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•RESEARCH PAPER•

Transient expression of a TaGRF4-TaGIF1 complex stimulates wheat regeneration and improves genome editing

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Genome editing is an unprecedented technological breakthrough but low plant regeneration frequencies and genotype dependence hinder its implementation for crop improvement. Here, we found that transient expression of a complex of the growth regulators TaGRF4 and TaGIF1 (TaGRF4-TaGIF1) increased regeneration and genome editing frequency in wheat. When we introduced synonymous mutation in the miR396 target site of *TaGRF4*, the resulting complex (mTaGRF4-TaGIF1) performed better than original TaGRF4-TaGIF1. Use of mTaGRF4-TaGIF1 together with a cytosine base editor targeting *TaALS* resulted in 2-9-fold increases in regeneration and transgene-free genome editing in 11 elite common wheat cultivars. Therefore, mTaGRF4-TaGIF1 will undoubtedly be of great value in crop improvement and especially in commercial applications, since it greatly increased the range of cultivars available for transformation.

mTaGRF4-TaGIF1, wheat, regeneration, genome editing, transient expression

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INTRODUCTION

Efficient CRISPR reagents delivery is a prerequisite for high-throughput genome editing. However, standard plasmid DNA delivery systems widely adopted in plants, i.e., *Agrobacterium*-mediated gene delivery and biolistic particle delivery, are restricted to a narrow range of plant genotypes that can regenerate (Zhang et al., 2020a). This impedes genome editing in both dicots and monocots. Regeneration of transformed or mutated explants and genotype restriction might, in principle, be improved by expressing plant genes involved in developmental reprogramming (Altpeter et al., 2016). All these genes are developmental regulators that can reprogram somatic cells to embryogenic cells, hence they boost the regeneration of transformed explants and so are referred to as boosters (Gao, 2021). Overexpression of boosters such as *WUSCHEL2* (*Wus2*) and *BABY BOOM* (*Bbm*) enhanced the regeneration of various transformation-recalcitrant genotypes and species (Lowe et al., 2018; Lowe et al., 2016; Mookkan et al., 2017). Therefore, the development of advanced transformation technologies ought to be facilitated by using appropriate boosters to promote plant regeneration.

GROWTH-REGULATING FACTOR (GRF) proteins are plant-specific transcription factors that affect cell proliferation and size (Kim, 2019; Liebsch and Palatnik, 2020; Omidbakhshfard et al., 2015; Ma and Liu, 2018; Hu et al., 2021). Each GRF protein interacts with a corresponding co-

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activator, known as GRF-INTERACTING FACTOR (GIF), to form a functional transcriptional complex (GRF-GIF) *in vivo* (Kim, 2019; Shimano et al., 2018). These complexes regulate the meristem-forming potential of proliferative and formative cells during organogenesis (Kim, 2019; Shimano et al., 2018). MicroRNA396 (miR396) post-transcriptionally represses major GRF members and fine tunes their expression (Debernardi et al., 2014; Debernardi et al., 2012; Rodriguez et al., 2010). Disrupting the miR396 target site of GRFs increases GRF transcripts, thus enhancing the levels and activities of GRF-GIF complexes (Che et al., 2015; Duan et al., 2015; Li et al., 2016; Li et al., 2018).

Recently, two studies have shown that expression of GRF genes greatly improves crop transformation (Luo and Palmgren, 2021). Debernardi and colleagues created a wheat GRF4-GIF1 chimera and showed that it substantially increased regeneration efficiency in wheat, rice and triticale. In addition, GRF4-GIF1 chimeras from citrus and grape enhanced the regeneration ability of citrus plants (Debernardi et al., 2020). Another study reported that ectopic expression of *Arabidopsis GRF5* and its orthologs enhanced regeneration and transformation in both monocot and dicot species, including maize, canola, soybean, sugar beet and sunflower (Kong et al., 2020). The T0 plants harboring the T-DNA and GRF genes had normal phenotypes in both studies; however, since the presence of foreign genes in commercial products has to be avoided, time and labour would be required to remove the transgenes.

Here, we report that transient expression of a TaGRF4-TaGIF1 complex dramatically improved regeneration in wheat, and most of the regenerated plants were transgene-free because the transgene was not integrated. Moreover, an improved TaGRF4-TaGIF1 complex (mTaGRF4-TaGIF1) with a mutation in the miR396 target site was even more effective. In combination with a cytosine base editor targeting *TaALS*, mTaGRF4-TaGIF1 stimulated regeneration and transgene-free editing in all of 11 elite common wheat cultivars, thus increasing the range of genotypes available for wheat transformation. Therefore, our GRF4-GIF1 transient expression approach holds great promise for practical breeding and plant commercialization.

