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Strategies for genotype-flexible plant transformation

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Recent advances in the genome-editing tools have demonstrated a great potential for accelerating functional genomics and crop trait improvements, but the low efficiency and genotype dependence in plant transformation hinder practical applications of such revolutionary tools. Morphogenic transcription factors (MTFs) such as *Baby boom*, *Wuschel2*, *GROWTH-REGULATING FACTOR5*, *GROWTH-REGULATING FACTOR4* and its cofactor *GRF-INTERACTING FACTOR1*, and *Wuschel-homeobox 5* related have been shown to greatly enhance plant transformation efficiency and expand the range of amenable species and genotypes. This review will summarize recent advancements in plant transformation technologies with an emphasis on the strategies developed for genotype-flexible transformation methods utilizing MTFs for both monocots and dicot plant species. We highlight several breakthrough studies that demonstrated a wide range of applicability.

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Introduction

Plant transformation is a complex process that involves the preparation of explants, delivery of genes of interest (GOI) into plant cells mainly via *Agrobacterium*- or biolistic-mediated methods, and the selection and regeneration of transgenic or gene-edited plants. Numerous factors are known to affect the success rate of plant transformation and they were well summarized in previous reviews [1–3•]. Among the challenges associated with plant transformation, low transformation rates and genotype dependence are considered two main factors that make plant transformation a bottleneck for gene editing or transgenic approaches [1,2].

Transformation rate ($\boldsymbol{T}$) can be expressed in an equation described in Figure 1. The $\boldsymbol{T}$ is considered a product of the multiplication of several factors, including the successful rate of DNA delivery into plant cells via either *Agrobacterium* or biolistic gun ($\boldsymbol{C_d}$), the percentage of cells that incorporate the introduced DNAs into plant genomes and express the selectable marker genes ($\boldsymbol{C_i}$), and the said cells maintain totipotency and capacities of regeneration and reproduction ($\boldsymbol{C_r}$). Compounded with these factors, one also must consider any known or unknown variables that are typically associated with the transformation processes ($\boldsymbol{V}$). These variables could be the quality of chemical reagents, non-optimal growth conditions, or even the skills of the researchers who perform the experiments. Transformation rates cannot be easily determined by one or two experimental outcomes, as the success rates can vary greatly between experiments and biological materials. It is usually reported in the publications as percentages of transformed plants out of starting explant materials used in the transformation experiments, or the T (percentage) is the number of transformants divided by the number of starting explants and multiplied by 100. For example, in *Agrobacterium*-mediated transformation using immature embryos, 10% transformation frequency usually refers to 10 transgenic T0 plants produced from 100 infected immature embryos [4].

Genotype dependence in plant transformation means that one can only achieve genetic transformation on limited numbers of plant species or varieties. In many instances, a successful protocol for one plant variety may not be transferrable to another one. For example, the protocol for maize inbred B104 cannot be applied to B73 [5]. Different strategies have been developed to overcome genotype dependence and low transformation rates, which are often caused by the limited capacity of regeneration during tissue culture known as recalcitrance. Some of the successful approaches are: 1) use of less genotype-dependent explant tissues, for example, meristematic tissues [6], 2) exogenous application of plant hormones to pretreat explants before gene delivery [7], and 3) ectopic overexpression of morphogenic transcription factors (MTFs) [8••–11••].

Immature embryos are commonly used as explants for many monocot crops such as maize, wheat, barley,

Figure 1

$$T = V(C_d \times C_i \times C_r)$$

Where,

T	*transformation rate (percentage of transformed cell or tissue)*
V	*known or unknown variables associated with the transformation procedures*
C_d	*percentage of cells that have received deliberately introduced DNA molecules containing a selectable marker gene*
C_i	*percentage of cells that have incorporated the introduced DNA molecules into the genomes and are expressing the selectable marker gene*
C_r	*percentage of cells that maintain cell totipotency or the ability to regenerate*

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Equation of plant transformation frequency.

