# Use of GRF-GIF chimeras and a ternary vector system to improve maize (*Zea mays* L.) transformation frequency

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## SUMMARY

Maize (*Zea mays* L.) is an important crop that has been widely studied for its agronomic and industrial applications and is one of the main classical model organisms for genetic research. *Agrobacterium*-mediated transformation of immature maize embryos is a commonly used method to introduce transgenes, but a low transformation frequency remains a bottleneck for many gene-editing applications. Previous approaches to enhance transformation included the improvement of tissue culture media and the use of morphogenic regulators such as *BABY BOOM* and *WUSCHEL2*. Here, we show that the frequency can be increased using a pVS1-VIR2 virulence helper plasmid to improve T-DNA delivery, and/or expressing a fusion protein between a *GROWTH REGULATING FACTOR* (*GRF*) and *GRF-INTERACTING FACTOR* (*GIF*) protein to improve regeneration. Using hygromycin as a selection agent to avoid escapes, the transformation frequency in the maize inbred line B104 significantly improved from 2.3 to 8.1% when using the pVS1-VIR2 helper vector with no effect on event quality regarding T-DNA copy number. When combined with a novel fusion protein between ZmGRF1 and ZmGIF1, transformation frequencies further improved another 3.5- to 6.5-fold with no obvious impact on plant growth, while simultaneously allowing efficient CRISPR/Cas9-mediated gene editing. Our results demonstrate how a GRF-GIF chimera in conjunction with a ternary vector system has the potential to further improve the efficiency of gene-editing applications and molecular biology studies in maize.

## SIGNIFICANCE STATEMENT

Maize is an important crop due to its widespread use as a staple food and feed for livestock and is a commonly used model organism, but its genetic transformation remains a limiting factor. In this study, we show a significant improvement in maize transformation frequency using a ternary vector system and/or a novel *ZmGRF1-GIF1* morphogenic regulator. This technical advance will aid future molecular biology studies and gene-editing applications in maize.

# INTRODUCTION

Maize (*Zea mays* L.) is set to become the world's most cultivated crop in the coming decade and is an important model organism for plant genetics (Andorf *et al.*, 2019; Erenstein *et al.*, 2022). However, the production of maize will face growing challenges due to ecological factors like land degradation, water scarcity, and climate change. CRISPR/Cas9-mediated gene editing revolutionized molecular and cellular biology research and facilitates the modification of crops to meet evolving agricultural requirements (Shi *et al.*, 2017; Gao and Chu, 2020). While gene editing strategies, such as multiplex gene editing (Lorenzo *et al.*, 2023), base and prime editing (Zong *et al.*, 2017; Jiang *et al.*, 2020), gene targeting (Barone *et al.*, 2020), chromosome engineering (Rönspies *et al.*, 2021), CRISPR library screens (Gaillochet *et al.*, 2021), and promoter tuning (Rodríguez-Leal *et al.*, 2017; Claeys *et al.*, 2023) have become more intricate, crop transformation remains a bottleneck.

*Agrobacterium*-mediated transformation is generally the favored method of maize transformation, since biolistics comes with drawbacks like genome damage (Liu *et al.*, 2019) and a greater likelihood of truncated or multicopy inserts (Kausch *et al.*, 2021). However, *Agrobacterium*-mediated transformation is constrained by the fact that only few genotypes are both susceptible to *Agrobacterium* infection and capable of efficient regeneration, such as the inbred lines B104 and A188. In traditional transformation methods plants are regenerated from embryogenic callus (Ishida *et al.*, 1996; Coussens *et al.*, 2012). Optimization of tissue culture media and the rapid induction of somatic embryos led to methods in which the callus step was reduced in time (Masters *et al.*, 2020; Aesaert *et al.*, 2022; Kang *et al.*, 2022). These faster methods also reduced the labor associated with tissue culture, but are associated with higher percentages of escapes (non-transgenic regenerants) when using the herbicide selection marker *BIALAPHOS RESISTANCE* (*BAR*, Aesaert *et al.* (2022); Kang *et al.* (2022)).

Because some *virulence* (*vir*) genes show gene dosage effects (Jin *et al.*, 1987), adding additional copies of *vir* genes has been used as a strategy to improve the delivery of the T-DNA by *Agrobacterium*. This led to the development of the superbinary vector pSB1, which carries a set of *vir* genes from the hypervirulent Ti plasmid pTiBo542 (Komari, 1990). van der Fits *et al.* (2000) showed that placing *virG* on a third compatible vector improved the transformation frequency in *Catharanthus roseus* L. and tobacco (*Nicotiana tabacum* L.). Later, more extensive ternary vector systems were established that improve the maize transformation frequency by generating virulence helper plasmids that contain a high copy number origin of replication (ori) and a set of *vir* genes from the hypervirulent Ti plasmid pTiBo542 (Anand *et al.*, 2018; Zhang *et al.*, 2019; Kang *et al.*, 2022).

A breakthrough in monocot transformation was the discovery that the morphogenic regulators (MRs) *BABY BOOM* (*ZmBBM*) and *WUSCHEL2* (*ZmWUS2*) can reprogram somatic cells to undergo somatic embryogenesis, which allowed the transformation of species and genotypes otherwise non-transformable (Lowe *et al.*, 2016). However, these introduced MRs have to be excised again using a Cre/LoxP system to regenerate healthy, fertile T0 transgenic plants without developmental defects (Lowe *et al.*, 2016). This can be partly circumvented using tissue- and developmental stage-specific promoters to drive the expression of the MRs (Lowe *et al.*, 2018), with additional Cre/LoxP-mediated excision further improving the fertility of regenerated plants (Aesaert *et al.*, 2022). Similarly, a *WUSCHEL* homolog in wheat (*Triticum aestivum* L.*)*, *TaWOX5*, was found to improve the wheat transformation frequency without developmental defects (Wang *et al.*, 2022). Also in wheat, it was discovered that the *TaGRF4-GIF1* chimera, a fusion between the transcription factor *GROWTH REGULATING FACTOR 4* (*TaGRF4*) and its cofactor *GRF-INTERACTING FACTOR 1* (*TaGIF1*), improved the transformation frequency in both monocots and dicots, and allowed the transformation of otherwise recalcitrant genotypes without pleiotropic effects (Debernardi *et al.*, 2020). Since then, *GRF-GIF*

chimeras have also been used to transform watermelon, lettuce and sorghum (Feng *et al.*, 2021; Bull *et al.*, 2023; Li et al., 2023), and overexpression of *ZmGRF5-LIKE* genes has been shown to increase transformation frequency in maize (Kong *et al.*, 2020).

In this study, we successfully used hygromycin B (HygB) as a selection agent for the transformation of the public maize inbred line B104 to eliminate escapes associated with a shortened tissue culture. By integrating HygB selection with the pVS1-VIR2 ternary helper vector (Zhang *et al.*, 2019), we achieved a notable increase in the average transformation frequency from 2.3% to 8.1%. Furthermore, we demonstrate that a novel *ZmGRF1-GIF1* chimera combined with pVS1-VIR2 leads to an additional 3.5- to 6.5-fold enhancement in the transformation frequency compared to the ternary vector system alone. Additionally, the *ZmGRF1-GIF1* chimera proves to be compatible with CRISPR/Cas9 technology when applied for editing the visual marker *chloroplast signal RECOGNITION PARTICLE 43* (*cpSRP43*).

# RESULTS

## Hygromycin as an effective selection agent for maize B104 transformation

Recently, we reported that using optimized tissue culture media increased the *Agrobacterium*-mediated transformation frequency of the maize B104 inbred line and reduced the time and labor associated with tissue culture (Aesaert *et al.*, 2022). However, the one-month time reduction on selective media was associated with a high number of escapes when using the herbicide phosphinothricin (PPT) in combination with the *BIALAPHOS RESISTANCE* (*BAR*) selection marker, with two out of three regenerated plantlets being non-transgenic. In the same study, we tested *HIGHLY-RESISTANT ACETOLACTATE SYNTHASE* (*HRA*; Green *et al.* (2009)) and the herbicide imazapyr as an alternative selection system in combination with the *ZmBBM* and *ZmWUS2* MRs. This eliminated all escapes, but transformation without MRs and imazapyr selection only yielded low transformation frequencies of 0-0.9% (Aesaert *et al.*, 2022). This encouraged us to further improve the method by evaluation of other selection agents and/or regimes. Here, we report the successful use of HygB as a selection agent in combination with the *Escherichia coli HYGROMYCIN B PHOSPHOTRANSFERASE* (*HPT*, Gritz and Davies (1983)).

To test the use of *HPT* for transformation and gene editing in maize, we initially used *HPT* driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter (p35S) and *A. tumefaciens NOPALINE SYNTHASE* terminator (tnos). We constructed the pMHb-pZmUBI-zCas9-tnos-pOsU3-BsaI-ccdB-BsaI-scaffold binary vector (Figure S1) that contains the *HPT* cassette and the maize codon-optimized *zCas9*-coding sequence (Xing *et al.*, 2014), driven by the maize *UBIQUITIN-1* promoter (pZmUBI) and terminated by tnos. In addition, it contains a pOsU3 module that allows direct cloning of the single guide RNA (sgRNA) spacer. For cloning multiple sgRNAs, pMHb-zCas9-AG-tdT was constructed that next to the zCas9 module also contains the tandem dimer Tomato (tdTomato) fluorescent reporter driven by the *Brachypodium distachyon* L. EF1α promoter (Coussens *et al.*, 2012), also terminated by tnos (Figure S1).

