used in this study.

Name	Sequence	Gene
Fw-GRF4a	GGGGACAAGTTTGTACAAAAAAGCTGCCACCATGGCGATGCCGTATGCCTCT	GRF4
Rev-GRF4a	GGGGACCACTTTGTACAAGAAAGCTGAACGGTACATYTCGCCGGCGAACAG	
Fw-GIF1a	GGGGACAAGTTTGTACAAAAAAGCTGCCACCATGCAGCAGCAACACCTGATG	GIF1
Rev-GIF1a	GGGGACCACTTTGTACAAGAAAGCTGAACGGCTTCCTTCCTCCTCGGT	
Fw-GRF4a	GGGGACAAGTTTGTACAAAAAAGCTGCCACCATGGCGATGCCGTATGCCTCT	GRF4-GIF1
Rev-GRF4b	GGCAGCGGCCGCGTACATYTCGCCGGCGAACAG	
Fw-GIF1b	GCGGCCGCTGCCATGCAGCAGCAACACCTGATG	
Rev-GIF1b	GGGGACCACTTTGTACAAGAAAGCTGAACGCTAGCTTCCTTCCTCCTCGGT	
Fw_HindIII	GCCACTCAGCAAGCTTTGCAGCGT	Ubi::GRF4-term
Rev-HindIII	TCACGCTGCAAAGCTCTAATTCCCGATCTAGTAAC	
Fw_ZmUbi-AscI	GGATCTGCAGGCGCGTGCAGCGTGACCCGGTCGTG	Ubi::GRF4-GIF1-term
Rev_NosTerm-AscI	TGCACTGCAGGCGCGCTAATTCCCGATCTAGTAAC	
QT1-F-GG	ACTTGATGAGGAACTGGACCAAGG	Q gene gRNA
QT1-R-GG	AAACCCTTGGTCCAGTTCCTCATC	
QT1check-F	TGAGCGACTACGAGGAGGAT	Q gene genotyping
QT1check-R	CAGCTGCCCTGTCACATCTA	
Fw-rGRF-Vvi	TCTAGAAAACCGGTCGAATCACAAACTA	rGRF-GIF
Rev-rGRF-Vvi	TCGACCGGTTTTCTAGAACGGTTGCGG	
Fw-GRF-Vvi	GGGGACAAGTTTGTACAAAAAAGCTGCCACCATGAAGCAAAGCTTTGTGG	
Rev-GIF-Vvi	GGGGACCACTTTGTACAAGAAAGCTGAACGTCAATTCCCATCTTCAGCA	
pLC41_1064	TCGCTTATTTAAAGGGCGAAT	Transgenic plants genotyping
pLC41_1061	AGCGCGCAAACTAGGATAAA	

Supplementary Table 2. Grain measurements in *GRF4-GIF1* T1 transgenic plants and their sister negative controls in a growth chamber (16 h light at 22 °C and 8 h darkness at 18 °C, light intensity 260 μM m^{-2} s^{-1}). Two statistical analyses are presented: 1) A more conservative test using the averages of the families from each event as experimental units (5 negatives *vs*. 3 positives). 2) A more liberal test using the individual plants as experimental units (38 negatives *vs*. 16 positives). Parameters from individual plants were obtained from an average of 23 grains estimated from a Marvin Grain Analyzer. The JD561 numbers indicate independent transformation events with the same *GRF4-GIF1* construct.

GRF4-GIF1 (JD561)	Plants	Spikelets / spike	Grains / spike	TGW(g)	Area (mm^2)	Width (mm)	Length (mm)
Negative (JD561#2-1)	8	13.25	26	41.48	17.75	3.31	7.43
Negative (JD561#12-1)	9	12.78	23	57.04	20.85	3.69	7.80
Negative (JD561#20-6)	4	11.50	23	55.92	20.07	3.66	7.63
Negative (JD561#21-1)	12	11.67	27	55.50	19.74	3.60	7.63
Negative (JD561#23-8)	5	12.80	26	51.81	19.27	3.55	7.43
Positive (JD561#13-1)	9	9.67	18	59.68	22.18	3.67	8.31
Positive (JD561#20-11)	2	10.00	13	57.09	21.60	3.69	8.08
Positive (JD561#23-6)	5	11.00	21	60.66	21.38	3.68	8.00
Weighted Avg. negatives	38	12.39	25.10	52.47	19.56	3.56	7.60
Weighted Avg. transgenic	16	10.13	19.10	59.66	21.86	3.68	8.18
% increase		-18.3%	-23.9%	13.7%	11.7%	3.2%	7.7%
Two side *t*-test (family as e.u)		0.007	0.007	0.131	0.022	0.239	0.003
Two side *t*-test (plant as e.u.)		3.3E-04	8.9E-05	2.9E-03	5.7E-06	1.5E-02	1.5E-08