sorghum, and oats. For these species, using the right size of immature embryos (1.5–2 mm long) is critical for efficient transformation. For dicotyledonous plant species, young leaves, stems, or roots can be used for transformation and regeneration. Some explants may be more amenable to regeneration. For example, epicotyl and internodal segment-based methods were more efficient and less genotype-dependent than the conventional hypocotyl-based methods in canola transformation [6]. Plant hormones are commonly used for in vitro plant tissue cultures to promote plant regeneration [7]. Recently, auxin has been implicated to modify chromatin environments to allow the expression of totipotency-related transcription factors [12], which in turn contribute to totipotency and pluripotency acquisition and regeneration [13••]. Consistent with this implication, Zobrist et al. [14] observed that highly regenerable embryogenic callus was induced from teosinte seedlings germinated on a medium amended with a synthetic auxin, picloram. Importantly, recent studies demonstrated that exogenous application of auxins and cytokinins in the tissue culture media could induce somatic embryogenesis on maize immature embryos without the long tissue culture period, significantly shortening the time to generate transgenic plants [15,16]. However, the most robust and widely applicable technology would be the utilization of MTFs, such as *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) [8••,17•,18•], *GROWTH-REGULATING FACTORs* (*GRF*s) [9••,10••], and a *Wuschel* family transcription factor *Wuschel-related homeobox 5* (*WOX5*) [11••]. Overexpression or inducible expression of these MTFs have greatly improved not only the transformation frequencies but also the range of transformable species and genotypes [3•,19•,20]. In dicots, MTFs also provided tissue culture-free options for gene editing and transformation by stimulating direct shoot regenerations [21••,22••].

In this review, we will first summarize recently developed strategies for plant genotype-flexible transformation. Especially, MTF-assisted plant transformation methods will be reviewed with efficient vector designs utilized in those studies. We will then discuss how different aspects of plant transformation can be optimized to overcome genotype dependence and recalcitrance, and lastly address briefly how future research can further enhance the transformation capacity.

Key factors affecting plant genetic transformation

Explant selection and preparation

It is well known that explant tissue types and physiological status play a critical role in successful transformation. Depending on the plant species and genotypes, many different types of tissues can be used as explants, including immature embryos, pollens, cotyledonary nodes, primary leaf nodes, epicotyls, hypocotyls, axillary buds, internodal segments, stem tips, young leaf segments, and root segments. For many cereal crop species, immature embryos are most efficiently transformable, but their size (1.5–2.0 mm) and growth conditions (seasonable variation) influence the outcome of transformation [16]. For these species, obtaining high-quality immature embryos is a key factor and it remains challenging for most academic labs to produce immature embryos year-round. To substantially increase the transformation capacity, it is important to develop more robust transformation protocols that can utilize readily available explant tissues, such as mature seeds, leaf segments, and internodal segments [8••,23–25].

Gene delivery methods

For gene delivery, *Agrobacterium*- and biolistic-mediated methods are most widely used for plants. *Agrobacterium* strains have been engineered for enhanced plant transformation [26•]. *Agrobacterium*-mediated transformation efficiency has been substantially increased by using strains harboring additional booster plasmids, such as super-binary vectors [27] or ternary helper plasmids [15,28•,29], which carry extra copies of virulence genes. Recently, an innovative approach was developed by implementing *Pseudomonas* effector proteins to suppress plant immune response during *Agrobacterium* infection. Engineered *Agrobacterium* strains that transport individual effector proteins AvrPto, AvrPtoB, or HopAO1 into plant cells via a Type III Secretion System greatly enhanced wheat, alfalfa, and switchgrass transformation by about 250–400% [30•]. Similar approaches could be developed to further engineer *Agrobacterium* strains to

broaden their host range and enhance transformation efficiency.

Nonetheless, because *Agrobacterium* species include plant pathogens, transgenic or gene-edited plants produced via *Agrobacterium*-mediated transformation undergo more stringent regulations. In addition, due to the limited host range of *Agrobacterium* strains, plant species or genotypes of interest may not be efficiently transformed with currently available disarmed *Agrobacterium* strains. To overcome these limitations, other nonpathogenic bacteria have been tamed and engineered for plant transformation. Recently, *Ensifer adhaerens*, *Ochrobactrum haywardense*, and *Rhizobium etli* strains have been successfully used to transform several plant species [31•]. Especially, *O. haywardense* H1 strain has been engineered to efficiently transform soybean and it demonstrated higher transformation frequencies than other tested *Agrobacterium* strains AGL1 and LBA4404Thy- [32•]. It needs to be noted that these nonpathogenic bacteria still require *Agrobacterium* gene delivery machinery (i.e. the Type IV Secretion System encoded by the tumor-inducing plasmids) for plant transformation. As more and more bacterial genome sequences are available, comparative genomics studies may provide insight into novel bacterial species and gene delivery machinery with distinct or expanded host ranges [33,34].