In 2020 and 2021, we performed 48 maize transformation experiments using HygB as selection agent (Data S1). The binary vectors used were either pHbm42GW7 (Karimi *et al.*, 2013), pP-U1-U9, pHb-U1-U9 or the newly constructed CRISPR/Cas9 vectors (Figure S1). For each experiment, we started with on average 355 immature embryos (IEs), isolated from three to eight different ears. Transformation was identical to Method 2 in Aesaert *et al.* (2022), but with HygB selection. A gradual increase in concentration of HygB is preferred when using IEs as explants (Ishida *et al.*, 1996). Here, we opted for a selection scheme starting with 15 mg/L for seven days in selection I medium, followed by 40 mg/L in the following maturation I and maturation II media (Protocol S1). Finally, during rooting on

regeneration II medium, 20 mg/L was used. From these 48 experiments, we obtained on average 11.6 T0 transformants of which 8.0 were independent, with only transgenic plants derived from different IEs considered as independent. When defining the transformation frequency conservatively as the number of independent transgenics per 100 IE explants, this resulted in an average transformation frequency of 2.3%. As observed before (Aesaert *et al.*, 2022), the transformation frequency was variable between experiments ranging from 0.0% to 7.9% (Figure 1, Data S1). Also within single experiments, a large variation from 0.0% to 15.4% was observed between ears (Figure 1, Data S1).

In conclusion, although the average transformation frequency using HygB selection was lower compared to PPT selection (2.3% vs 4,2%, Aesaert *et al.* (2022)), the near elimination of escapes facilitated downstream processing of regenerated plants, reducing labor at this point in the transformation pipeline.

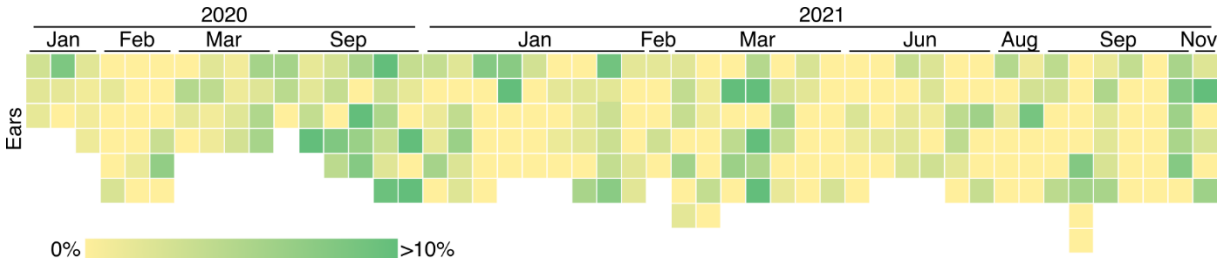


**Figure 1.** Ear-to-ear variability in maize transformation using HygB as selection agent. Heat map showing the transformation frequency (independent transgenics) per ear in 48 chronologically ordered experiments during 2020 and 2021. For each experiment, embryos were derived from three to eight ears.

## Use of the ternary helper vector pVS1-VIR2 improves transient and stable transformation

A limiting factor in *Agrobacterium*-mediated plant transformation is the efficacy of the T-DNA delivery. This has been improved by the development of ternary vector systems that contain not only the disarmed Ti and binary vector, but also a third compatible vector. This helper vector carries an extra set of *vir* genes and a high copy number ori (Anand *et al.*, 2018). Zhang *et al.* (2019) constructed a new set of binary vectors, designated as pGreen3 (pG3). pG3-type vectors contain oriV of the replication origin of pRK2, but not *trfA*, *trfB* and *parDE*. The latter elements are provided *in trans* on the ternary helper vector pVS1-VIR2 to allow replication (Figure 2a). This configuration is hypothesized to lead to higher plasmid stability (Zhang *et al.*, 2019). Here, we evaluated extensively if the ternary helper vector pVS1-VIR2 can be used to improve maize B104 transformation in combination with HygB selection. As the *vir* genes present in pVS1-VIR2 are homologous with the *vir* genes present in EHA105, we used the recombinase-deficient EHA105 *recA*$^-$ strain we recently constructed using base editing (Rodrigues *et al.*, 2021) to minimize risk of recombination. EHA105 *recA*$^-$ was equipped with pVS1-VIR2 and subsequently with a compatible pG3 binary vector.

We constructed new pG3 binary vectors that are both compatible with pVS1-VIR2 (Zhang *et al.*, 2019) and the GreenGate Golden Gate cloning approach we use (Lampropoulos *et al.*, 2013). In addition, we further optimized our maize *HPT* selection marker cassette based on a configuration successfully used in wheat (Hayta *et al.*, 2019). *HPT* containing the castor bean *CATALASE-1* intron (Wang *et al.*, 1997) is driven by the rice (*Oryza sativa* L.) *ACTIN1* promoter (pOsAct1, Mcelroy *et al.* (1991)) and terminated by the 35S terminator (t35S, Figure 2b). pOsAct1 shows a high and constitutive expression in maize (Zhong *et al.*, 1996) and results in higher transformation frequencies compared to viral promoters (Beringer *et al.*, 2017). Moreover, the use of a heterologous promoter sequence from a monocot other than maize is favored to avoid gene silencing of endogenous genes (Hoengenaert *et al.*, 2022).

We first cloned the reporter gene *β-GLUCURONIDASE* with intron IV2 of the potato (*Solanum tuberosum* L.) gene *ST-LS1* (*GUSint*) under control of the ZmUBI promoter and 35S terminator in the newly constructed pVS1-VIR2-compatible binary vector pG3HI-AG (Figure 2b). We then compared this vector in the ternary vector system to our control binary vector pBb7m24GW-pZmUBI::*GUSint*:t35S, which has been used for many years to monitor transient transformation (Coussens *et al.*, 2012). Eighty-one and 90 IEs were isolated from nine ears and transformed with pZmUBI::*GUSint*:t35S in the binary or ternary vector system, respectively. A marked effect was seen with a higher number of transformed cells per embryo for all nine ears using the ternary vector system (Figure 2c-d), with a 9- to 155-fold increase in the area of transformed cells depending on the ear (Figure 2e, Data S2). The ear expressing on average the lowest GUSint transformed with the ternary helper vector, was still higher than the most highly expressing ear transformed with the binary vector (Figure 2e, Data S2). Employing the ternary vector system, 89/90 IEs displayed an area of transiently transformed cells of over 0.05%, roughly correlating with >10 transformed cells, compared to 40/81 with the binary vector system.

To evaluate the effect of using a ternary vector system for stable transformation, we used the ZmPLTP promoter, which is specifically expressed in the scutellum epithelium (Lowe *et al.*, 2018) to drive YFP, C-terminally fused to the N7 NLS of the Ankyrin protein encoded by AT4G19150, and terminated by the G7 terminator. This transcriptional unit was combined with pOsAct1::*HPTint*:t35S in either a pPZP200 backbone, which is routinely used in our transformation pipeline, or in the new pVS1-VIR2-compatible pG3 backbone (Figure 2b). The backbones of these binary vectors differ in the ori, with the pPZP200 and pG3 backbones harboring the pVS1 and the split pRK2 ori, respectively. The pVS1 and pRK2 ori have been reported to yield a similar transformation frequency in maize when used in a binary vector (Oltmanns *et al.*, 2010). In total, 242 and 128 embryos from six ears were transformed with the binary and ternary vector systems, respectively. We recovered transgenic events from both experiments, with the binary vector system showing a 7.9% transformation frequency, whereas the ternary vector system had a frequency of 32% or a 4.1-fold increase (Figure S2).

Next, 13 and 11 independent T0 transgenics transformed with, respectively, the binary and ternary vector systems were randomly selected and the T-DNA copy number was determined using digital PCR (dPCR). This revealed a similar distribution of copy number, with 15.4% and 27.3% of plants having only a single copy of the HygB transgene (Figure 2f). Prompted by this successful result, we performed 19 experiments using the ternary vector system over the course of two years. On average, we observed a transformation frequency of 8.1%, or a 3.5-fold increase compared to the average obtained with the binary system (2.3%, Figure 2g-h, Data S3). A dPCR was used to determine the HygB transgene copy number and presence of vector backbone in 147 regenerants from 10 experiments. This allowed us to determine the escape rate using HygB as a selection agent with the ternary vector system. 96.6% (142/147) scored positive for the presence of the T-DNA, resulting in a negligible escape rate of only 3.4% (Data S4). Out of the 129 transgenics tested for the presence of both T-DNA and plasmid backbone, 23.3% were identified as high-quality events, having one copy of the T-DNA and no backbone (Data S4).

In conclusion, by combining the pVS1-VIR2 ternary helper vector with HygB selection, we were able to significantly increase the transformation frequency from 2.3% to 8.1% with hardly any escapes without an increase in T-DNA copy number and one-fifth of transgenics of high quality.