Supplementary Table 3. Regeneration frequencies for different *GRF-GIF* combinations compared with empty vector in tetraploid wheat Kronos. The number of embryos used is indicated below each frequency. Regeneration frequencies were estimated as the number of calluses showing at least one regenerating shoot / total number of inoculated embryos. The blue "x" indicate the experiments included in the statistical analyses presented in the different figures and supplementary figures. All experiments in this Table used the regular 91 d protocol and *Agrobacterium* strain EHA105.

		GRF4-	Fig.	GRF4 &	Fig.			Fig.	GRF4-		Fig.	GRF5	GRF1	GRF9	Fig.
Exp.	pLC41	GIF1	1D	GIF1	1E	GIF1	GRF4	1F	GIF2	GIF3	1G		-GIF1		S1
1-a	0.04 25	0.90 48	x												
1-b	0.08 25	0.96 25	x												
2		0.27 60		0.06 32	x										
3		0.91 79		0.77 83	x										
3b[a]		0.60 47		0.14 41	x										
4	0.13 53	0.70 50	x	0.46 48	x	0.57 47									
6	0.20 20	0.65 20	x	0.50 20	x	0.35 20									
22	0.16 24	0.82 28	x			0.16 24	0.64 25	x							
25	0.00 15	0.70 20	x			0.40 15	0.06 15	x							
25b[a]	0.00 15	0.35 17	x			0.00 15	0.00 15	x							
26	0.08 24	0.55 20	x			0.20 20	0.20 20	x							
26b[b]	0.06 16	0.31 16	x			0.10 10	0.12 16	x							
12	0.00 10	0.50 10	x						0.20 10	0.20 10	x				
13	0.17 24	0.72 25	x						0.50 24	0.46 24	x				
24	0.10 10	0.50 10	x						0.50 10	0.30 10	x	.	0.10 10	0.30 10	x
17	0.00 21	0.67 21	x									0.57 21	0.16 19	0.19 21	x
18	0.20 24	0.88 24	x									0.70 23	0.79 24	0.76 21	x
28	0.02 44	0.56 43	x									0.21 42	0.16 45	0.06 18	x

"-" indicates a fused protein or chimera, "&" indicates individual genes induced by separate promoters.

[a] No embryo dissection.

[b] No cytokinin.

Supplementary Table 4. Regeneration frequencies in plants transformed with the *Ubi::GRF4-GIF1* chimera or the empty vector pLC41. **A**) Tetraploid and hexaploid wheat commercial cultivars. **B**) Triticale breeding line UC3190. EHA105 and AGL1 are two different *Agrobacterium* strains (no differences were observed between the two strains). The number of embryos used for each genotype is indicated below the regeneration frequency.

A. Wheat

Desert King (4x)	Exp1 EHA105	Exp2 EHA105	**Average**	**SE**
pLC41	0.05 20	0 50	**0.025**	0.025
Ubi::GRF4-GIF1	0.80 20	0.46 50	**0.630**	0.170

Fielder (6x)	Exp1 UCD EHA105	Exp2 UCD EHA105	UCD **Average**	UCD **SE**
pLC41	0.05 49	0 10	**0.025**	0.025
Ubi::GRF4-GIF1	0.58 67	0.5 10	**0.540**	0.040

Fielder (6x)	Exp1 JIC AGL1	Three Fielder experiments **Average**	 **SE**
pAGM8031	0.33 81	0.127	0.103
Ubi::GRF4-GIF1	0.775 40	0.618	0.082

Cadenza (6x)	Exp1 AGL1	Exp2 AGL1	Exp3 EHA105	**Average**	**SE**
pLC41	0 19	0 23	0 25	**0.000**	0.000
Ubi::GRF4-GIF1	0.20 12	0.17 24	0.20 25	**0.190**	0.010

Hahn (6x)	Exp1 EHA105	Exp2 EHA105	Exp3 EHA105	Exp4 AGL1	**Average**	**SE**
pLC41	0 31	0 48	0 69	0 25	**0.000**	0.000
Ubi::GRF4-GIF1	0.03 37	0.04 50	0 51	0.28 25	**0.088**	0.087

Supplementary Table 4B. Triticale

Triticale UC3190 (6x)	Exp9 EHA105	Exp11 EHA105	Exp15 EHA105	**Average**	**SE**
pLC41	0 45	0 21	0 42	**0.000**	0.000
Ubi::GRF4-GIF1	0.05 45	0.13 22	0.14 43	**0.107**	0.028