Biolistic-mediated delivery utilizes high-pressure helium gas to accelerate heavy metal particles, such as gold, coated with different forms of GOI, such as DNA, mRNA, protein, or ribonucleoprotein (RNP). In principle, the biolistic-mediated method has not changed over the past decades. For more efficient transformation, however, the biolistic delivery method needs improvements for both delivery efficiency and consistency. One of the main problems associated with biolistic delivery was the huge shot-to-shot variations, which limit the use of biolistic delivery methods for quantitative comparative analyses required for gene-editing reagent optimization. Recently, Miller et al. used a double-barrel device to load two samples on the same macrocarrier and this approach allowed the inclusion of an internal standard [35]. Although the double-barrel device did not significantly improve gene delivery efficiency per se, the use of an internal standard drastically reduced the shot-to-shot variations enabling more robust comparative analyses, such as guide RNA efficiency testing [35].

For the plant species that can regenerate from protoplasts, chemicals such as polyethylene glycol is used to deliver either DNA or RNPs [36]. It is noteworthy to mention that alternative gene delivery methods have been developed using various nanoparticles [37], but these methods still suffer low transformation frequency and require substantial improvements before they can be practically used for plant transformation and gene editing.

Plant regeneration

The most challenging step in plant transformation would be the regeneration of transformed cells into whole plants. Plant cells have totipotency that refers to the ability to dedifferentiate and redifferentiate into different tissues, organs, or whole organisms [38]. Different strategies have been developed to enhance plant regeneration, including the exogenous application of plant hormones [39•,40•], wounding [39•,41], and ectopic expression of various MTFs [3•,19•,20] (Figure 1 in reference [3•] provides a good summary on how plant hormones, wounding, and MTFs affect plant regeneration). The most successful strategies involve the overexpression of one or more MTFs that can promote shoot regeneration or somatic embryogenesis. In the sections below, we will review these seminal studies and discuss how they can be adopted and optimized for different plant species or genotypes.

Selection reagents

Selectable marker genes provide efficient means to enrich transformed cells by providing tolerance to antibiotics or herbicides included in the culture media. Different selectable marker genes have been used for plant transformation [42], and efficient marker gene and selective reagent dosage should be tested and optimized to minimize escapes without compromising regeneration efficiency. For instance, Kang et al. recently reported that a widely used marker gene *bar* and selection reagent bialaphos was not efficient in a rapid maize transformation protocol, which skips a lengthy selection and callus proliferation step, resulting in a high rate of escape (66.7%) [15]. For such modified transformation protocols, other selectable marker genes such as *HIGHLY-RESISTANT ACETOLACTATE SYNTHASE* (*HRA*) and *neomycin phosphotransferase II* (*NPTII*) could be more efficient in reducing escape rates [8••,17•,18•].

Recent advances in morphogenic transcription factor-mediated plant transformation

The most robust and widely applicable strategies for genotype-flexible transformation would be MTF-assisted methods. Many individual MTFs have been tested for their ability to induce shoot or somatic embryogenesis on various plant species [3•,19•,20]. A short list of commonly used and tested MTFs is summarized in Table 1. In the sections below, however, we focus on five recent seminal studies that demonstrated a great potential for highly efficient and genotype-flexible transformation [8••–11••,48••].

Baby boom and Wuschel2

In 2016, scientists from Corteva Agrisciences demonstrated that ectopic coexpression of maize *Bbm* and *Wus2* genes promotes direct somatic embryogenesis and

Table 1

Morphogenic transcription factors used for plant transformation.