RESULTS

Comparison of the effects of wild type TaGRF4-TaGIF1 and mutated TaGRF4-TaGIF1 on regeneration and genome editing of common wheat

Debernardi et al. (2020) compared the effect of having *TaGRF4* and *TaGIF1* fused in a chimera versus having each of the genes expressed separately under its own promoter in a single construct. They found that the forced proximity of the two proteins in a chimera increased their ability to induce regeneration. Therefore, we used *TaGRF4* and *TaGIF1* as a complex in common wheat, but with two modifications. First, the miR396 target site in *TaGRF4* in the TaGRF4-TaGIF1 complex was mutated (mTaGRF4-TaGIF1) (Figure 1A and B). Second, the transient expression strategy (Zhang et al., 2016), which imposed no selection pressure throughout the whole tissue culture procedure, was used to generate transgene-free products in the T0 generation (Figure 1D). TaGRF4-TaGIF1 and mTaGRF4-TaGIF1 were each transformed into the common wheat cultivars Bobwhite and Kenong 199 (Figure 1B), along with the vector pUBI-A3A-sgTaALS containing a single-guide RNA (sgRNA) for the acetolactate synthase gene (*TaALS*) (Figure 1C), in which substitution of Pro174 confers resistance to the herbicide nicosulfuron (Zong et al., 2018). The combination of pUBI-A3A-sgTaALS and pUBI-GFP was used as a control. After six weeks on non-selective medium, numbers of regenerated plants and *TaALS* mutations were analyzed.

We found that immature embryos of both Bobwhite and Kenong 199 transformed with TaGRF4-TaGIF1 produced more regenerated plants (508.0% and 654.5%, (No. of regenerated shoots/total immature embryos bombarded× 100%) respectively) than the control groups (81.0% and 136.9%, respectively) (Figure 2A, Table 1 and Table S1 in Supporting Information). Moreover, mTaGRF4-TaGIF1 gave even higher regeneration ratios (Figure 2A, Table 1 and Table S1 in Supporting Information). We also examined editing efficiencies at the *TaALS* target site by PCR-RE assays and Sanger sequencing. In agreement with the regeneration ratios, the Bobwhite and Kenong 199 plants transformed with TaGRF4-TaGIF1 complex had higher mutation frequencies (32.7% and 103.4%, (No. of mutants/total immature embryos bombarded×100%) respectively) than those transformed with the control construct (9.9% and 17.7%, respectively) (Figure 2B, Table 1 and Table S1 in Supporting Information). The number of mutants using the mTaGRF4-TaGIF1 complex was about 2-fold higher than with the TaGRF4-TaGIF1 complex, and 6-10-fold higher than in the control groups (Figure 2B, Table 1 and Table S1 in Supporting Information). Thus, the TaGRF4-TaGIF1 complex increased both regeneration frequency (RF) and genome-editing efficiency, and mutation of the miR396 target site further enhanced the activity of the TaGRF4-TaGIF1 complex.

Transgene-free wheat mutants have been efficiently achieved by transient expression of TaGRF4-TaGIF1 and mTaGRF4-TaGIF1

Since the plasmids in these experiments were delivered using the transient expression approach (Figure 1D), there was a high probability that the TaGRF4-TaGIF1 and base editor DNA constructs would not have been integrated into the