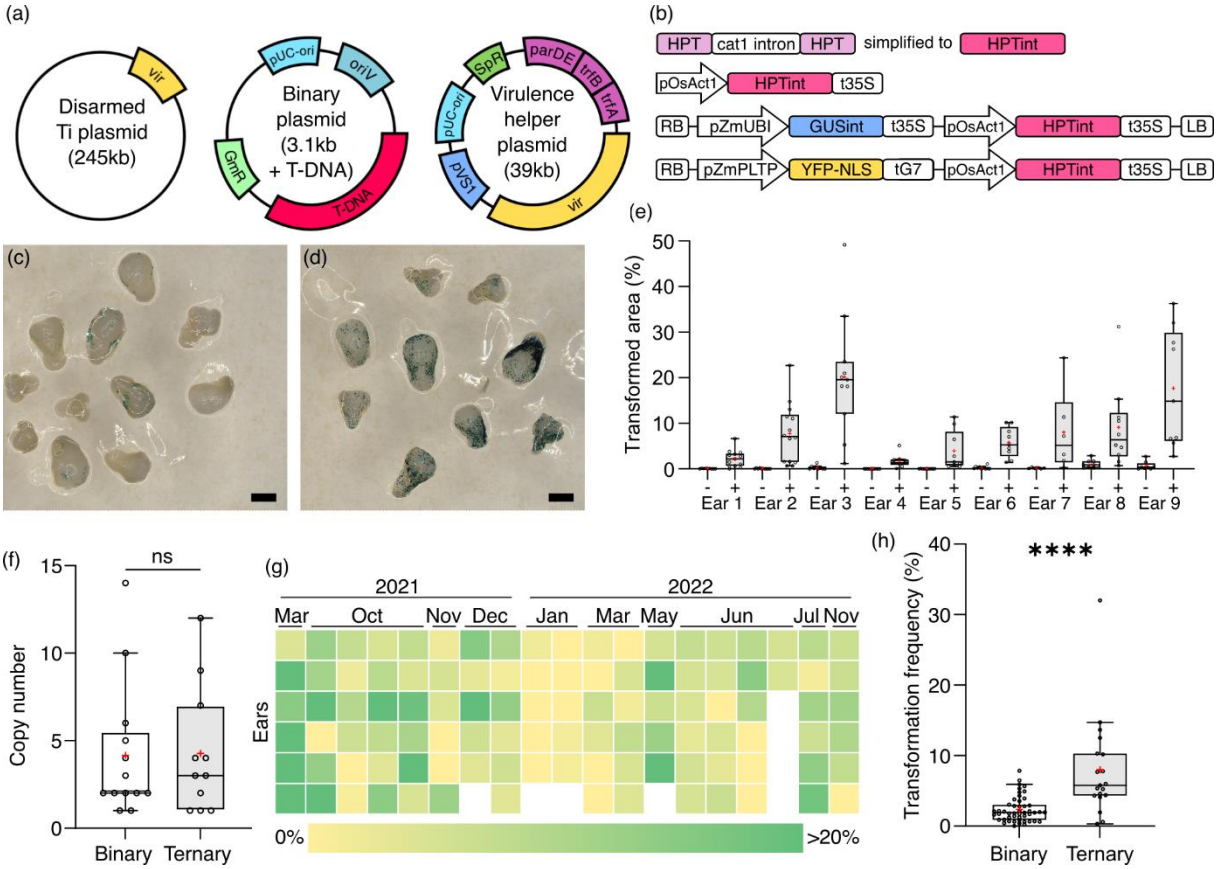


**Figure 2.** Maize transformation using the pVS1-VIR2 ternary vector system. (a) Simplified representation of the ternary vector system with the disarmed Ti plasmid (pTiBo542ΔT-DNA), a pGreen3-type binary vector, and the pVS1-VIR2 ternary helper vector. pUC-ori, *E. coli* ColE1 ori. The pRK2 ori is split with oriV on the binary vector and *trfA*, *trfB* and *parDE* on the ternary helper so the binary vector cannot replicate in *A. tumefaciens* without pVS1-VIR2. The ternary helper vector contains the pVS1 ori for replication in *A. tumefaciens*. vir, virulence gene cluster; GmR, gentamycin resistance cassette; SpR, spectinomycin resistance cassette. (b) Diagrams of gene cassettes and T-DNAs used for transformation. HPT, *HYGROMYCIN B PHOSPHOTRANSFERASE*; cat1 intron, castor bean *CATALASE-1* intron; pOsAct1: rice *ACTIN1* promoter; t35S, CaMV 35S terminator; RB, right border; pZmUBI, maize *UBIQUITIN* promoter; GUSint, *β-GLUCURONIDASE* with intron IV2 of the potato gene *ST-LS1*; LB, left border; pZmPLTP, maize *PHOSPHOLIPID TRANSFER PROTEIN* promoter; YFP-NLS, *YELLOW FLUORESCENT PROTEIN* with C-terminal N7 nucleotide localization signal; tG7, G7 terminator. (c-d) X-Gluc staining of maize B104 IEs transformed with pZmUBI::*GUSint* in a binary (c) or ternary (d) vector system setup. Scalebars = 1 mm. Blue spots are indicative of plant cells transiently expressing *GUSint.* (e) Quantification of the IE area transiently expressing *GUSint* from nine ears using a binary (-) or ternary (+) vector system, $P < 0.001$ (Linear Mixed Model). (f) T-DNA copy number of independent transgenics transformed with a pPZP200-type binary vector (n=13) or a pVS1-VIR2 ternary helper vector with a pG3-type compatible binary vector (n=11), ns, not significant ($P = 0.9391$, unpaired Student's *t*-test). (g) Heatmap of maize transformation frequency (independent transgenics) per ear in 19 chronologically ordered experiments performed using the ternary vector system and HygB as selection agent. (h) Transformation frequency of binary vs ternary vector systems over multiple experiments. n = 48 for the binary vector, n = 19 for the ternary vector system. ****, $P < 0.0001$ (Generalized Linear Model). In all boxplots, center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, averages are indicated by a red cross.

## A maize GRF-GIF chimera improves transformation

The higher increase in transient *GUSint* expression (Figure 2e) with the ternary vector system does not translate into an equal increase in stable transformation frequency (Figure 2h), indicating other limiting factors such as T-DNA integration, and regeneration. Regeneration improvements have been made using MRs such as *ZmBBM* and *ZmWUS2* driven by tissue- and developmental stage-specific promoters (Lowe *et al.*, 2018). However, resulting transgenic plants may still show pleiotropic effects, necessitating the excision of the MRs to ensure fertility and transmission of gene edits or transgenes (Aesaert *et al.*, 2022). In wheat, expression of a chimeric protein obtained by the fusion of TaGRF4 and its cofactor TaGIF1 improved the transformation frequency without pleiotropic effects (Debernardi *et al.*, 2020). In order to test the *TaGRF4-GIF1* chimera in maize, we cloned it under control of pZmUBI and tnos in pHb-U1-U9 with the pOsAct1::*HPTint*:t35S selection marker described previously. As control, we used pZmUBI::*GUSint*:t35S in pHb7m24GW (Figure S1). We transformed 212 and 214 embryos derived from six ears using pZmUBI::*GUSint*:t35S and pZmUBI::*TaGRF4-GIF1*:tnos and obtained 3 (1.4%) and 7 (3.3%) independent T0 plants, respectively, yielding a 2.3-fold increase in transformation frequency (Figure S3). Although statistically not significant, the *TaGRF4-GIF1* chimera showed promise to improve the transformation frequency in maize.

To test our hypothesis that a maize *GRF-GIF* chimera would outperform the wheat *GRF-GIF* chimera in maize, we cloned a fusion of the closest homologs of *TaGRF4* and *TaGIF1*, *ZmGRF1* and *ZmGIF1* (Figure S4), under control of pZmUBI and tnos in pG3HI-AG, a vector containing pOsAct1::*HPTint:t35S* compatible with the ternary vector system (Figure 3a). As controls, we made identical constructs but with *GUSint* and *TaGRF4-GIF1* in pG3HI-AG. While no increased number of somatic embryos were observed, as is the case with BBM-WUS, *ZmGRF1-GIF1*- and *TaGRF4-GIF1*-regenerating calli were larger and more plants regenerated from a single callus compared to the control, resulting in a "bushy" phenotype (Figure 3b-c). However, some of these calli had slower or less root formation (Figure 3c), limiting the transfer to soil to a fraction of the regenerating plantlets. Starting from 396, 371 and 321 embryos transformed with *GUSint*, *TaGRF4-GIF1* and *ZmGRF1-GIF1* derived from six different ears, 54 (13.6%), 121 (34.5%) and 152 (47.4%) independent T0 plants (derived from discrete IEs), respectively, were transgenic and transferred to soil, resulting in a significant 2.5-fold and 3.5-fold increase in transformation frequency compared to the control (Figure 3d).

Ten randomly picked *GUSint* and *ZmGRF1-GIF1*, and nine *TaGRF4-GIF1* T0 plants were grown to maturity to assess fertility by reciprocal backcrosses to the wild type. Usually, T0 plants are backcrossed to wild-type ears to segregate somaclonal variation that may arise during tissue culture (Bregitzer *et al.*, 2008). Seven *TaGRF4-GIF1* and nine *ZmGRF1-GIF1* T0 plants were male fertile and had a similar seed set as the control (Figure 3e-f). Male sterility was correlated with the presence of a higher T-DNA copy number (Figure 3e). When the T0 ear of *TaGRF4-GIF1* and *ZmGRF1-GIF1* plants was used in a backcross with wild-type pollen, there was a slightly reduced seed set compared to the control (Figure S5). In conclusion, *ZmGRF1-GIF1* outperformed *TaGRF4-GIF1* in maize and improved the transformation frequency by 3.5-fold, without major pleiotropic effects, and maintaining fertility.

When we repeated this experiment with the same *Agrobacterium* strains, using embryos from six ears, 15 independent T0 plants were obtained from 236 embryos transformed using *GUSint* (6.4%), 10 independent T0 plants from 251 embryos using *TaGRF4-GIF1* (4.0%) and 20 independent T0 plants from 254 embryos using *ZmGRF1-GIF1* (7.9%; Figure S6). Hence, no significant increase, nor decrease in transformation frequency was observed in this experiment ($P > 0.05$, Generalized Linear Model). Nevertheless, strengthened by the promising results of the previous experiments, we continued testing *ZmGRF1-GIF1*, but now focusing on functional plasmids with use for gene editing.