Supplementary Table 5. Regeneration frequencies in wheat transformation with *Agrobacterium*

Wheat methods	Explant	Average efficiency	Marker	Agro strain	Cultivars
This study without *GRF4-GIF1*	immature embryos	8.3 / 2.5 % 12.7 % / 0.0 %	HPT	EHA105	Kronos (4x) / 1 other (4x) Fielder (6x) / 2 other (6x)
GRF4-GIF1	immature embryos	65.1 / 63.0 % 61.8 / 13.9 %	HPT	EHA105	Kronos (4x) / 1 other (4x) Fielder (6x) / 2 other (6x)
Cheng et al., 1997 [2]	immature embryos	2.2 %	NPT	C58 (ABI)	Bobwhite (6x)
Khanna HK, Daggard GE 2003 [3]	immature embryos	3.9 %	PPT	LBA4404	Veery5 (6x)
Wu et al., 2003 [4]	immature embryos	9.5 / 4.5 %	PPT	AGL1	Bobwhite / 3 other (6x)
Cheng et al., 2003 [5]	immature embryos	1.1%	NPT II	C58 (ABI)	Bobwhite (6x)
Hu et al., 2003 [6]	immature embryos	4.4 %	Glyphosate	C58 (ABI)	Bobwhite (6x)
Przetakiewicz et al., 2004 [7]	immature embryos	12.6 / 2.3 %	NPT II	EHA101 / LBA4404	Kontesa, Torka & Eta (6x)
Mitic et al., 2004 [8]	immature embryos	0.6 %	PPT / HPT	AGL1 / LBA4404	Vesna (6x)
Wu et al., 2008 [9]	immature embryos	3.0 %	PPT	AGL1	Ofanto (4x)
Risacher et al., 2009 [10]	immature seeds *in planta*	5.0 %	NPT II	EHA105	NB1 (6x)
He et al., 2010 [11]	immature embryos	6.3 %	PPT	AGL1	Stewart (4x)
Bińka et al., 2012 [12]	immature embryos	3.4 %	NPT / PPT	EHA101/AGL1	Kontesa, Torka (6x)
Hensel et al., 2017 [13]	immature embryos	5 to 15 % [a]	HPT	AGL1	Bobwhite (6x)
Hayta et al., 2019 [14]	immature embryos	19 %	HPT	AGL1	Fielder (6x)
Proprietary Japan Tobacco [b]					
Ishida et al., 2015 [c] [15]	immature embryos	76.2 / 60.8 %	PPT / HPT	EHA101/EHA105	Fielder (6x, PPT *vs* HPT)
Richardson et al., 2014 [16]	immature embryos	40.9 / 12.1 % 50.8 / 26.0 %	PPT	AGL1	Fielder / 9 other (6x) Kronos / 1 other (4x)
Wang et al., 2017 [17]	immature embryos	45.3 / 10.8 %	PPT	C58C1	Fielder / 17 other (6x)

[a] Only range provided

[b] At UCD, we purchased the JT license and received training at their company. However, without the *GRF4-GIF1*, we have not been able to obtain the high regeneration efficiencies reported in Ishida et al. 2015 [15] (likely because we use a wider range of embryo sizes collected from plant grown under different conditions)

[c] Report by the Japan Tobacco company in a non-peer reviewed journal

Supplementary Table 6. Regeneration frequencies in rice (*Oryza sativa*) cultivar Kitaake. Experiments 1 and 6 utilized the wheat-optimized vector pLC41 with or without the *Ubi::GRF4-GIF1* chimera. Experiments 2-5 used pCAMBIA1300, a vector frequently utilized in rice transformation, with or without the *Ubi::GRF4-GIF1* chimera. In each of these three experiments, calli generated from the same seed stock were inoculated with *Agrobacterium* containing the designated vector construct. In experiment 2, pCAMBIA1300-sgRNA refers to the pCAMBIA1300 vector carrying *Ubi::GRF4-GIF1* chimera plus a sgRNA targeted to gene *OsKitaake06g041700* encoding a TYROSYLPROTEIN SULFOTRANSFERASE (TPST). In experiments 3 and 4, pCAMBIA1300-gus refers to the control pCAMBIA1300-gus without the chimera. All experiments employed *Agrobacterium* strain EHA105. The number of calli used for each genotype is indicated below the regeneration frequency