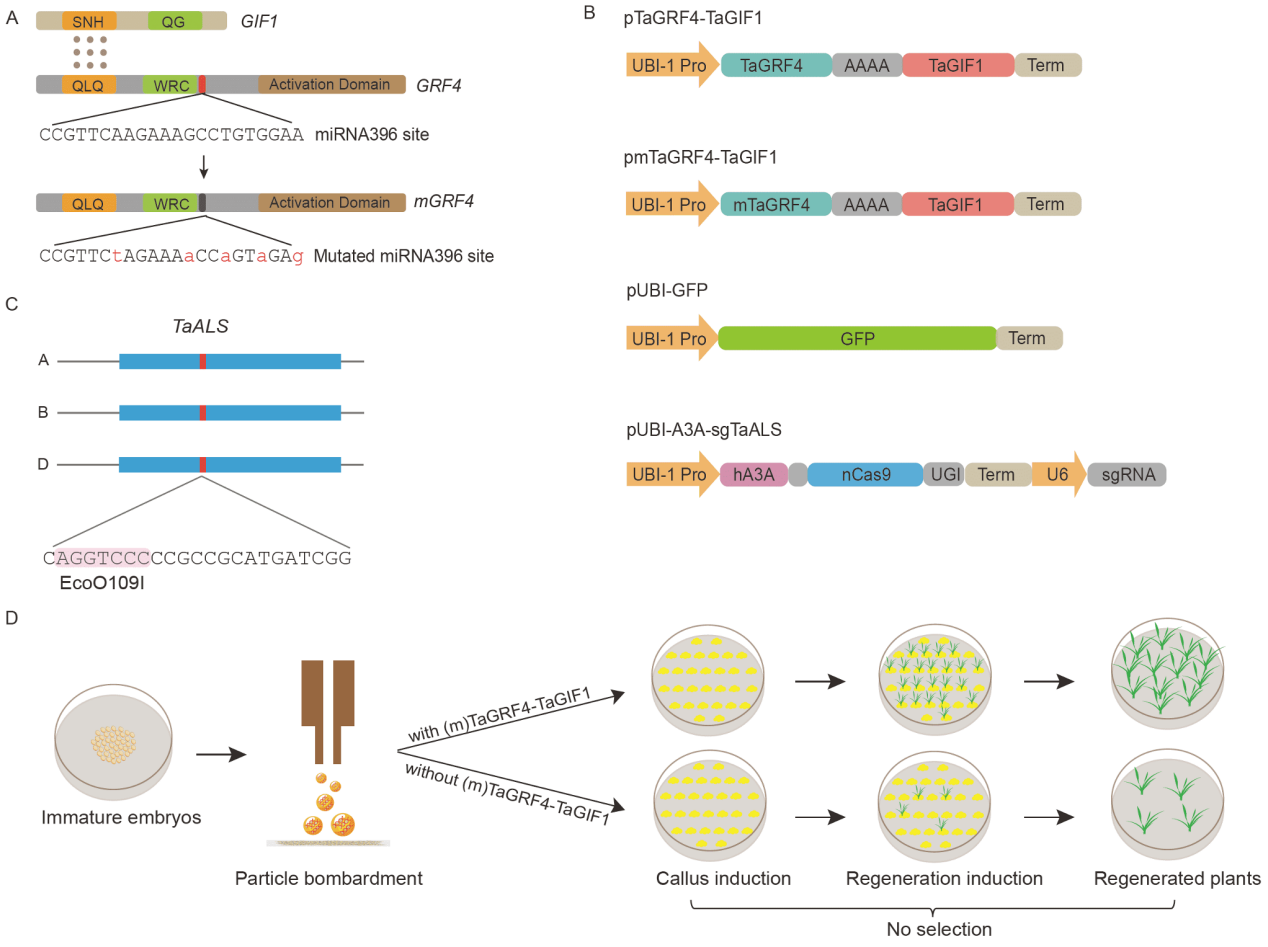

Figure 1 Schematic of vector, targeting constructs and transgene-free genome editing in wheat. A, Schematic representation of common wheat *GIF1*, *GRF4*, and mutated *GRF4*. The dotted lines indicate the interaction between the SNH and QLQ domains. *mTaGRF4* was created by introducing five single-site mutations into the miRNA396 target site of *TaGRF4*. B, Schematic representations of constructs pTaGRF4-TaGIF1, pmTaGRF4-TaGIF1, and the base editor, pUBI-A3A-sgTaALS. pUBI-GFP is a control construct. C, Schematic of the *TaALS* sgRNA target sites in the common wheat genome. The sgRNA target sites within the conserved region of common wheat *TaALS* homologous genes were targeted by the base editing system, and the EcoO109I restriction enzyme site in the sgRNA target sequence was used to detect mutations. D, General procedure for transgene-free genome editing in common wheat by transient expression of a cytosine base editor.

genome of the mutant plants. PCR amplification assay was used to investigate the presence of plasmid DNAs in the regenerated T0 mutants. Six primer sets (three each for pUBI-A3A-sgTaALS and pmTaGRF4-TaGIF1) were used to amplify discrete regions in the TaGRF4-TaGIF1 and base editor constructs, which altogether represented all major parts of the constructs (Figure 3A and Table S2 in Supporting Information). The two vectors (pUBI-A3A-sgTaALS and pTaGRF4-TaGIF1 /pmTaGRF4-TaGIF1) were found to be absent in 47.1%–55.4% of the Bobwhite and Kenong 199 mutants treated with TaGRF4-TaGIF1, mTaGRF4-TaGIF1, or the control construct (Figure 3B, Table 1 and Table S1 in Supporting Information). Also, the total numbers of transgene-free mutants in the mTaGRF4-TaGIF1 treated groups for both Bobwhite and Kenong 199 were 6-11-fold higher than in the controls (Table 1 and Table S1 in Supporting Information). Moreover, when we grew transgene-free mutants of Kenong 199 treated with mTaGRF4-TaGIF1 in a greenhouse, they displayed no obvious abnormalities throughout the whole of development (Figure 3C). Evidently, transient expression of mTaGRF4-TaGIF1 not only increased the number of transgene-free mutants but also had no side-effects on phenotype.