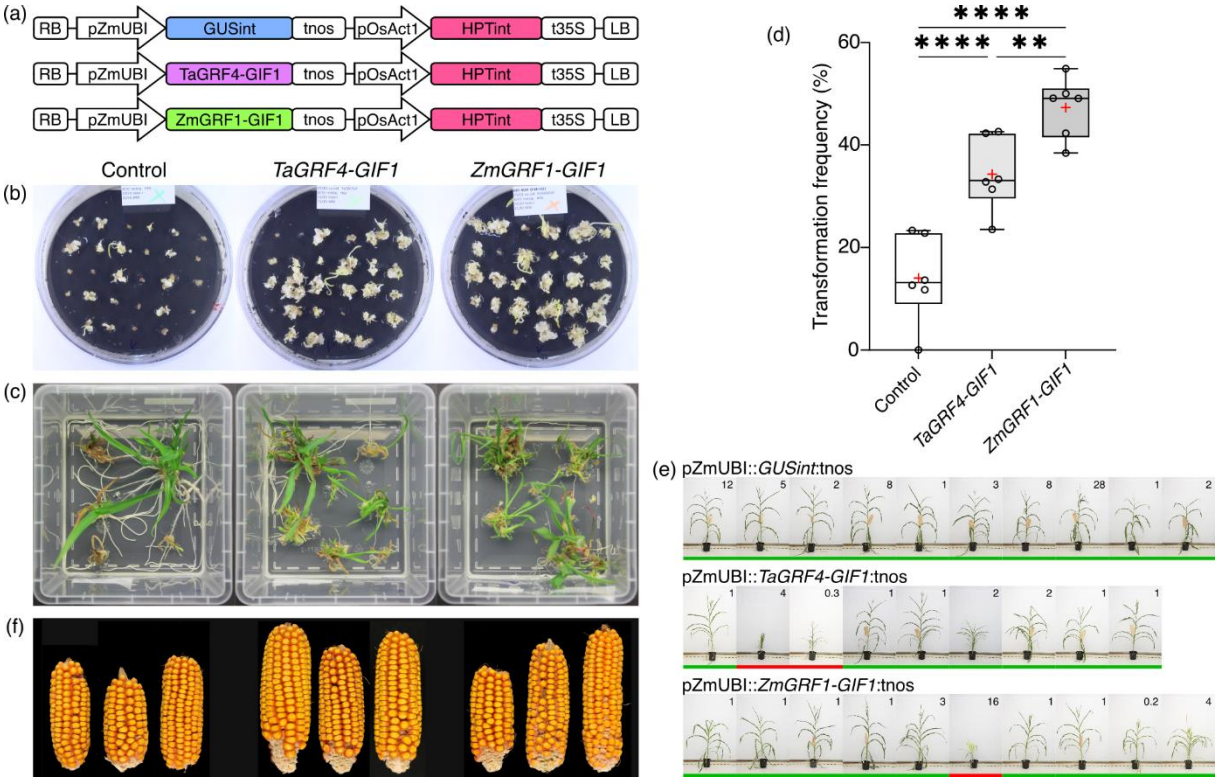


**Figure 3.** Transformation using *GRF-GIF* chimeras. (a) Diagrams of T-DNAs used for transformation. RB, right border; pZmUBI, maize *UBIQUITIN* promoter; GUSint, *β-GLUCURONIDASE* with intron IV2 of the potato gene *ST-LS1*; tnos, *A. tumefaciens NOPALINE SYNTHASE* terminator; pOsAct1, rice *ACTIN* promoter; HPTint, *HYGROMYCIN B PHOSPHOTRANSFERASE* with castor bean *CATALASE-1* intron; t35S, CaMV 35S terminator; LB, left border. (b) Representative pictures of regenerating calli after two weeks on maturation I medium. (c) Representative pictures of regenerated plantlets after five days on regeneration II medium. (d) Transformation frequency using *GRF-GIF* chimeras. The number of independent transgenics scoring positive for *HPTint* by detached leaf assays are plotted per starting IE per ear. ****, $P < 0.0001$; **, $P < 0.01$ (Generalized Linear Model). Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, averages are indicated by a red cross. (e) Mature T0 plants at fertile stage. In the top right, the T-DNA copy number is shown. The green lines below the pictures indicate male fertile plants, the red ones are sterile. (f) Representative pictures of cobs after a backcross to the wild type using T0 pollen.

## Efficient gene editing using the ZmGRF1-GIF1-zCas9 vector

As a proof of concept that the *ZmGRF1-GIF1* chimera can be used in combination with gene editing approaches, we constructed a new vector that contained *ZmGRF1-GIF1* controlled by the switchgrass (*Panicum virgatum* L.) *UBIQUITIN* promoter, a visual marker pBdEF1α::*YFP-NLS*:tBdUBI1, *zCas9* with both an N- and C-terminal bipartite NLS, which improves editing efficiency (Gaillochet *et al.*, 2023), the pOsAct1::*HPTint*:t35S resistance cassette, and a sgRNA expressed by pOsU3 targeting *cpSRP43*, a visual marker gene in Arabidopsis that upon knockout results in a pale leaf phenotype, named *chaos* (Klimyuk *et al.* (1999); Figure 4a-b). As control, an identical vector was made in which *ZmGRF1-GIF1* was replaced by *GUSint*. From three ears, 159 embryos were transformed using the control vector and 156 embryos using the *ZmGRF1-ZmGIF1-zCas9* vector, yielding 10 and 62 T0 plants, respectively, of which seven (4.4%) and 31 (19.9%) were independent, resulting in a significant 4.5-fold increase in transformation frequency using *ZmGRF1-GIF1* (Figure 4c). Regenerating calli of *ZmGRF1-GIF1* were

again larger compared to the control and showed a bushy phenotype, although this was less prominent than in the previous experiment (Figure S7). Editing frequency was extremely high, all ten control plants and 56/57 phenotyped *ZmGRF1-GIF1* plants showed varying degrees of the pale leaf phenotype, ranging from mosaic leaves with few pale stripes to completely pale leaves (Figure 4d, Data S5). When considering independent *ZmGRF1-GIF1* transgenics, 22/31 contained homozygous or transheterozygous mutations at the *cpSRP43* locus (Figure 4e-g, Data S5).

A question in the field remains whether different plants regenerating from the same IE are dependent, that is whether the different plants originate from the same transformed cell in the scutellum epithelium. To answer this question, we determined the copy number of the T-DNA and vector backbone for all control and *ZmGRF1-GIF1* transgenics, and combined this data with the edit made at the *cpSRP43* locus (Data S5). For example, plant 897-518-Wa, b and c regenerated from the same IE, displayed the same T-DNA and backbone copy number, along with a transheterozygous A/T insertion. This suggests they derived from the same transformed cell and exhibit dependence of each other. Conversely, plants 897-518-Ya and Yb regenerated from a single IE and possessed distinct T-DNA and backbone copy numbers. Additionally, 897-518-Ya carried a transheterozygous C/T insertion, while 897-518-Yb had a transheterozygous A/G insertion. This indicates that these plants did not regenerate from the same transformed cell and are therefore independent of each other. When calculating the transformation frequency, we have only considered transgenic plants as independent when derived from different IE (in the absence of molecular information). In this way, 7/10 transgenic control T0 plants were considered independent, while in fact 8/10 were independent based on dPCR and sequencing data. This increases the transformation frequency of the control only modestly from 4.4% to 5.0%. For *ZmGRF1-GIF1*, with 31/62 transgenic T0 plants considered independent, dPCR and sequencing data increases the number of independent plants to 51/62, and the transformation frequency therefore increases from 19.9% to 32.7%. Overall, our results show a 6.5-fold increase in transformation frequency in this experiment when using *ZmGRF1-GIF1* compared to the control ($P < 0.0001$, Generalized Linear Model).

In the first transformation experiment using GRF-GIF chimeras, we observed a high number of single T-DNA copy events in the 10 randomly chosen plants for both GRF-GIF constructs when compared to the control (Figure 3e). When comparing the distribution of the T-DNA copy number of all independent ZmGRF1-GIF1-Cas9 and GUSint-zCas9 plants, no apparent difference in copy number is observed (Figure S8). In total, 93 T0 plants transformed with *ZmGRF1-GIF1* from multiple experiments were analyzed using dPCR to determine the HygB copy number and presence of vector backbone. We identified 4.3% as escapes, and 24.7% as high-quality events. These are similar rates as observed without the use of a *GRF-GIF* chimera (3.4%, $P = 0.7378$; 23.3%, $P = 0.7515$, Fisher's exact tests), indicating that use of the *ZmGRF1-GIF1* chimera does not influence T-DNA copy number.

We next investigated if the CRISPR/Cas9-induced edits at the *cpSRP43* locus are inherited. We genotyped T1 seedings from a back-cross of wild type B104 with three independent lines of *ZmGRF1-GIF1-zCas9* and *GUSint-zCas9*. Of the T1 null segregants, 64 to 100% and 67 to 100% of *ZmGRF1-GIF1-zCas9* and *GUSint-zCas9* plants respectively, contained a heterozygous edit at the *cpSRP43* locus (Table 1). Observed edits largely corresponded with the indels also detected in the parental T0 plants (Data S5, Data S6). Differences between the T0 and T1 generation, and absence of edits in some T1 null segregants, can be attributed to mosaic editing in the T0 plant. Finally, T1 plants that still contain the T-DNA were found to show a *cpSRP43* full knockout phenotype, indicating strong transgenerational editing activity (Figure S9).