MTF	Plants	Mechanism	Abnormal phenotype
AGL15	*Arabidopsis* [43], cotton [44], and soybean [43]	Embryogenesis	Yes
BBM	*Arabidopsis* [45], cacao [46], canola [45], maize [8••], poplar [47], rice [48••], sweet pepper [49], and tobacco [50]	Oragnogenesis/ embryogenesis	Yes
BBM/WUS	Maize [4,8••,17,51,52], rice [8••], sorghum [8••,53,54], sugarcane [8••], switchgrass [24], and tef [25]	Embryogenesis	Yes
CUC1/2	*Arabidopsis* [55]	Organogenesis	Yes
ESR1/2	*Arabidopsis* [56,57]	Organogenesis	No
GRF4–GIF1	Lemon[9••], rice[9••], and wheat [9••]	Organogenesis/ embryogenesis	No
GRF5	Maize [10••], soybean [10••], sugar beet [10••], and sunflower [10••]	Organogenesis/ embryogenesis	No
IPT	Aspen[58], lettuce [59], and tobacco [59]	Organogenesis	No
IPT/WUS2	*Arabidopsis* [21••], *Nicotiana benthamiana* [21••], tobacco [21••], and tomato [21••]	Organogenesis	No
Kn1/NTH	Tobacco [60,61], orange[62], and lemon [62]	Organogenesis	Yes
MPΔ	*Arabidopsis* [63]	Organogenesis	Yes
PLT5	Bok choy [22••], Pei-Tsai [22••], pepper [22••], and tomato [22••]	Organogenesis	No
RKD4	Phalaenopsis (orchid) [64]	Embryogenesis	No
STM	*Arabidopsis* [65]	Embryogenesis	NA
STM/WUS2	Grape[21••], potato [21••], and tobacco [21••]	Organogenesis	No
WOX2 + 8 or 2 + 9	Tobacco[66]	Organogenesis	Yes
WOX5	Barley [11••], maize [11••], rye [11••], triticale [11••], and wheat [11••]	Embryogenesis	No
WUS	Coffee [67], cotton [68], maize [18•], poplar [48••], and tobacco [69]	Embryogenesis	Yes