The use of mTaGRF4-TaGIF1 expands the scope of genome editing in common wheat cultivars

We tested whether the mTaGRF4-TaGIF1 complex improved regeneration rates and genome editing efficiencies in nine common wheat cultivars that are widely grown in China. The pUBI-A3A-sgTaALS vector containing an sgRNA for *TaALS* (Figure 1C), was delivered with mTaGRF4-TaGIF1 into immature embryos of nine major Chinese common wheat cultivars according to the procedure in Figure 1D. The regeneration frequencies of the nine cultivars transformed with mTaGRF4-TaGIF1 (9.9%–440.8%)

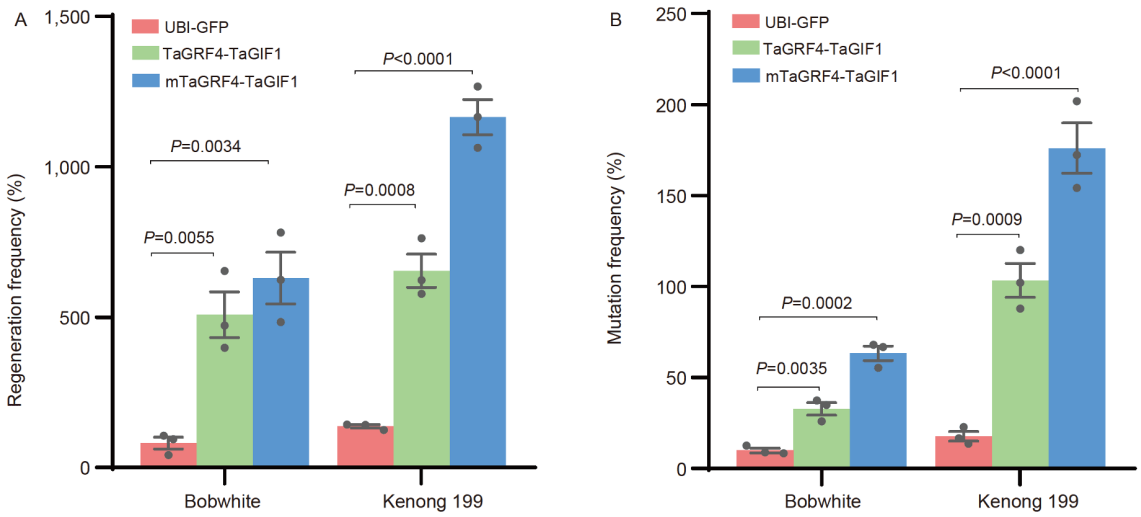

Figure 2 Comparison of the effects of TaGRF4-TaGIF1 and mTaGRF4-TaGIF1 on regeneration and genome editing of the two common wheat cultivars Kenong 199 and Bobwhite. A, Comparison of the effects of TaGRF4-TaGIF1 and mTaGRF4-TaGIF1 on regeneration frequencies of Bobwhite and Kenong199. RF=No. of regenerated shoots/total immature embryos bombarded×100%. B, Comparison of the effects of TaGRF4-TaGIF1 and mTaGRF4-TaGIF1 on genome editing frequencies in Bobwhite and Kenong199. MF (mutation frequency)=No. of mutants/total immature embryos bombarded×100%. Values and error bars are means±SEM of three independent experiments.

Table 1 Effect of the TaGRF4-TaGIF1 and mTaGRF4-TaGIF1 complexes on regeneration and genome editing efficiencies of common wheat cultivars Bobwhite and Kenong 199[a)]

Targeted gene	Common wheat cultivar	Treatment	No. bombarded immature embryos	No. regenerated plants/RF (%)	No. mutants/MF (%)	No. transgene-free mutants/TFF (%)
TaALS(C to T)	Bobwhite	pUBI-GFP (CK)	333	266 (81.0±19.8)	33 (9.9±1.3)	17 (50.9±5.5)
		TaGRF4-TaGIF1	277	1,507 (508.0±75.9)	100 (32.7±3.45)	47 (47.1±5.9)
		mTaGRF4-TaGIF1	343	2,080 (630.1±86.2)	216 (63.3±4.0)	103 (47.5±2.6)
	Kenong 199	pUBI-GFP (CK)	307	419 (136.9±5.7)	55 (17.7±2.6)	28 (52.8±8.4)
		TaGRF4-TaGIF1	325	2,095 (654.5±55.5)	331 (103.4±9.3)	170 (51.6±1.8)
		mTaGRF4-TaGIF1	328	3,796 (1,165.4±58.8)	577 (176.1±13.9)	324 (55.4±6.6)

a) RF (regeneration frequency)=No. of regenerated plants/total immature embryos×100%. MF (mutation frequency)=No. of mutants/total immature embryos×100%. TFF (transgene-free frequency)=No. of transgene-free mutants/total mutants×100%. The numbers and means for each treatment were calculated from data collected from three replicates of the experiment. Data are means±SEM (n=3).