In conclusion, *ZmGRF1-GIF1* was successfully combined with CRISPR/Cas9, with 71% of independent T0 plants having both alleles edited and these edits being inherited to the T1 generation, while improving the transformation frequency 6.5-fold in this experiment.

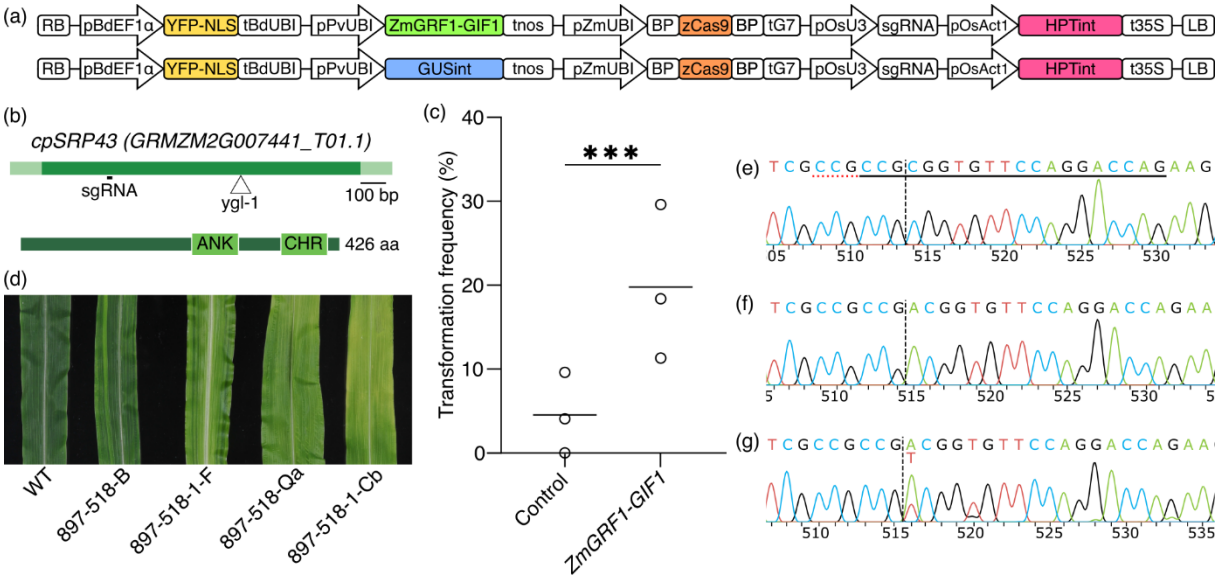


**Figure 4.** Transformation and gene editing of a visual marker in B104 using the *ZmGRF1-GIF1-zCas9* vector. (a) Diagrams of T-DNAs used. RB, right border; pBdEF1α, *B. distachyon ELONGATION FACTOR-1 ALPHA* promoter; YFP-NLS, *YELLOW FLUORESCENT PROTEIN* with C-terminal N7 nucleotide localization signal; tBdUBI1, *B. distachyon UBIQUITIN* terminator; pPvUBI, switchgrass *UBIQUITIN* promoter; GUSint, *β-GLUCURONIDASE* with intron IV2 of the potato gene *ST-LS1;* tnos, *A. tumefaciens NOPALINE SYNTHASE* terminator; pZmUBI, maize *UBIQUITIN* promoter; BP, bipartite nuclear localization signal; zCas9, maize codon optimized Cas9; tG7, G7 terminator; pOsU3, rice U3 promoter; pOsAct1, rice *ACTIN* promoter; HPTint, *HYGROMYCIN B PHOSPHOTRANSFERASE* gene with castor bean *CATALASE-1* intron; t35S, CaMV 35S terminator; LB, left border. (b) Genomic structure of *cpSRP43* with location of the sgRNA, ygl-1 locus (Guan *et al.*, 2016), and protein domains. ANK, four ankyrin repeats; CHR, three chromodomains. (c) Transformation frequency in B104 using the *ZmGRF1-GIF1* chimera. The number of independent transgenics scoring positive for *HPTint* by dPCR are plotted per starting immature embryo. ***, P < 0.001 (Generalized Linear Model). Center lines show the means. (d) Leaves of maize B104 T0 plants transformed with the *ZmGRF1-ZmGIF1-zCas9* vector with mosaic (897-518-B, 1-F) and homozygous (897-518-Qa, 1-Cb) mutations in *cpSRP43* compared to the wild-type leaf. (e-g) Chromatograms of the wild-type B104 (e) and T0 plants 897-518-1-Cb (f) and 897-518-Ha (g) *cpSRP43* sequence. The spacer region and PAM site are underlined in black and a red dotted line, respectively; the cut site is indicated with the vertical black dotted line.

**Table 1.** Inheritance of CRISPR/Cas9-induced edits in T1 null segregants. The number of T1 null segregants with an edit at the *cpSRP43* locus is given for three independent *ZmGRF1-GIF1-zCas9* and *GUSint-zCas9* lines after a backcross of the T0 parent to the wild type.

| Independent events | Edited null segregants | Total analyzed | % Edited |
|---|---|---|---|
| *ZmGRF1-GIF1-zCas9* | | | |
| 897-518-G ♂ x WT ♀ | 11 | 12 | 92 |
| 897-518-Ha ♂ x WT ♀ | 18 | 18 | 100 |
| 897-518-Ia ♂ x WT ♀ | 9 | 14 | 64 |
| *GUSint-zCas9* | | | |
| 898-519-B ♂ x WT ♀ | 8 | 8 | 100 |
| 898-519-E ♂ x WT ♀ | 9 | 12 | 75 |
| 898-519-La ♂ x WT ♀ | 4 | 6 | 67 |

## DISCUSSION

Gene-editing strategies to study and engineer crops have become more sophisticated and employ technologies such as multiplex gene editing (Lorenzo *et al.*, 2023), base and prime editing (Zong *et al.*, 2017; Jiang *et al.*, 2020), CRISPR library screens (Gaillochet *et al.*, 2021), gene targeting (Barone *et al.*, 2020) and promoter tuning (Rodríguez-Leal *et al.*, 2017; Claeys *et al.*, 2023). Many of these technologies require a large number of primary transformants to be screened in order to find the desired complex gene modifications. Here, we show that combining a *ZmGRF1-GIF1* morphogenic regulator with a ternary vector system further improves the transformation frequency in the maize inbred line B104.

Recent optimization of tissue culture media and conditions have allowed to drastically reduce the time and effort associated with maize transformation (Lowe *et al.*, 2018; Aesaert *et al.*, 2022; Kang *et al.*, 2022). However, these methods are associated with a high number of escapes when using the herbicide PPT in combination with the *BAR* selection marker (Aesaert *et al.*, 2022; Kang *et al.*, 2022). PPT is an analog of glutamate and functions as a glutamine synthetase inhibitor. Applying PPT leads to a lack of glutamine and an accumulation of ammonia, which are toxic for plant cells due to the inhibition of photosynthesis (Sauer *et al.*, 1987). The *BAR* selection marker codes for an enzyme that acetylates PPT, granting resistance to transformed cells. Here, we evaluated *HPT* as an alternative selectable marker in combination with the antibiotic HygB and found that this combination eliminates nearly all escapes even when using a shortened tissue culture phase. HygB inhibits the elongation step of polypeptide synthesis, and *HPT* detoxifies HygB by phosphorylation (González *et al.*, 1978). The HygB/*HPT* system has been used before in maize (Ishida *et al.*, 1996) and is a very commonly used system for wheat transformation (Hayta *et al.*, 2019). Interestingly, a similar approach was recently undertaken by others, replacing PPT/*BAR* with geneticin/*NEOMYCIN PHOSPHOTRANSFERASE II* (Lee *et al.*, 2023). Escape rates were also low using the antibiotic geneticin, with a mode of action similar to HygB also affecting polypeptide synthesis (Padilla and Burgos, 2010). While concerns have been raised regarding the use of high concentrations of Hygromycin B on T0 plant health (Yadava et al., 2017), we have not observed obvious detrimental effects. Although the HygB/*HPT* system came with a trade-off of a reduced transformation frequency, the elimination of escapes reduced the overall workload, especially in experiments using the ternary vector system.