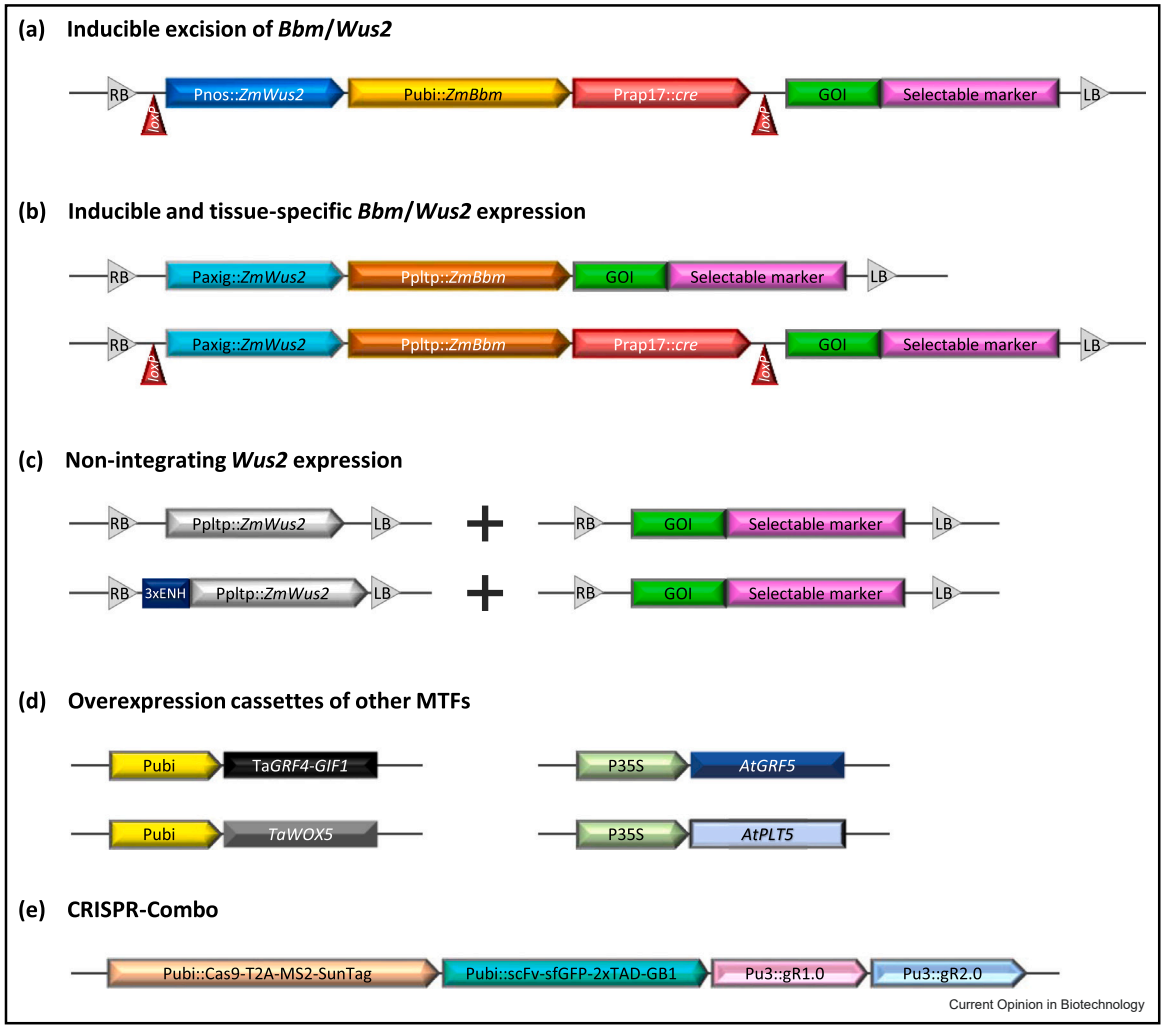
AGL15: AGAMOUS-LIKE15
CUC1/2: CUP-SHAPED COTYLEDON1/2
ESR1/2: ENHANCER OF SHOOT REGENERATION1/2
Kn1/NTH: Knotted1/Nicotiana tabacum homeobox (Knotted1-type class 1 homeobox protein)
MPΔ: Truncated version of MONOPTEROS, without the conserved C-terminal domains involved in Aux/IAA interaction
RKD4: RWP-RK domain-containing 4

greatly enhanced *Agrobacterium*-mediated transformation efficiencies of four monocot species: maize, sorghum, rice, and sugarcane [8••]. Recalcitrant genotypes were readily transformed using the constructs carrying maize *Bbm*/*Wus2* gene expression cassettes. Moreover, young maize leaf segments were transformed with an average of 45% frequency (based on embryogenic calli formation), showing the potential for wider applications [8••]. One major limitation of *Bbm*/*Wus2* is that their constitutive overexpression inhibits the regeneration and production of fertile plants. To overcome the pleiotropic deleterious effects of the constitutive overexpression of *Bbm* or *Wus2*, three different approaches have been developed. The first approach is to remove the *Bbm*/*Wus2* cassettes in the T-DNA of transformed embryogenic callus using an inducible promoter-driven *cre*/*loxP* system (Figure 2a) [4,8••,51]. The inducible excision method requires special treatment of the embryogenic callus tissues, such as heat [4] or desiccation [8••], to activate *cre* expression to mediate recombination between the two *loxP* sites flanking the *Bbm*, *Wus2*, and *cre* genes. Since transgenic events with multiple T-DNAs would suffer a lower excision rate, it is important to obtain high-frequency single-copy events and establish an efficient excision protocol to maximize the regeneration capacity acquired by *Bbm*/*Wus2*. The second approach uses inducible or tissue-specific expression of *Bbm*/*Wus2* (Figure 2b, top) [17•,52] with inducible excision afterward (Figure 2b, bottom) [25,53]. This approach using auxin-inducible (Paxig) and scutellum-specific (Ppltp) promoters does not require excision but would only be used for certain explants, such as immature embryos, in which both promoters have sufficient strength to drive *Bbm*/*Wus2* expression. The third approach uses a nonintegrating *Wus2* strategy (Figure 2c) [18•]. It uses two separate *Agrobacterium* strains: one carrying GOI and the other carrying the *Wus2* cassette with a strong constitutive promoter. WUS2 proteins either expressed from the nonintegrating T-DNA or diffused from neighboring cells trigger somatic embryogenesis [18•]. It is critical to find an optimal ratio of the two *Agrobacterium* strains to ensure sufficient *Wus2* expression without too much cointegration with GOI. This method, however, would not work for certain genotypes or explants that require both *Bbm* and *Wus2* for efficient regeneration [8••].