were significantly greater than those transformed with the pUBI-GFP control (0–187.3%) (Figure 4A and B, Table 2 and Table S3 in Supporting Information). In particular, mTaGRF4-TaGIF1 greatly improved the regeneration and editing of Xiaoyan 54, Zhoumai 28, Jimai 20, Jimai 22 and Shannong 20, most of which regenerated inefficiently without the help of booster (Figure 4A and B, Table 2 and Table S3 in Supporting Information). Using PCR amplification and Sanger sequencing (Table S2 in Supporting Information), mutations in sites in *TaALS* were detected in all nine cultivars transformed with mTaGRF4-TaGIF1, at frequencies ranging from 1.2% to 8.1%, compared with 0–3.9% in the control groups (five of the cultivars yielded no mutants in the controls) (Figure 4C and Table 2). Moreover, the homozygous mutant frequencies (HMF) in the nine cultivars transformed with mTaGRF4-TaGIF1 ranged from 10%–55.6%, compared with 0–15.4% in the controls (Table 2 and Table S4 in Supporting Information). We also identified transgene-free mutants in all nine common wheat cultivars transformed with mTaGRF4-TaGIF1 (22.2%–66.7%). In contrast, transgene-free mutants were only found in Shannong 116, Zhoumai 27 and Zhongmai 175 transformed with the control plasmid, and they were present at significantly lower frequencies (Table 2). Collectively, the findings show that mTaGRF4-TaGIF1 improves regeneration and editing efficiencies in all nine common wheat cultivars.

DISCUSSION

Genome-editing technology already shows great potential in agriculture, but it is still limited by the low plant regeneration frequencies and genotype dependence (Debernardi et al., 2020; Gao, 2021; Zhang et al., 2020b). The use of plant development regulators greatly increases regeneration and transformation frequencies in many cereal and commercial