The development of superbinary vectors such as pSB1 played a pivotal role in the early successes to transform monocot crops (Hiei *et al.*, 1994). These superbinary vectors harbor a 'Komari fragment', a 15-kb region containing the *virB*, *virC* and *virG* operons from the *Agrobacterium tumefaciens* Ti plasmid pTiBo542 (Komari, 1990). Use of these superbinary vectors increases the overall virulence, broadens the host range of *Agrobacterium*, and also helps to transfer larger T-DNAs (Zhang *et al.*, 2020). More recently, this technology was improved by placing the *vir* genes *in trans* on a ternary helper vector,

also known as accessory plasmid or virulence helper (Anand *et al.*, 2018). Several research groups have now reported the construction of such ternary helper vectors. These can contain also the additional operons *virD* and *virE*, and may differ from each other in bacterial resistance marker and origins of replication (Anand *et al.*, 2018; Zhang *et al.*, 2019; Kang *et al.*, 2022). As an alternative to superbinary vectors, early progress in maize transformation was made using the *Agrobacterium* strain EHA101 and its derivatives, EHA105 and AGL1 (Frame *et al.*, 2002). These strains have a C58 chromosomal background, complemented with a disarmed pTiBo542 Ti plasmid (Hood *et al.*, 1993; De Saeger *et al.*, 2021). Superbinary and ternary helper vectors are mostly used in the LBA4404 *Agrobacterium* background (Zhang *et al.*, 2020), as it is hypothesized that the combination of *vir* genes from both pTiBo542 and pTi4404 have synergistic effects (Zhang *et al.*, 2020). Here, we have explored the use of the helper pVS1-VIR2 containing a pTiBo542 fragment in combination with the pTiBo542ΔT-DNA-containing strain EHA105 *recA*$^{-}$, both with identical *vir* genes. We observed a marked increase in transient expression of the *GUSint* reporter gene, and in stable transformation. These results suggest that the mechanism by which ternary helper vectors increase the transformation frequency is at least partially related to virulence gene dosage. This does not exclude any additional synergy in combining *vir* genes from different Ti plasmids, which will be interesting to study for example using minimal synthetic Ti plasmids (Thompson *et al.*, 2023). Application of the pVS1-VIR2 ternary vector system resulted in an increased number of cells per embryo expressing the T-DNA *GUSint* reporter gene, but also in more embryos that show any *GUSint* expression. The latter is important because competition has been observed between neighboring somatic embryos, likely limiting the amount of transgenic plants that can be obtained per IE explant (Lowe *et al.*, 2018). Hence, having more embryos overall responsive to both transformation and tissue culture is important to increase the transformation frequency.

In addition, we define maize transformation frequency as the number of independent T0 plants obtained per starting IE (Aesaert *et al.*, 2022). In the absence of any molecular characterization, we consider T0 plants only independent if they are derived from different IE explants. This definition is useful as it is not always straightforward to rule out if multiple T0 plants regenerating from a single IE are not clonal. When combining the ternary vector system and a *ZmGRF1-GIF1* chimera, a single IE however often leads to non-clonal T0 plants. This prompted us to examine all T0 plants at the molecular level, revealing multiple independent events, increasing our transformation frequency from 19.9% to 32.7%. Although many publications define the transformation frequency as the total number of transformants divided by the number of starting explants and multiplied by 100 (Lee and Wang, 2023), we here use a more conservative definition, because many of the obtained plants are still clonal based on our analysis.

Expression of *ZmBBM* and *ZmWUS2* improves the transformation frequency via direct somatic embryogenesis of transformed cells (Lowe *et al.*, 2018). This can be attributed to the endogenous functions of these MRs: BBM is suggested to signal fertilization and induce and/or maintain zygotic embryo development (Boutilier *et al.*, 2002; Khanday *et al.*, 2023), and WUS maintains the stem cell pool during the development of the shoot apical meristem (Somssich *et al.*, 2016), while ectopic *WUS* expression can also trigger somatic embryogenesis (Zuo *et al.*, 2002). As MRs, *ZmBBM* and *ZmWUS2* are typically expressed using tissue- and developmental stage-specific promoters in maize to ensure normal development and fertility of regenerated plants (Lowe *et al.*, 2018), further improved by excision of this cassette (Hoerster *et al.*, 2020; Wang *et al.*, 2020; Aesaert *et al.*, 2022). Whereas high expression of *ZmBBM* and/or *ZmWUS2* in maize elicits the immediate formation of somatic embryos on the scutellum epithelium of immature maize embryos, we did not observe such phenotype for expression of the *GRF-GIF* chimera from the strong constitutive promoters ZmUBI or PvUBI. Hence, the *GRF-GIF* chimera may function later during the maturation of somatic embryos into plantlets, instead

of promoting *de novo* somatic embryogenesis. This seems likely given the known function of *ZmGRF1* and *ZmGIF1* in plant development. GRFs are transcription factors that interact with members of the GIF family of transcriptional coregulators (Liebsch and Palatnik, 2020). GIF proteins do not contain a DNA-binding domain and act as adaptor proteins with the SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeling complex (Vercruyssen *et al.*, 2014; Nelissen *et al.*, 2015). In general, GRF/GIF complexes act as positive regulators of plant growth by regulating the balance between determinacy and indeterminacy of meristems (Nelissen *et al.*, 2015; Rodriguez *et al.*, 2015; Zhang *et al.*, 2018). *ZmGRF1* is mainly expressed in shoots and young leaves, and promotes cell division in the leaf growth zone together with *ZmGIF1*, which is expressed in ears, immature tassels, shoots, leaves and roots (Zhang *et al.*, 2008; Nelissen *et al.*, 2015). Molecular analysis of regenerated plants showed that indeed more independent plants can regenerate from a single transformed IE, while there is no apparent increase in number of somatic embryos formed, supporting the hypothesis that *GRF-GIF* chimeras promote the maturation of somatic embryos into a plantlet. Since BBM-WUS and GRF-GIF likely function during distinct regeneration processes, there is a potential to combine both systems, which could lead to additive or even synergistic effects (Chen *et al.*, 2022).

Our results illustrate the variability in frequency that is encountered when transforming maize using IE explants. Large variations are observed between experiments carried out by the same experienced operators on different days, and between IE donor plants within the same experiment, even when using ears from side-by-side grown plants from an inbred line in controlled greenhouse conditions, a phenomenon which has also been observed by others (Lowe *et al.*, 2018; Masters *et al.*, 2020; Aesaert *et al.*, 2022). The origin of this variation is not well understood, both minor insect infestations which could lead to sub-optimal competence for *Agrobacterium* infection, and slight physiological differences between plants due to position effects in the greenhouse have been suggested as possible causes (Aesaert *et al.*, 2022). This may also help explain why in one experiment both the *ZmGRF1-GIF1* and *TaGRF4-GIF1* chimeras had no effect on transformation frequency. This brings up the issue of the importance of repeatability and reproducibility in transformation research, which is not always reported in the literature, showing in many cases single successful experiments, despite the historical importance of reproducibility of maize transformation methods (Kausch *et al.*, 2021)**.** Here, we report the performance of the use of *HPT* over 48 experiments, the use of *HPT* combined with the pVS1-VIR2 ternary vector system over 19 experiments, and the use of *GRF-GIF* chimeras over four experiments, strengthening our message, while managing expectations.

In recent research relying on the genetic transformation of maize, only a few lines were used, such as Hi-II (Chen *et al.*, 2018; Feng *et al.*, 2018; Liu *et al.*, 2021) and the inbred lines B104 (Char *et al.*, 2017; Lee *et al.*, 2019; Gong *et al.*, 2022; Laureyns *et al.*, 2022; Lorenzo *et al.*, 2023) and A188 (Doll *et al.*, 2019; Zhang *et al.*, 2021). There is however an interest to expand the number of transformable lines to include alternative inbred lines, especially from other heterotic groups. To improve transformation frequency in other inbred lines, the MRs *ZmBBM* and *ZmWUS2* have been successfully used (Lowe *et al.*, 2018; Masters *et al.*, 2020), and the ZmGRF1-GIF1 chimera could potentially also allow transformation of lines generally regarded as transformation-recalcitrant. This could allow trait validation in genotypes with different morpho-physiological conditions. However, the production of IEs is still a practical bottleneck in setting up transformation experiments for alternative inbred lines due to greenhouse growth performance, IE quality, flowering time or space restrictions. Some of these can likely be addressed in the future using alternative explants (Wang *et al.*, 2023).

The discovery and optimization of the use of MRs such as BBM-WUS has been a major breakthrough to improve the efficiency of transforming crop species. Similarly, it was reported that expression using the constitutive BdEF1 promoter of ZmGRF5-LIKE1 and ZmGRF5-LIKE2, belonging to a different GRF

subfamily as ZmGRF1, led to a three-fold increase in transformation frequency in the inbred A188 (Kong *et al.*, 2020). The *GRF-GIF* chimera approach arises as a promising alternative to BBM-WUS. It can be driven by a constitutive promoter without the need of excision to yield fertile plants and improves transformation frequencies in both monocotyledonous and dicotyledonous species such as wheat, rice, sorghum, citrus, lettuce and watermelon (Debernardi *et al.*, 2020; Li et al., 2023; Feng *et al.*, 2021; Bull *et al.*, 2023). It was recently reported that in sorghum, a TaGRF4-OsGIF1 chimera combined with pVS1-VIR2 yielded the highest transformation frequencies (Li et al., 2023). Moreover, *GRF-GIF* chimeras can help to expand the range of transformable genotypes. It would be interesting to explore the use the *GRF-GIF* chimera to further improve the regeneration of other maize inbred lines and other species, such as barley and soybean. To further improve transformation frequencies, miR396a-resistant *GRF-GIF* variants can be used (Debernardi *et al.*, 2020; Feng *et al.*, 2021; Bull *et al.*, 2023). However, there may be a need for excision of the chimera because miR396a-resistant *GRF1*-expressing plants display female fertility issues (Nelissen *et al.*, 2015). Alternatively, expression of the chimera could be boosted using stronger constitutive promoters. Furthermore, the *GRF-GIF* chimera may also help to improve the transformation of other explant material such as maize leaves, in combination with other MRs such as *ZmBBM* and *ZmWUS2* (Wang *et al.*, 2023), or more recently described MRs such as *TaWOX5* (Wang *et al.*, 2022), *DOF* (Liu *et al.*, 2023) and *TaLAX1* (Yu *et al.*, 2023). Finally, a promising method to explore is the combination of *GRF-GIF* with the altruistic transformation strategy, in which *ZmWUS2* is co-expressed transiently (Hoerster *et al.*, 2020; Kang *et al.*, 2023). This may be more convenient compared to stacking even more MRs on the same T-DNA, as this would create large T-DNAs which are intrinsically transformed with lower frequencies (Hamilton, 1997; Park *et al.*, 2000; Xi *et al.*, 2018).