Growth-regulating factors

GRFs are small transcription factors that play critical roles in plant growth and development [70]. These

Figure 2



Schematic diagrams of MTF constructs. **(a)** Constitutive expression of maize *Wus2/Bbm* and inducible excision via *cre/loxP* recombination system [8••]. **(b)** Inducible (Paxig) and scutellum tissue-specific (Ppltp) expression of *Wus2/Bbm* without (top) or with (bottom) inducible excision [4,17•,52]. **(c)** Nonintegrating *Wus2* delivery using two T-DNAs [18•]. **(d)** Constitutive expression cassettes of MTFs without deleterious pleiotropic effects, wheat *GRF4–GIF1* chimera [9••], *Arabidopsis GRF5* [10••], wheat *WOX5* [11••], and *Arabidopsis PLT5* [22••]. **(e)** CRISPR-Combo cassettes for gene activation and editing [48••]. Pnos, nopaline synthase promoter; Pubi, ubiquitin promoter; Prap17, *rap17* gene promoter; GOI, gene of interest; RB and LB, right and left T-DNA borders; Paxig, maize Axig1 promoter; Ppltp, maize phospholipid transferase promoter; 3xENH, three caulimoviral enhancers from the Fig Wart Mosaic Virus, the Peanut Chlorotic Streak Virus, and the Mirabalis Mosaic Virus; P35S, CaMV 35S promoter; T2A, self-cleaving peptides; MS2-SunTag and 2xTAD activator form MCP-SunTag-2xTAD activator complex; ScFv, single-chain antibody fragment that binds the GCN4 motif present in the SunTag; sfGFP, superfold GFP; GB1, B1 domain of *Streptococcal* protein G; Pu3, U3 promoter; gR1.0, sgRNA with regular scaffold for gene editing; gR2.0 contains two MS2 RNA aptamers to recruit the MCP-SunTag-2xTAD activator.

plant-specific transcription factors have various numbers of paralogs in each plant species, typically 8–20 in each land plant genome. Multiple *GRFs* are regulated by microRNA miR396, and this plays a key role in the GRF-mediated growth and developmental regulation. GRFs form complexes with their cofactors called GRF-interacting factors to exert regulatory effects [70].

Debernardi and colleagues demonstrated that constitutive overexpression of wheat *GRF4* and a cofactor *GRF-INTERACTING FACTOR1* (*GIF1*) significantly enhanced wheat regeneration, but it was the GRF4–GIF1 fusion protein (Figure 2d) that demonstrated even more promising results in *Agrobacterium*-mediated transformation: transformation frequencies were increased by up to 7.8-fold in wheat, rice, and lemon [9••]. In the same study, GRF5–GIF1 chimera also enhanced wheat regeneration efficiency but GRF9–GIF1 or GRF1–GIF1 did not have a significant impact. It is noted that unlike *Bbm/Wus2*, overexpression

of *GRF4–GIF1* chimera did not have noticeable deleterious effects and the transgenic wheat plants were normal and fertile [9••]. Additional improvement was made by engineering the *GRF4* coding sequence: because *GRF4* is negatively regulated by miR396, Qiu and colleagues were able to further improve wheat regeneration and cytosine base editing efficiencies by 2–9-fold in 11 elite wheat cultivars by introducing a synonymous point mutation at the miR396 target site [71•]. Similar approaches can be applied to other GRFs to enhance the efficacy of these MTFs.

