**Genotype-independent plant transformation**

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**Abstract**

Plant transformation and regeneration remain highly species- and genotype-dependent. Conventional hormone-based plant regeneration via somatic embryogenesis or organogenesis is tedious, time-consuming, and requires specialized skills and experience. Over the last 40 years, significant advances have been made to elucidate the molecular mechanisms underlying embryogenesis and organogenesis. These pioneering studies have led to a better understanding of the key steps and factors involved in plant regeneration, resulting in the identification of crucial growth and developmental regulatory genes that can dramatically improve regeneration efficiency, shorten transformation time, and make transformation of recalcitrant genotypes possible. Co-opting these regulatory genes offers great potential to develop innovative genotype-independent genetic transformation methods for various plant species, including specialty crops. Further developing these approaches has the potential to result in plant transformation without the use of hormones, antibiotics, selectable marker genes, or tissue culture. As an enabling technology, the use of these regulatory genes has great potential to provide the application of advanced breeding technologies such as genetic engineering and gene editing for crop improvement in transformation-recalcitrant crops and cultivars. This review will discuss the recent advances in the use of regulatory genes in plant transformation and regeneration, and their potential to facilitate genotype-independent plant transformation and regeneration.

**Introduction**

Plant transformation and regeneration are highly species- and genotype-dependent and are often the principal bottlenecks in applying genetic engineering and gene editing for crop trait improvement [1–4]. Plant transformation starts with delivering genes of interest into single regeneration-competent or embryogenic stem cells, typically achieved through Agrobacterium-mediated or biolistics-based methods. In vitro plant regeneration is a process of generating a whole plant from a single cell derived from various explants such as leaf, cotyledon, hypocotyl, root, microspore, and immature embryo, and usually involves the formation of callus from explants cultured on a callus-inducing medium (CIM). Callus is a highly heterogeneous group of cells with organized structures similar to lateral root primordia [5], most of which are regeneration-incompetent cells with a limited number of cells capable of proliferating. The foundation of plant regeneration lies in the totipotency of plant cells, which is the ability of a somatic or meristematic cell to regenerate into an entire plant [6]. The successful regeneration of a singular transformed cell into a fully functioning plant dictates the success of plant transformation.

Regeneration-competent cells can originate from the proliferation of pre-existing undifferentiated meristematic cells within explants that will go through direct organogenesis to develop into plantlets. This process permits the co-cultivation of explants with *Agrobacterium* for the delivery of the genes of interest into the regeneration-competent cells, e.g. the *Agrobacterium*-mediated transformation of cotyledonaly nodal regions of soybean where axillary meristem is located. In many plant species, however, regeneration-competent cells originate from reprogrammed differentiated somatic cells via a dedifferentiation process to regain the capacity for proliferation competence or pluripotency, i.e. the ability of plant embryogenic or stem cells to develop into all shoot and root cell types [7]. Acquisition of pluripotency converts somatic cells to regeneration-competent cells for callus induction. Thus, *Agrobacterium*-mediated gene delivery can be performed at different stages from isolated explants such as leaf discs or immature embryos to the resulting callus.

Through somatic embryogenesis and *de novo* organogenesis, transformed regeneration-competent cells develop into somatic embryos and shoot/root apical meristems (SAMs/RAMs), respectively, which develop
Figure 1. Key steps and factors in exogenous hormone-induced plant regeneration. Aerial explants go through the sequential steps of elimination of leaf identity, establishment of root identity, establishment of shoot identity, followed by organogenesis (step 1) or somatic embryogenesis (steps 2 and 3). Wounding, epigenetic modifications, growth regulatory genes, and developmental regulatory genes are the four classes of crucial regeneration-promoting factors. Boxed arrows, key steps. Dark red, regeneration-promoting factors. Blue, key genes for each factor. Green, plant growth hormones. Orange circle, somatic embryo.

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into plantlets [6–12] (Figure 1). Somatic embryogenesis starts with the formation of an embryo-like structure from embryogenic cells like those in calli that further develops into a whole plant. Organogenesis may result in the formation of shoots and roots directly from explants (i.e. direct organogenesis) or start with the formation of SAM development from callus, and then the SAM develops into shoots that subsequently generate roots (i.e. indirect organogenesis) [13].