In summary, our results demonstrate that a *ZmGRF1-GIF1* chimera combined with a ternary vector system and an *HPT* selection marker greatly improves B104 transformation frequency and we show it can be used for gene editing strategies.

# EXPERIMENTAL PROCEDURES

## Plant material and growth conditions

Seeds of the maize inbred lines B104 were originally obtained from the USDA National Plant Germplasm System (Accession no. PI 594047). A single seed was placed in a pre-wetted Jiffy-7® pellet and kept in controlled growth room conditions (300 µm $m^{-2}$ $s^{-1}$, 16-h light/8-h dark, 24°C). Seedlings were transferred to 10-L pots with professional potting mixture (Van Israel nv) containing controlled release fertilizer (2.0 kg $m^{-3}$, Osmocote®, Scotts International B.V., Heerlen, the Netherlands, NPK 12/14/24) and moved to the greenhouse (300 µm $m^{-2}$ $s^{-1}$, 16-h light/8-h dark, 26°C/22°C) until grown to maturity.

## Bacterial strains

DH5α competent cells (Invitrogen) and *ccdB* Survival™2 cells (Invitrogen) were used for cloning. For transformation, hypervirulent disarmed *A. tumefaciens* strains EHA105 (Hood *et al.*, 1993), EHA105 *recA*$^-$ (Rodrigues *et al.*, 2021) and EHA105 *recA*$^-$ pVS1-VIR2 (Addgene #134745) were used.

## sgRNA design

We used B73 gene model GRMZM2G007441 (PLAZA 3.0, Proost *et al.* (2015)) for *cpSRP43* and designed a sgRNA with high predicted selectivity and specificity (CTGGTCCTGGAACACCGCGG) using CRISPOR (Concordet and Haeussler, 2018). The target region in wild-type B104 was amplified and sequenced, no mismatches between B73 and B104 were found, so the selected spacer could be used.

## Vector construction

All plasmids used in this study are listed in Table S1, primers in Table S2 and modules produced by gene synthesis in Table S3. All plasmids created for this research are available on gatewayvectors.vib.be.

For cloning of expression vectors, we made use of Golden Gibson cloning (GGIB, Jacobs and Karimi, unpublished), a variation of 3G assembly (Halleran *et al.*, 2018). Elements were combined into GGIB shuttle vectors using Golden Gate cloning (Lampropoulos *et al.*, 2013). Shuttle vectors contain unique nucleotide sequences (U-sites, Torella *et al.* (2014)) flanked by I-SceI restriction sites that can be combined in a destination vector using Gibson assembly (Gibson *et al.*, 2009).

***Binary vector system***

To create the destination vector for the transformation using the binary vector system in which one sgRNA could be cloned, shuttle vectors were first constructed using Golden Gate cloning. pGG-A-pZmUBI-B, pGG-B-linker-C, pGG-C-zCas9-D and pGG-D-tnos-G were cloned into pGGIB-U1-AG-U2, yielding pGGIB-U1-pZmUBI-zCas9-tnos-U2. pGG-A-linker-F and pGG-F-pOsU3-BbsI-ccdB-BbsI-scaffold-G were cloned into pGGIB-U2-AG-U3, yielding pGGIB-U2-pOsU3-BbsI-ccdB-BbsI-scaffold-U3. These shuttle vectors were assembled together with pGGIB-U3-L3-U9 into pHb-U1-U9, yielding pMHb-pZmUBI-zCas9-tnos-pOsU3-BsaI-ccdB-BsaI-scaffold. For the other vector in which multiple sgRNAs could be cloned, pGGIB-U3-pBdEF1α.2-TdTomato-tnos-U4 was constructed using Golden Gate cloning from pGGIB-U3-AG-U4, pGG-A-pBdEF1α.2-B, pGG-B-linker-C, pGG-C-TdTomato-D, pGG-D-linker-E, pGG-E-tnos-F and pGG-F-linker-G, and assembled with pGGIB-U1-pZmUBI-zCas9-tnos-U2, pGGIB-U2-AG-U3 and pGGIB-U4-L4-U9 into pHb-U1-U9 to create pMHb-zCas9-AG-tdT.

***Ternary vector system***

The *HYGROMYCIN B PHOSPHOTRANSFERASE* coding sequence with the castor bean *CATALASE-1* intron was cloned flanked by Greengate C and D sites using ICSL11059 (Addgene #68263; Lawrenson *et al.* (2015)) as template to yield pGG-C-HPTint-D. The rice actin promoter (pOsAct1) was flanked by A and C sites, and a BsaI site was removed to yield pGG-A-pOsAct1-C. Using GreenGate cloning, pGG-A-OsAct1-C, pGG-C-HPTint-D and pGG-D-t35S-G were cloned into pGGIB-U8-AG-U9 to yield pGGIB-U8-pOsAct1-HPTint-t35S-U9.

The pGreen3-compatible destination vectors containing the *E. coli* pUC ori, *A. tumefaciens* pRK2 oriV, kanamycin or gentamycin resistance, pTiT37 LB and RB were created using gene synthesis (GenScript). Subsequently a Golden Gibson module was inserted between the LB and RB, generating pLAPAU20 and pG3-U1-AG-U9, respectively. The sfGFP sequence was PCR amplified from pEN-L1-PJ23119-Bsa1-PglpT-sfGFP-TrrfB-BsaI-Scaf-L2 using CROPGEN776 and 777, cloned into the pJET1.2/blunt Cloning Vector (ThermoFisher) and subsequently into pGGIB-U6-AG-U7, yielding pGGIB-U6-A-sfGFP-G-U7. Using a Gibson reaction, pG3-U1-AG-U9 was combined with pGGIB-U1-L1-U6, pGGIB-U6-A-sfGFP-G-U7, pGGIB-U7-L7-U8 and pGGIB-U8-pOsAct1-*HPTint*-t35S-U9 to yield pG3HI-AG.

For cloning of the vector used in the X-Gluc assay, pGG-A-pZmUBI.2-C, pGG-C-GUSint-D and pGG-D-t35S-G were cloned into pLAPAU20 to generate pLAPAU20-pZmUBI.2-GUSint-t35S. To create the vectors used to compare transformation frequencies between the binary and ternary vector system, pGG-F-pOsAct1-*HPTint*-t35S-G was first created by PCR amplification of pGGIB-U8-pOsAct1-*HPT*int-t35S-U9 and cloned into a BsaI-digested GreenGate pGGF000 entry vector. Subsequently, pGG-A-pZmPLTP-C, pGG-C-YFP-D, pGG-D-linker-NLS-E, pGG-E-tG7-F and pGG-F-pOsAct1-*HPTint*-t35S-G were cloned into pP-U1-U9 and pG3-U1-AG-U9, yielding pP-pZmPLTP-YFP-linker-NLS-tG7-pOsAct1-*HPTint*-t35S and pG3-pZmPLTP-YFP-linker-NLS-tG7-pOsAct1-*HPTint* -t35S, respectively.

Gibson assembly was used to create destination vectors for transformation using the ternary vector system. pG3HI-AG was assembled from pG3-U1-AG-U9, pGGIB-U1-L1-U6, pGGIB-U6-A-sfGFP-G-U7, pGGIB-U7-L7-U8 and pGGIB-U8-pOsAct1-*HPTint*-t35S-U9. pG3HI-zCas9-tdT-AG was assembled from pG3-U1-AG-U9, pGGIB-U1-pZmUBI.2-zCas9-tnos-U2, pGGIB-U2-L2-U3, pGGIB-U3-pBdEF1α.2-TdTomato-tnos-U4, pGGIB-U4-L4-U6, pGGIB-U6-A-sfGFP-G-U7, pGGIB-U7-L7-U8 and pGGIB-U8-pOsAct1-*HPTint*-t35S-U9. pGGIB-U1-pZmUBI.2-zCas9-tnos-U2 was constructed from pGG-A-pZmUBI.2-C, pGG-C-zCas9-D and pGG-D-tnos-G into pGGIB-U1-AG-U2.

***GRF-GIF vectors***

The coding sequence of *TaGRF4-GIF1* was PCR amplified from pJD553 (Addgene #160392, Debernardi *et al.* (2020)) flanked by C and D overhangs while simultaneously adding a stop codon using CROPGEN750 and 751 and cloned into pGGC000 to yield pGG-C-TaGRF4-GIF1-D. pGG-A-pZmUBI.2-C, pGG-C-TaGRF4-GIF1-D and pGG-D-tnos-G were cloned into pHb-U1-U9 using Golden Gate cloning, yielding pHb-pZmUBI.2-TaGRF4-GIF1-tnos. The coding sequence of ZmGRF1-GIF1 was created using gene synthesis (GenScript) and cloned into pGG-C000, yielding pGG-C-ZmGRF1-GIF1-D. pG3HI-pZmUBI.2-GUSint-tnos, pG3HI-pZmUBI.2-TaGRF4-GIF1-tnos and pG3HI-pZmUBI.2-ZmGRF1-GIF1-tnos were created using Golden Gate cloning using pG3HI-AG, pGG-A-pZmUBI.2-C, pGG-C-GUSint-D, pGG-C-TaGRF4-GIF1-D, pGG-C-ZmGRF1-GIF1-D and pGG-D-tnos-G.