Rice Kitaake	Exp1	Exp2-4	Exp5	Exp6		
No. calli inoc.	n=85	n=100 x 3	n=50	n=50	**Average**	**SE**
No *GRF4-GIF1*	0.118	0.235	0.22	0.24	**0.2033**	0.028
	pLC41	pCAMBIA1300-gus	pCambia1300	pLC41		
Ubi::GRF4-GIF1	0.353	0.460	0.44	0.46	**0.4283**	0.025
	pLC41	pCAMBIA1300-sgRNA	pCambia1300	pLC41		

Two sided paired *t*-test *GRF4-GIF1 vs.* control: $P < 0.0001$ (n = 4 experiments)

Supplementary Table 7. Regeneration frequencies in *Citrus*. Experiments 1 to 3 used a *GRF-GIF* chimera based on *Citrus* sequences whereas experiments 4 to 6 used a *GRF-GIF* chimera based on *Vitis* sequences. In addition, the last three experiments included a second *Vitis* construct with mutations in the miR396 binding site (*rGRF4-GIF1*) that precludes its cleavage. The number of epicotyls used for each genotype is indicated below the regeneration frequency.

Carrizo	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6	**Average**	**SE**
Empty vector	0.04 45	0.00 38	0.12 56	0.02 65	0.09 32	0.02 40	**0.05**	0.02
Citrus GRF-GIF	0.15 45	0.39 41	0.16 69	-	-	-	**0.21**	0.09
Vitis GRF-GIF	-	-	-	0.07 59	0.25 31	-	**0.16**	0.09
Vitis rGRF4-GIF1	-	-	-	0.20 66	0.61 31	0.30 40	**0.37**	0.12

Supplementary Table 8. Comparisons of *GRF4-GIF1* with transformation technologies using different morphogenic genes.

Technology	Ref.	Advantages	Disadvantages / limitations
GRF4-GIF1	This one	1. Publicly available for research 2. No developmental defects 3. Expands the range of genotypes that can be transformed 4. Rapid transformation protocol (60 days in wheat) 5. Robust regeneration efficiencies under broader set of protocols, including embryogenic and organogenic methods 6. Simple to implement and combine with gene editing 7. Efficient selection without selectable markers (wheat) 8. Tested in monocot and dicot species	1. No tested yet in mature tissues in monocots 2. Transgene incorporated together with the *GRF4-GIF1* chimera [a]. 3. Only tested in protocols that require in vitro tissue culture
Bbm-Wus2 (CORTEVA)	[18-20]	1. High regeneration efficiencies in maize 2. Rapid transformation protocol (35 days maize) 3. Expanded range of maize germplasm that can be transformed 4. Works in mature tissues 5. Advanced vectors worked well in sorghum, Indica rice, and sugar cane	1. Proprietary (but available for research) 2. Protocol optimized for maize. Use of specific maize promoters required to avoid regeneration problems and developmental defects 3. Tested only in monocots 4. If the BBM-WUS2 is not excised it induces developmental defects. Vectors with a CRE-LOX system are available 5. Only tested in methods that require in vitro tissue culture
De novo meristem induction Fast-TrACC *Wus2*/*ipt*	[21]	1. Sidesteps the need for tissue culture 2. Co-delivery of developmental regulators and guide RNAs can generate edited shoots in plants constitutively expressing Cas9 3. It worked in *Benthamiana* soil grown plants	1. Tested only in dicot plants (*Benthamiana*, tomato, potato, grape) 2. Fertile plants showed only in *Benthamiana* 3. Many edited plants show developmental defects and failed to produce seeds 4. Specific developmental regulators need to be defined in each new species 5. Needs transgenic plants previously transformed with Cas9

[a] This is not a problem for gene editing because both the CRISPR-Cas9 and the GRF4-GIF1 constructs are segregated out after editing. Although the presence of the *GRF4-GIF1* is not associated with developmental defects, the user can separate the transgene from the *GRF4-GIF1* chimera by using a line previously transformed with the *GRF4-GIF1* without a selectable marker, and then retransforming the same line with the desired transgene. Since the transgene and the *GRF4-GIF1* construct are incorporated in different loci, they can be segregated apart.

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University of California Davis
Commercial use terms for the GRF-GIF technology to improve regeneration efficiency

The University of California, Davis (UCD) is currently allowing for-profit entities to evaluate and use the GRF-GIF technology to improve regeneration of transgenic plants under a 1-year non-commercial material transfer agreement. For use greater than one year or for commercial use, UCD, subject to approval from the Howard Hughes Medical Institute (HHMI), plans to broadly and non-exclusively license the technology for a flat annual fee, no royalty, and without field restriction.

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