Since various factors affect callus formation and plant regeneration, conventional plant transformation requires optimizing many external factors including explant types, plant growth regulators (mainly auxin and cytokinin that are natural or synthetic chemicals exogenously applied to modify plant growth), basal media composition, pH, light conditions, and transgenic plant selection strategies. However, recent advances in the understanding and identification of plant growth and developmental regulatory genes (also called morphogenic genes) have revealed that many of these genes are involved in the regulation of biosynthesis and signaling pathways of auxin and cytokinin and thus control the plant regeneration process [2–5,10,12,14] (Figure 1; Table 1). Plant growth regulatory genes are involved in the biosynthesis, perception and transduction of plant growth hormones that are endogenously biosynthesized within plants and regulate plant growth. Plant developmental regulatory genes are the transcriptional factors or signaling molecules that control cell fate and thus regulate plant development (e.g. organ formation) by regulating the expression of various genes. The use of growth and developmental regulatory genes could significantly improve regeneration/transformation efficiency, speed up the transformation process, and enhance the application of gene editing in various crops [15–18] (Table 1). This review will discuss: i) recent advances in the identification of the functions of growth and developmental regulatory genes and their use in plant transformation; ii) the potential of these advances to create novel approaches for genotype-independent plant transformation and regeneration; and iii) how these advances can be harnessed to provide a better understanding of plant transformation from the molecular and developmental biology perspectives.

Key steps and factors in CIM-induced plant regeneration

Exogenous hormone-induced regeneration from aerial explants starts with the elimination of leaf identity in above-ground or aerial explants [19, 20] and then root and shoot identities [21, 22] needs to be re-established in both aerial and root explants, followed by de novo plant regeneration via somatic embryogenesis or organogenesis (Figure 1). The elimination of leaf identity is mainly achieved via epigenetic changes (see below). The establishment of lateral root identity occurs in pericycle-like cells of aerial explants or xylem-pole pericycle cells of root explants, permitting the middle cell layer of the pericycle-like cells to obtain pluripotency and develop into pericycle founder cells for callus induction [14, 22]. This includes the activation of expression of ABERRANT LATERAL ROOT FORMATION 4 (ALF4), the gene required for the first asymmetric division of pericycle cells during lateral root initiation, and RAM developmental regulatory genes such as PLETHORA 1 (PLT1) and PLT2, WUSCHEL-
### Table 1. Summary of plant growth and developmental regulatory genes studied in plant transgenic research

| Gene Cassette | Transformed Species | Explant | Hormone | Regeneration<sup>a</sup> | Regenerated Plant | Transform. Efficiency | Abnormal Phenotype | Ref |
|---------------|---------------------|---------|---------|--------------------------|-------------------|---------------------|-------------------|-----|
| 35S:AtWUS     | Gossypium hirsutum  | Hypocotyl| 2,4-D, kinetin | Embryogenesis | × | N.A. | ✓ | [23] |
| P<sub>G10-90·XVE-O<sub>LexA</sub>-min35S:AtWUS | Nicotiana tabacum | Leaf | BAP, NAA | Organogenesis | ✓ | N.A. | ✓ | [24,25] |
| P<sub>G10-90·XVE-O<sub>LexA</sub>-min35S:AtWOX5 or 2/8 or 2/9 | Coffea canephora | Leaf | BAP, IAA | Embryogenesis | ✓ | N.A. | ✓ | [26,27] |
| 35S:AtK14·AtR KD4-GR | Coffea canephora | Leaf | BAP, IAA | Organogenesis | ✓ | N.A. | ✓ | [28] |
| 35S:AtBBM; 35S:BnBBM | Nicotiana tabacum | Leaf | IAA | Organogenesis | ✓ | N.A. | ✓ | [29] |
| 35S:BnBBM; HaUbi:BnBBM | Brassica napus | Microspore | N.A. | Embryogenesis | × | N.A. | ✓ | [30] |
| 35S:TcBBM-GR | Theobroma cacao | Cotyledonb | 2,4-D, TDZ, kinetin | Embryogenesis | × | N.A. | ✓ | [31] |
| 35S:AtHSP18.2:FLP-35S:BcBBM | Populus tomentosa | Leaf | 2,4-D, BAP | Embryogenesis | ✓ | N.A. | × | [32] |
| 35S:PaHAP3A; PG10·XVE-OLexA·min35S:PaHAP3A | Picea abies | Embryonic cell lines | N.A. | Embryogenesis | × | N.A. | ✓ | [33] |
| 35S:AtCUC1 or 2 | Arabidopsis thaliana | Seedling | IBA, IAA, 2,4-D, kinetin | Embryogenesis | ✓ | N.A. | ✓ | [34] |
| 2 × 3SS:AtGRF5; 2 × 3SS:BuGRF5-L | Beta vulgaris | Cotyledon, hypocotyl | BAP, NAA | Organogenesis | ✓ | N.A. | ✓ | [35] |

<sup>a</sup>Regeneration refers to the ability of the gene cassette to promote regeneration of the plant from the transgenic tissue.