Arabidopsis GRF5 is one of the MTFs that can promote organogenesis in multiple dicot plant species (Figure 2d) [10••]. GRF5 also interacts with GIF1 and promotes cell division and leaf development. Scientists from the KWS research group showed that when overexpressed using strong constitutive promoters, *Arabidopsis GRF5* and its orthologs enhanced regeneration efficiencies in canola, soybean, sugar beet, sunflower, and maize [10••]. The application of *GRF5* seems, however, less straightforward. For instance, overexpression of *Arabidopsis GRF5* significantly improved transformation frequencies for recalcitrant sugar beet varieties, but sugar beet *GRF5* did not show significant enhancements [10••]. In addition, although *GRF5* overexpression did not show deleterious impacts in sugar beets and canola, some of the *GRF5*-overexpressing soybean shoots did not develop normal roots [10••]. Given the various number of *GRFs* in each genome and their functional diversifications [70], overexpression of *GRF5* in heterologous systems might require additional validation and optimization steps. As demonstrated by Debernardi et al., overexpressing the *GRF5–GIF1* fusion protein would be more efficient for plant regeneration and transformation [9••]. While *Arabidopsis GRF5* did not have a significant impact, maize *GRF5-LIKE* genes, which are orthologous to wheat *GRF1* [9••], significantly improved maize inbred A188 transformation efficiency [10••]. Because A188 is a transformable inbred without MTFs, it is yet to be tested if *GRF5-LIKE* genes can be useful for recalcitrant maize inbred lines such as B73 or other monocot species.

Wuschel-related homeobox 5

Ectopic overexpression of wheat *WOX5* (Figure 2d), a *WUSCHEL* family transcription factor [72], greatly improved regeneration efficiency and increased transformation frequencies for various monocot species and genotypes, including a total of 47 different varieties from wheat, barley, rye, triticale, and maize [11••]. In addition, unlike *Wus2*, overexpression of wheat *WOX5* did not inhibit shoot or root regeneration and the transgenic wheat plants had slightly wider leaves providing a screenable phenotype to identify *WOX5*-expressing plants [11••]. Because *WOX5* enabled the transformation of multiple recalcitrant genotypes of wheat, barley, and maize, wheat *WOX5* has great potential to be used for the genotype-flexible transformation of many other monocot species.

CRISPR-Combo

Clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated protein9 (Cas9) represents the most powerful and efficient gene-editing tool developed thus far. Pan and his colleagues recently developed a versatile system called CRISPR-Combo (Figure 2e) that simultaneously activates endogenous genes and edits target genes [48••]. In *Arabidopsis*, CRISPR-Combo-mediated activation of a florigen gene *FT* induced early flowering, providing an efficient method to accelerate breeding time and enrich transgenic plants. In poplar, activation of endogenous *Wus* not only reduced the time to generate rooted plants by half, but also doubled the rooting rate to nearly 100% [48••]. In addition, target gene-editing efficiency was 100% with more than 75% carrying homozygous mutations. In rice, activation of the rice *Bbm1* gene boosted regeneration without exogenous application of hormones in the culture media. Edited plants were enriched on the hormone-free medium, indicating that CRISPR-Combo-mediated *Bbm1* activation can not only boost transformation frequency but also improve targeted gene-editing efficiency [48••]. Although CRISPR-Combo has not been tested for the transformation of recalcitrant species or genotypes, its flexibility and multiplexing capability would allow to evaluate different combinations of endogenous MTFs to develop species- and genotype-optimized gRNA designs.

Tissue culture-free transformation

Tissue culture-free transformation or *in planta* transformation provides a less genotype-dependent option, but *in planta* transformation methods have been limited to *Arabidopsis* and a few species in the Brassicaceae family [73]. Two recent studies tested various MTFs to promote shoot regeneration in dicot plants [21••,22••]. By injecting *Agrobacterium* suspension onto the wound sites in dicot plants, Maher et al. discovered that a combination of maize *Wus2* and *Arabidopsis SHOOT MERISTEMLESS* (*STM*) or a cytokinin biosynthesis gene *isopentenyl transferase* (*IPT*) induced shoot apical meristems and transgenic shoots were produced on *Nicotiana benthamiana*, tomato, grape, and potato [21••]. Using similar approaches, Lian et al. tested more MTFs and found that *Arabidopsis PLETHORA5* (*PLT5*) can promote *in planta* transformation efficiencies on snapdragons (*Antirrhinum majus*) and tomato plants, likely via activation of auxin and cytokinin biosynthesis [22••]. In addition, although *in planta* transformation was not successful, overexpression of *PLT5* also allowed tissue culture-based transformation of recalcitrant Bok choi and sweet pepper (*Capsicum annuum*), further demonstrating the applicability of *PLT5* for dicot transformation [22••].