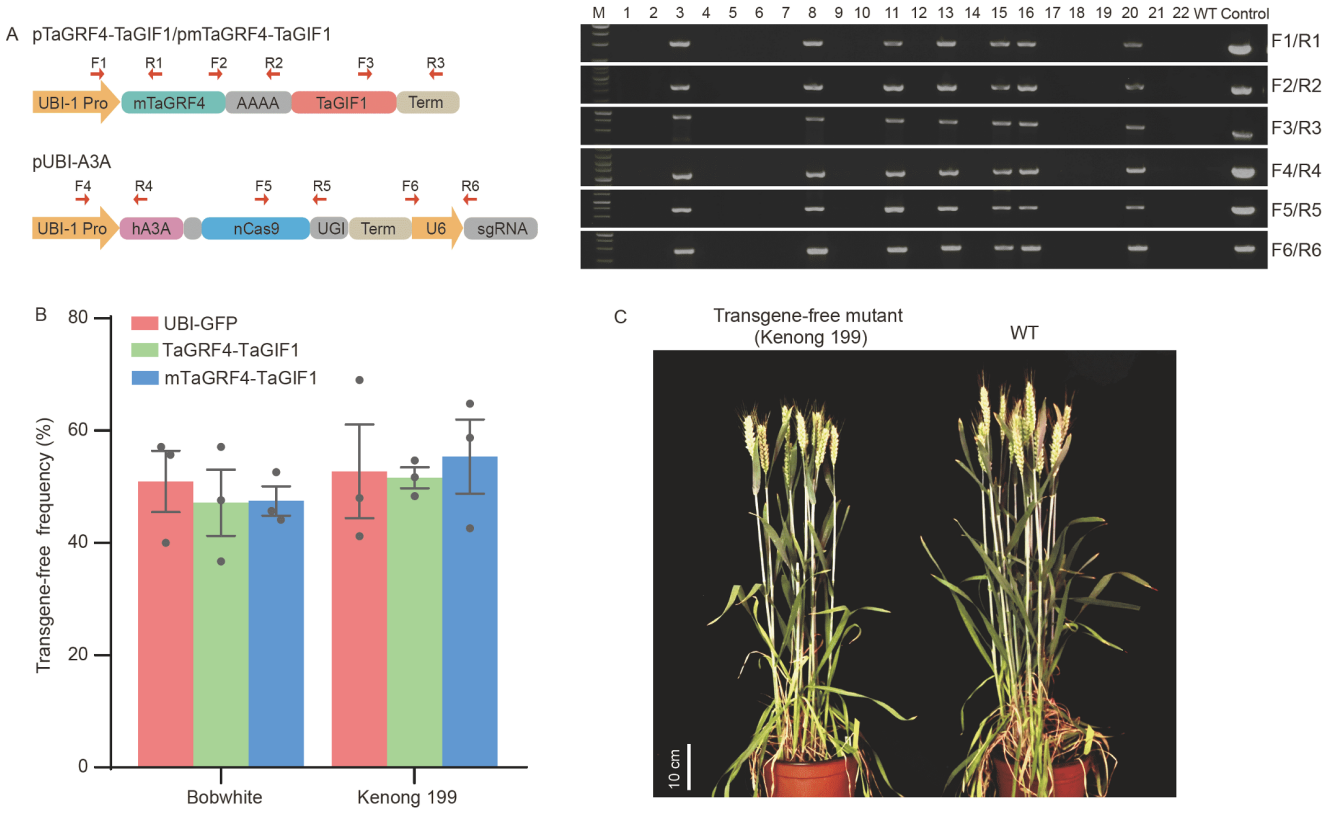

Figure 3 Identification of transgene-free mutants of Kenong 199 and Bobwhite. A, Primer sets for detecting transgene-free mutants, and the outcome of tests on 22 representative *TaALS* mutant plants (Kenong 199). B, Frequencies of transgene-free mutants in Bobwhite and Kenong199 transformed with TaGRF4-TaGIF1, mTaGRF4-TaGIF1 and UBI-GFP (control construct), respectively. Values and error bars are means±SEM of three independent experiments. C, Normal appearance of transgene-free mutant plants regenerated from Kenong 199 immature embryos transiently-expressing mTaGRF4-TaGIF1 and the cytosine base editor A3A-PBE.

crops, and so facilitates genome editing in these crops (Gordon-Kamm et al., 2019). One way to stimulate monocot transformation in a broad range of genotypes is to overexpress maize *Wus2* and *Bbm*. This combination of morphogenic regulators enhances transformation in maize, sugarcane, *indica* rice, and sorghum (Lowe et al., 2016). However, side-effects were observed in transgenic plants in which *Wus2* and *Bbm* were integrated (Lowe et al., 2016). Recently, two studies have shown that *GRFs* alone or in chimeras with *GIFs* dramatically boost regeneration in a broad range of species, and integration of these genes has no obvious effects on plant development (Debernardi et al., 2020; Kong et al., 2020). Since genetically-modified plants are strictly regulated and difficult to commercialize, laborious and time-consuming transgene elimination is needed (Chen et al., 2019; Kausch et al., 2019). The present work has demonstrated that transient expression of mTaGRF4-TaGIF1 together with genome editing components can also greatly improve regeneration ability and genome editing efficiency in immature embryos of common wheat, and greatly increased the proportion of transgene-free mutants in nine elite commercial cultivars.

It has been shown that *Agrobacterium*-mediated T-DNA transformation involves transient expression of the T-DNA for 36–48 h post infection followed by stable integration of the T-DNA (Gelvin, 2003; Yoshioka et al., 1996). By taking advantage of this phenomenon, a study using non-integrating *ZmWus2* gene enhanced maize transformation frequency (Hoerster et al., 2020). The method involved the use of two *Agrobacterium* strains, one containing *ZmWus2* and the other containing a selectable marker gene. Co-infection of immature embryos with these two strains in an appropriate ratio resulted in rapid somatic embryos formation from the scutella and recovery of T0 plants containing only the selectable marker gene. This outcome demonstrated that transiently expressed WUS2 was able to stimulate growth (Hoerster et al., 2020), which is consistent with our observation that transient expression of TaGRF4-TaGIF1 or mTaGRF4-TaGIF1 boosts regeneration. Based on these results, we believe that boosters transiently expressed during tissue culture can help to activate transcriptional cascades that promote cell fate reprogramming and cell proliferation, and lead eventually to plant regeneration in wheat. We found that mTaGRF4-TaGIF1 with a mutation of the miR396 target site was more effective in improving regeneration ability and genome editing frequency than TaGRF4-TaGIF1 itself. The