The tBdUBI1 sequence was PCR amplified from pGG-D-tBdUBI1-G, flanked by E and F overhangs while adding stop codons to the sequence using CROPGEN1095 and 1096, purified using the Zymo Gel DNA Extraction Kit (Zymo Research) and assembled into digested pGG-E000, yielding pGG-E-tBdUBI1-F.

For cloning the sgRNA targeting *cpSRP43*, two complementary oligos with 4-bp overhangs, CROPGEN25 and 26, were annealed and inserted via a Golden Gate reaction with BbsI (NEB) and T4 ligase (Thermo) in pGG-A-pOsU3-BbsI-ccdB-CmR-BbsI-B. Gibson shuttle vectors were constructed using Golden Gate cloning of pGG-A-pBdEF1α.2-B, pGG-B-linker-C, pGG-C-YFP-D, pGG-D-linker-NLS-E, pGG-E-tBdUBI1-F and pGG-F-linker-G into pGGIB-U3-AG-U4 to yield pGGIB-U3-pBdEF1α.2-YFP-linkerNLS-tBdUBI1-U4; pGG-A-pPvUBI2-B, pGG-B-Linker-C, pGG-C-ZmGRF1-GIF1-D and pGG-D-tnos-G into pGGIB-U4-AG-U5 to yield pGGIB-U4-pPvUBI2-ZmGRF1-GIF1-tnos-U5; pJET-A-BsaI-sfGFP-BsaI-G into pGGIB-U4-AG-U5 to yield pGGIB-U4-A-sfGFP-G-U5; pGG-A-pZmUBI.2-B, pGG-B-BP_NLS-C, pGG-C-zCas9_noNLS_nostop-D, pGG-D-BP_NLS_star-E, pGG-E-tG7-F and pGG-F-linker-G into pGGIB-U6-AG-U7 to yield pGGIB-U6-pZmUBI.2-BP-zCas9-BP-star-tG7-U7; pGG-A-pOsU3-sgRNA(chaos)-B and pGG-B-linker-G into pGGIB-U7-AG-U8 to yield pGGIB-U7-pOsU3s-sgRNA(chaos)-U8. Vectors used for transformation were then created using Gibson assembly of pGGIB-U1-L1-U3, pGGIB-U3-pBdEF1α.2-YFP-linkerNLS-tBdUBI1-U4, pGGIB-U4-pPvUBI2-ZmGRF1-GIF1-tnos-U5, pGGIB-U5-L5-U6, pGGIB-U6-pZmUBI.2-BP-zCas9-BP-star-tG7-U7, pGGIB-U7-pOsU3-sgRNA(chaos)-U8 and pGGIB-U8-pOsAct1-HPTint-t35S-U9 into pG3-U1-AG-U9 to yield pG3-YFP-ZmGRF1-GIF1-zCas9-chaos-HPTint. To create the control vector, pGGIB-U4-A-sfGFP-G-U5 was used instead to yield pG3-YFP-sfGFP-zCas9-chaos-HPTint in which pGG-A-pPvUBI2-B, pGG-B-Linker-C, pGG-C-GUSint-D and pGG-D-tnos-G were cloned using Golden Gate to yield pG3-YFP-GUSint-zCas9-chaos-HPTint.

Lastly, a new destination vector was constructed for efficient gene editing in maize. First, pGGIB-U7-A-sfGFP-G-U8 was made from pGGIB-U7-AG-U8 and pJET-A-BsaI-sfGFP-BsaI-G, and pGGIB-U5-pPvUBI2-ZmGRF1-GIF1-tnos-U6 was made from pGGIB-U5-AG-U6, pGG-A-pPvUBI2-B, pGG-B-Linker-C, pGG-C-ZmGRF1-GIF1-D and pGG-D-tnos-G by Golden Gate cloning. These shuttle vectors were assembled together with pGGIB-U1-L1-U5, pGGIB-U6-pZmUBI.2-BP-zCas9-BP-star-tG7-U7 and pGGIB-U8-pOsAct1-*HPTint*-t35S-U9 into pG3-U1-AG-U9 using Gibson assembly to create pG3HI-ZmGRF1-GIF1-zCas9-A-sfGFP-G.

### Maize transformation

Maize transformation was performed as described in Aesaert *et al.* (2022) with an altered selection scheme using HygB (Protocol S1 and Figure S10). Routine transformation experiments using the binary and ternary vector systems were performed by the same experienced operators, limiting variation between experiments. Because the number of escapes is negligible, all obtained plants from routine transformation experiments were considered as transgenic.

### X-Gluc assay and quantification

Histochemical 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) assay was performed six days after inoculation as described in Coussens *et al.* (2012). Images were taken using a Leica S9D microscope (Leica Microsystems) and the area of blue spots was quantified using ImageJ (Schindelin *et al.*, 2012; Rodrigues *et al.*, 2021).

### Genotyping

For genomic DNA (gDNA) extraction, a piece of leaf of approximately 1.5 $cm^2$ was cut and placed in a 2-mL tube together with two 4-mm metal balls. After snap-freezing in liquid nitrogen, tissue was crushed using a Retsch® Mixer Mill MM 400 at 20 Hz for one minute and gDNA was isolated from the crushed, frozen tissue using the Wizard® Genomic DNA Purification kit (Promega). PCR amplification of gDNA samples was performed using the GoTaq® Flexi kit (Promega) and PCR products were run on a 1% agarose gel. When DNA sequencing was required, PCR products were purified using magnetic beads (HighPrep™ PCR Clean-up System, Magbio), sequenced using a Mix2Seq Kit (Eurofins Genomics) and sequences were analyzed using ICE (Synthego).

As a low-labor alternative, detached leaf assays were performed based on Ishida *et al.* (2007). Leaf pieces of approximately 1 $cm^2$ were cut and placed in leaf assay medium containing HygB (Protocol S1). Sensitivity to HygB was scored after 4 days.

### Digital PCR

Droplet digital PCR was used to determine the copy number and presence of backbone in plants transformed with *YFP-NLS* using a binary or ternary vector system as described in Aesaert *et al.* (2022). For all other copy number analyses, nanoplate-based dPCR was used. gDNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo), diluted to 5 ng/µL and added together with primers and probes to the QIAcuity Probe PCR Kit Master Mix (Qiagen) and subsequently digested with CviQI (NEB) for 10 min at 24°C. PCR and analysis was performed using the Qiagen QIAcuity One 5-plex platform and an 8.5K 24-well or 96-well Nanoplate. PrimeTime™ Probes (IDT, Leuven, Belgium) with 6-FAM, HEX and Cy5 fluorophores for selection marker, reference gene and backbone, respectively, were used to allow multiplexing. As single-copy reference genes *FPGS* (Zm00007a00000670, Manoli *et al.* (2012)) or *ADH1* (Collier *et al.*, 2017) were used. Primers and probes for reference genes, backbone (*GmR* or *aadA*) and selection marker (*HPT*/*HPTint*) are listed in Table S2.

## ACCESSION NUMBERS

*cpSRP43* (Zm00007a00019471), *ZmGRF1* (Zm00001eb251820, Zm00007a00010155), *ZmGIF1* (Zm00001eb056300, Zm00007a00006979), *TaGRF4* (TraesCS6D03G0604500), *TaGIF1* (TraesCS4A03G0657900).

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## AUTHOR CONTRIBUTIONS

W.V. and L.P. designed the research. G.C., S.A., W.V., J.H. and L.I. performed maize transformations. M.K., W.V. and J.H. designed constructs and performed cloning. G.C. performed X-Gluc assays. W.V. and G.C. performed dPCR. J.M.D. and L.P. supervised the research. W.V. and L.P. wrote the manuscript.

## CONFLICT OF INTEREST

J.M.D. is an inventor of the UC Davis patent application US 2023/0032478 A1 that describes the use of GRF–GIF chimeras to enhance regeneration frequency in plants.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. All plasmids created for this research are available on gatewayvectors.vib.be.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Destination vectors for the binary vector system used in maize transformation experiments.

**Figure S2.** Transformation frequency using the binary versus ternary vector system.

**Figure S3.** Transformation frequency using the *TaGRF4-GIF1* chimera.

**Figure S4.** Phylogenetic trees for GRF and GIF families for maize and wheat.

**Figure S5.** Cobs after reciprocal backcrosses of *GUSint*, *TaGRF4-GIF1* and *ZmGRF1-GIF1* T0 plants with the wild type.

**Figure S6.** Data of unsuccessful experiment using GRF-GIF chimeras.

**Figure S7.** Regenerating calli and plants using control and *ZmGRF1-GIF1-zCas9* vectors.

**Figure S8.** T-DNA copy number of control and *ZmGRF1-GIF1* T0 plants.

**Figure S9.** Transgenerational editing in T1 generation.

**Figure S10.** Overview of maize transformation procedure.

**Table S1.** Plasmids used in this study.

**Table S2.** Oligonucleotides used in this study.

**Table S3.** DNA sequences of elements synthesized in this study.

**Data S1.** Transformation frequencies using the binary vector system.

**Data S2.** Area of transient transformation of immature embryos.

**Data S3.** Transformation frequencies using the ternary vector system.

**Data S4.** Copy number analysis of T0 plants after transformation using the ternary vector system and HygB selection.

**Data S5.** Copy number and sequencing analysis of B104 *ZmGRF1-GIF1-zCas9* and control plants.

**Data S6.** Sequencing analysis of B104 T1 null segregants.

**Protocol S1.** Maize transformation protocol.

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