Tissue culture-free methods hold invaluable options for vegetatively propagated crops, such as potato and sweet potato, and further optimization of MTF-assisted transformation methods using transient expression systems would be highly desirable.

Future perspectives and conclusions

Plant transformation is an important tool for functional genomics and crop trait improvements. Recent advances in CRISPR technology and MTF-assisted transformation methods demonstrated great potential in overcoming one of the major bottlenecks in plant biotechnology, that is, genotype dependence in genetic transformation. Additionally, improving gene delivery efficiency using engineered *Agrobacterium* or novel nonpathogenic bacterial strains can enhance transformation capacity. As reviewed, multiple MTFs have promising impacts on promoting organogenesis or direct somatic embryogenesis in multiple monocot and dicot species. Clearly, however, there is unlikely a single MTF or MTF combos that can enable the universal transformation of all recalcitrant species or genotypes. There are still genotype-dependent variations in response to different MTFs.

How can we further expand transformable species and genotypes? First, novel MTFs can be screened for their capacity in improving regeneration efficiencies. This process would be accelerated by developing high-throughput assay systems. Second, existing MTF technologies should be further harnessed by optimizing the expression cassettes and combining synergistic MTFs. As exemplified in the *Bbm*/*Wus2* technologies for monocot transformation, combining synergistic MTFs with optimized expression cassettes can not only enhance transformation frequencies but also significantly expand the transformable genotypes and species [8••,17•,18•]. The efficiency of GRFs can be further enhanced by producing fusion protein with a cofactor [9••] and abolishing the negative regulation by miR396 via synonymous substitution at the target site [71•]. In addition, expanding the use of the nonintegrating *Wus2* strategy [18•] would be useful for gene-editing applications because it does not require excision of the *Wus2* cassette for regeneration, and thus can be readily combined with existing genome-editing vector constructs. Third, other regulatory pathways influencing plant regeneration competence can be explored. For instance, it is well known that regeneration competence decreases as plants get older and this is mediated by miR156 [13••]. The level of miR156 decreases as plants mature, leading to increased expression of its target gene *SQUAMOSA PROMOTER BINDING-LIKE9*, a negative regulator of the type-B *Arabidopsis Response Regulator* (*ARR*) genes. Therefore, overexpression of miR156 might enhance shoot regeneration competence by enhancing responsiveness to cytokinin via elevated expression of type-B *ARR* genes [74]. Fourth, epigenetic-modifying agents, such as auxin [12,13••] and 5-Azacytidine [75], can be applied to the plant transformation protocols to further enhance regeneration efficiencies. A high dose of auxin treatment can modify the chromatin status and may promote regeneration competence [12], whereas the DNA methylation inhibitor 5-Azacytidine can reduce the transgene-silencing to enhance transformation frequencies [75]. Lastly, single-cell multi-omics studies [76•] could provide useful insights into what transcriptomic and epigenetic modifications occur in the explant tissues over time or after the induction of various MTFs that enhance regeneration and transformation. Single-cell RNA-seq studies may identify key regulatory pathways or novel molecular markers for regeneration-competent cells, providing new tools to strategically design MTF expression cassettes or generate a high-throughput screening platform.

In conclusion, the strategies for enhancing plant genetic transformation efficiency are moving beyond the mere optimization of culture media composition and growth conditions. With the powerful tools of genomics, bioinformatics, and CRISPR technology, now is the time than ever, to investigate and understand the mechanisms of cell totipotency, pluripotency acquisition, and regeneration, as well as cell's ability to acquire and manage incoming genetic molecules such as nucleic acids and proteins.

CRediT authorship contribution statement

KL and KW prepared the figures and the table and wrote the manuscript.

Conflict of interest statement

Nothing declared.

Data Availability

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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