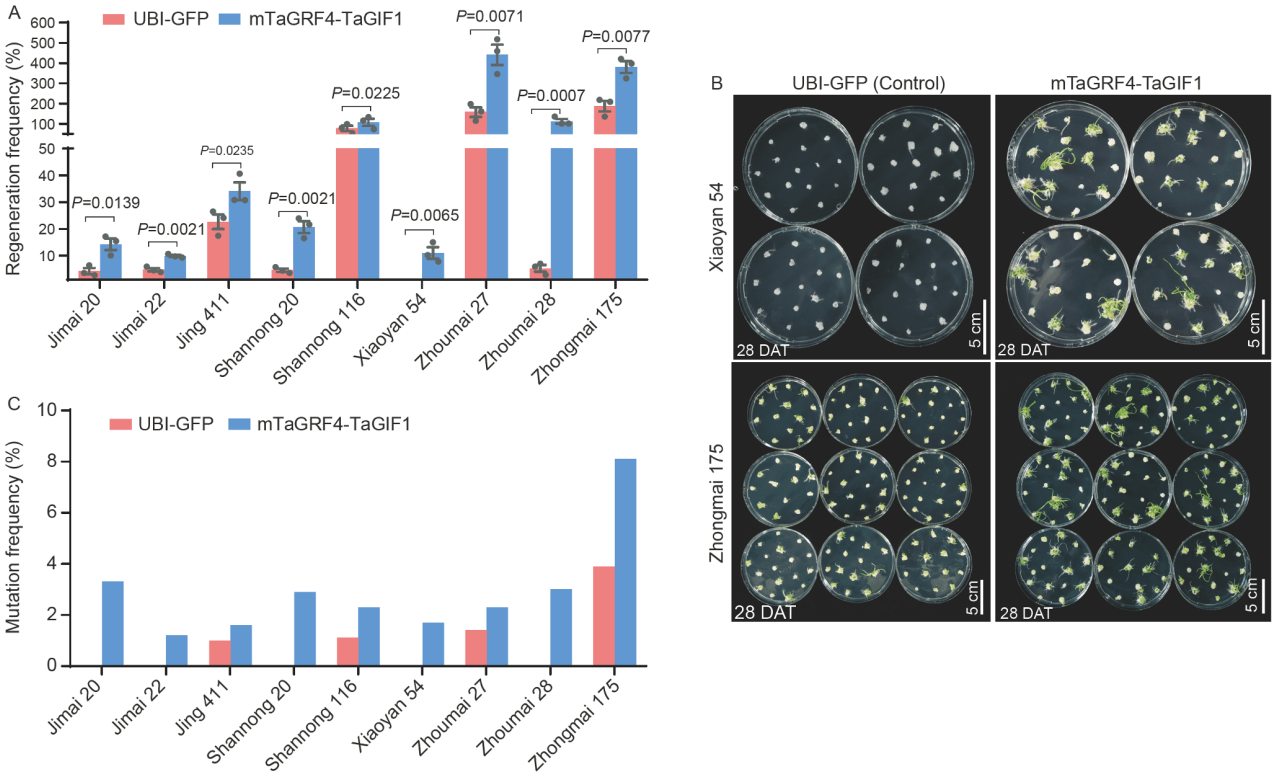

Figure 4 The effect of transient expression of mTaGRF4-TaGIF1 on regeneration and editing efficiencies in nine elite wheat cultivars. A, Regeneration frequencies in nine elite common wheat cultivars transformed with mTaGRF4-TaGIF1 and UBI-GFP (control construct), respectively. Values and error bars are means±SEM of three independent experiments. B, Regenerated plants of Xiaoyan 54 and Zhongmai 175 transformed with mTaGRF4-TaGIF1 and pUBI-GFP (control construct), respectively, 28 d after transformation (DAT). C, Mutation frequencies in the nine elite common wheat cultivars transformed with mTaGRF4-TaGIF1 and UBI-GFP (control construct), respectively.

Table 2 Effect of mTaGRF4-TaGIF1 on regeneration and genome editing efficiencies of nine elite common wheat cultivars

Targeted gene	Common wheat cultivar	Treatment	No. bombarded embryos	No. regenerated plants/RF (%)	No. mutants/MF (%)	No. homozygous mutants/HMF (%)	No. transgene-free mutants/TFF (%)
TaALS (C-to-T)	Jimai 20	UBI-GFP (CK)	335	14 (4.2±1.1)	0	0	0
		mTaGRF4-TaGIF1	270	37 (14.2±2.1)	9 (3.3)	5 (55.6)	2 (22.2)
	Jimai 22	UBI-GFP (CK)	272	13 (4.7±0.6)	0	0	0
		mTaGRF4-TaGIF1	344	34 (9.9±0.4)	4 (1.2)	0 (0.0)	1 (25.0)
	Jing 411	UBI-GFP (CK)	203	65 (18.8±2.7)	2 (1.0)	0	0
		mTaGRF4-TaGIF1	320	135 (34.0±3.2)	5 (1.6)	1 (25.0)	2 (50.0)
	Shannong 20	UBI-GFP (CK)	335	15 (4.5±0.6)	0	0	0
		mTaGRF4-TaGIF1	344	71 (20.7±2.2)	10 (2.9)	1 (10)	5 (50.0)
	Shannong 116	UBI-GFP (CK)	285	128 (66.3±8.2)	3 (1.1)	0	1 (33.3)
		mTaGRF4-TaGIF1	308	342 (101.7±18.4)	7 (2.3)	1 (14.3)	2 (28.6)
	Xiaoyan 54	UBI-GFP (CK)	320	0	0	0	0
		mTaGRF4-TaGIF1	343	36 (11.0±2.1)	6 (1.7)	1 (16.7)	3 (50.0)
	Zhoumai 27	UBI-GFP (CK)	286	441 (157.6±24.1)	4 (1.4)	0 (0.0)	1 (25.0)
		mTaGRF4-TaGIF1	343	1,578 (440.8±50.3)	8 (2.3)	1 (12.5)	5 (62.5)
	Zhoumai 28	UBI-GFP (CK)	308	16 (5.2±1.3)	0	0	0
		mTaGRF4-TaGIF1	331	370 (112.3±11.4)	10 (3.0)	2 (20.0)	4 (40.0)
	Zhongmai 175	UBI-GFP (CK)	335	614 (187.3±26.1)	13 (3.9)	2 (15.4)	8 (61.6)
		mTaGRF4-TaGIF1	332	1,265 (380.6±29.0)	27 (8.1)	10 (37.0)	18 (66.7)