<sup>b</sup>Cotyledon.
Table 1. Continued

| Gene Cassette | Transformed Species | Explant | Hormone | Regeneration | Regenerated Plant | Transform. Efficiency | Abnormal Phenotype | Ref |
|---------------|---------------------|---------|---------|--------------|--------------------|----------------------|-------------------|-----|
| 2 × 35S:AgGRF5; 2 × 35S:HaGRF5-L | Helianthus annuus | Cotyledon | BAP, NAA | Organogenesis | ✓ | ✓ | x | [55] |
| PeUbi4-2: GmGRF5-L | G. max | Primary node | IAA, kinetin, IBA, zeatin | Organogenesis | ✓ | ✓ | x | [55] |
| PeUbi4-2: BnGRF5-L | B. napus | Hypocotyl | 2,4-D, zeatin, kinetin | Organogenesis | ✓ | ✓ | x | [55] |
| B:nEF1 A:GRF5; B:nEF1 ZmGRF5- L1/2 | Z. mays | Immature embryo | 2,4-D, zeatin, IBA, BAP | Organogenesis | ✓ | ✓ | x | [55] |
| ZmUbi-GRF4-GIF1 | Triticum aestivum | Immature embryo | 2,4-D, zeatin | Organogenesis | ✓ | ✓ | x | [55] |
| O. sativa | Seed | 2,4-D, BAP, NAA | Organogenesis | ✓ | ✓ | x | [55] |
| C. lemon | Etiolated epicotyl | BAP, NAA, BA | Organogenesis | ✓ | ✓ | x | [55] |
| 35S:ipt in A: | N. tabacum | Leaf | – | Organogenesis | ✓ | N.A. | x | [55] |
| P. sieboldii × P. grandi-entata | P. tabacum | Stem | IBA | Organogenesis | ✓ | N.A. | x | [55] |
| 35S:GVG - 6 × UAS | N. tabacum; Lactuca sativa | Leaf | BAP, NAA | Organogenesis | ✓ | ✓ | x | [55] |
| -min35SS:pt | Cotyledon | NAA | Organogenesis | ✓ | ✓ | x | [55] |

Non-tissue culture – based plant transformation

| Nos ZmWUS2 -ZmUbi:ipt | A. thaliana, N. tabacum, Solanum lycopersicum | Agro transient | – | Organogenesis | ✓ | N.A. | x | [55] |
| Nos ZmWUS2 -ZmUbi:A:STM | S. tuberosum; N. tabacum; Vitis vinifera | Mature plant | – | Organogenesis | ✓ | N.A. | x | [55] |

Embryogenesis, somatic embryogenesis.
Staminodes were used as the explants to induce somatic embryos, and cotyledons from the somatic embryos were used for plant transformation.
Antibiotic selectable marker-free. ✓, yes. x, no.

RELATED HOMEBOX5 (WOX5), SHORT-ROOT (SHR), and SCARECROW (SCR) [14]. Kareem et al. [21] found that transcription factors PLT3, PLT5, and PLT7 activate the expression of PLT1 and PLT2 to establish pluripotency. Moreover, PLT3, PLT5, and PLT7 activate the expression of the shoot-promoting factor CUP-SHAPED COTYLEDON2 (CUC2) via PLT-mediated upregulation of auxin biosynthesis genes YUCCA1 (YUC1) and YUC4, leading to shoot regeneration [60].

There are many genetic and environmental factors that can affect plant regeneration; among them, these four types of regeneration-promoting factors play essential roles: wounding, epigenetic modifications, growth regulatory genes, and developmental regulatory genes (Figure 1). By inducing a series of physical and chemical changes in the detached explants, wounding is the primary external trigger of callus induction from explants [5, 14]. These changes start with the perception of damage-associated molecular patterns such as extracellular ATP [61, 62] and cell wall-derived oligogalacturonic acid [63], triggering cytoplasmic calcium signaling and a burst of reactive oxygen species [61, 62]. These local wounding signals are translated into long-distance signals such as the electrical signal of cation channel GLUTAMATE RECEPTOR-LIKEs, inducing epigenetic modifications, alternations in the synthesis and accumulation of cytokinin and free auxin, and transcriptional upregulation of growth and development regulatory genes [10, 64] (Figure 1). Transcriptional changes include the activation of expression of callus-inductive chromatin remodeling regulator genes POLYCOMB REPRESSIVE COMPLEX2 (PRC2; see below), cell cycle genes CYCLINs (CYCs) and CYCLIN-DEPENDENT KINASES (CDKs), cytokinin biosynthesis gene ISOPENTENYL TRANSFERASE (IPT), auxin biosynthesis gene YUC5, and AP2/ERF transcription factors WOUND-INDUCED DEdifferentiation1 ~ 4 (WIND1 ~ 4) and ENHANCER OF SHOOT REGENERATION1 (ESR1) [64, 65]. Among these, activation of expression of CYCs and CDKs triggers cells to reenter the cell cycle and reacquire cell proliferative competence, a central mechanism of callus induction [66], whereas WINDs promote callus induction by directly binding to the promoter of ESR1 and upregulating its expression [67].