a) RF (regeneration frequency)=No. of regenerated plants/total immature embryos×100%. MF (mutation frequency)=No. of mutants/total immature embryos×100%. HMF (homozygous mutant frequency)=No. of homozygous mutants/total mutants×100%. TFF (transgene-free frequency)=No. of transgene-free mutants/total mutants×100%. The numbers and means for each treatment were calculated from data collected from three replicates of the experiment. Data are means±SEM (n=3).

transient expression of mTaGRF4-TaGIF1, and its resistance to repression by miR396, not only minimized side-effects on plant morphology but also generated a large number of transgene-free mutants.

In summary, transient expression of an improved TaGRF4-TaGIF1 complex containing a mutation of the miR396 target site stimulated regeneration and improved genome editing in monocotyledonous common wheat. Moreover, the effect of the GRF-GIF1 complex was genotype-independent. Since the method yields transgene-free edited plants, this method should be beneficial for crop improvement and accelerate the commercialization of products.

MATERIALS AND METHODS

Plasmid construction

All plasmids were generated using restriction enzyme-based cloning and Gibson Assembly. *TaGRF4* and *TaGIF1* genes were synthesized commercially (Genewiz, Suzhou, China), and *mTaGRF4* was made by introducing five points mutation in miR396 binding site by normal PCR. *TaGRF4* and *mTaGRF4* were separately fused with *TaGIF1* by the "AAAA" liker using overlap PCR, the TaGRF4-TaGIF1 and mTaGRF4-TaGIF1 fusion sequences were cloned into the backbone of vector pJIT163 with a HindIII site. To construct the base editing vector pUBI-A3A-sgTaALS, we cloned TaU6-sgRNA scaffold DNA fragment into the previously described pA3A-PBE vector (Zong et al., 2018) with SpeI site by using ClonExpressII One Step Cloning Kit (Vazyme, Nanjing, China). The GFP fragment was cloned into pJIT163 with the HindIII site using ClonExpressII One Step Cloning Kit to generate the control vector pUBI-GFP. All primer sets and sequences synthesized by Beijing Genomics Institute (BGI) are listed in Table S2 in Supporting Information and Supplementary Sequence, respectively.

Plant growth

Seeds of the spring common wheat (*Triticum aestivum* L.) cv "Bobwhite" and winter common wheat "Kenong 199" were sown at weekly intervals in a mixture of soil and they were grown in controlled growth rooms at 24°C±1°C day and 15°C±1°C night temperatures. Nine Chinese elite common wheat cultivars grew in the field at the IGDB experimental station in Beijing under normal field management conditions.

Biolistic delivery of DNA constructs into wheat immature embryo cells

DNA of plasmids pTaGRF4-TaGIF1 or pmTaGRF4-TaGIF1 were co-transformed with pUBI-A3A-sgTaALS into immature embryos of common wheat via biolistic bombardment as previously described (Liang et al., 2017; Zhang et al., 2016; Zong et al., 2018). After bombardment, the embryos were cultured for plantlets regeneration on medium without selective agent.

Mutation identification by PCR-RE assays and Sanger sequencing

PCR-RE assays and Sanger sequencing were used to identify wheat mutants with C-to-T conversions in target regions, as described previously (Shan et al., 2013; Wang et al., 2014; Zong et al., 2017). we pooled the plantlets regenerated from a single immature embryo (each pool usually contained 5–10 plantlets) to detect the mutations using PCR-RE assays or Sanger sequencing using a conserved primer for *TaALS* gene. All the plantlets in the pools that giving positive signals were tested one by one with PCR-RE and subsequent Sanger sequencing using a conserved primer for *TaALS* gene.

Transgene-free mutant identification

Because the GRF4-GIF1 complex enhanced plant regeneration, it could increase the chance to obtain transgene-free mutants in the presence or absence of selection. Normal PCR was used to examine the presence of plasmid DNAs in all identified mutants of common wheat. A total of six primer sets (three for pUBI-A3A-sgTaALS, three for pmTaGRF4-TaGIF1) were used to examine transgene-free mutants in common wheat.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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