Epigenetic modifications of chromatin structure cause genome-wide changes in gene expression required for callus induction [11, 19, 20, 68]. This global reprogramming of epigenetic modifications includes changes in genome-wide DNA methylation (especially gene promoter DNA methylation), histone modifications in transcription start sites (TSSs) and other gene parts, and deposition of histone variants. For example,
He et al. [19] discovered that genome-wide reprogramming of histone H3 lysine 27 trimethylation (H3K27me3) is critical in the leaf-to-callus transition since the Arabidopsis thaliana prc2 (a key gene in establishing H3K27me3) mutants were defective in callus formation. The PRC2-mediated global epigenetic changes are directly involved in the elimination of the leaf identity in aerial explants by silencing leaf-regulatory genes while removing the repressive methyl marks on auxin pathway genes YUC4 and AUXIN/INDOLE-3-ACETIC ACID2 (AUX/IAA2) and root-regulatory genes WOX5 and SHR [19]. PRC2-dependent repressive histone modifications also control expression of wound-responsive WND genes [69], which promote callus induction by activating the cytokinin biosynthesis pathway via the upregulation of type-B ARABIDOPSIS RESPONSE MODIFIER (ARR-B) gene expression [70]. Lee et al. [20] found that AUXIN RESPONSE FACTOR7/9 (ARF7/9) and JUMONJI C DOMAIN-CONTAINING PROTEIN 30 (JMJ30) form the ARF-JMJ30 complexes to remove the methyl groups from H3K9me3 at lateral root identity. Lee et al. [71] revealed that the expression of chromatin modifier ARABIDOPSIS TRITHORAX4 (ATX4) is repressed during callus induction to eliminate leaf identity but is reactivated to facilitate shoot identity establishment by removing the methyl groups from H3K4me3 at shoot identity genes such as KNOTTED-LIKE HOMEobox GENE 4 (KNAT4) and YABBY 5 (YAB5). In addition, epigenetic reprogramming also provides local changes in the epigenetic states of key genes involved in callus induction and plant regeneration. These key genes include WIND3, BABY BOOM (BBM), LEAFY COTYLEDON1 (LEC1) and LEC2, and WOX5/11 genes [11]. All of these epigenetic modifications prepare explants for callus induction and are involved in all of the steps of callus induction and plant regeneration (Figure 1).

Cytokins and auxins are critical for and routinely used in callus induction and plant regeneration. A balanced cytokinin and auxin ratio promotes callus induction, while high and low cytokinin-to-auxin ratios tend to induce shoot and root formation, respectively [72]. For example, endogenous auxin production and enhanced cytokinin sensitivity promote pluripotency acquisition in the middle cell layer of pericycle cells for organ regeneration [22]. In the middle cell layer of pericycle cells, endogenous auxin production increases through induced expression of Tryptophan Mono-Transferase of Arabidopsis (TAA1) by WOX5, PLT1 and PLT2, while cytokinin sensitivity increases through repression of ARRs-A by WOX5 and type-B ARR12. Hu et al. [73] also found that elevated endogenous auxin levels in the basal end of citrus epicotyl cuttings inhibit in vitro shoot organogenesis in a cytokinin-dependent manner. Similarly, genetic components of the biosynthesis and signaling pathways of cytokinin and auxin regulate callus formation and plant regeneration [54, 74–77]. For example, cytokinin induces expression of the D-type cyclin CYCD3 gene, whose overexpression induces callus formation in the absence of cytokinin [78]. Cytokinin also induces expression of the transcription factor gene SHOOT MERISTEMLESS (STM), whose protein maintains cell division and inhibits cell differentiation in SAM and enhances cytokinin levels via activation of IPT7 [79]. The overexpressed A. tumefaciens IPT gene in transgenic tobacco and cucumber induces cytokinin biosynthesis, resulting in the promotion of shoot organogenesis [77]. In addition, YUC-mediated auxin biosynthesis increases the total auxin level in leaf explants, and when polar auxin transport delivers auxin to regeneration-competent cells, it triggers WOX11 and WOX12 expression [74, 80]. WOX11 and WOX12 directly bind to the promoters of WOX5 and WOX7 and induce their expression for rapid root primordia initiation and root identity establishment [74, 80]. Auxin also inhibits the expression of type-A ARR7 and ARR15 via the auxin response transcription factor MONOPTEROS (MP) [81]. ARR-As negatively regulate ARR-Bs expression and thus repress cytokinin-mediated signaling [82].

Plant developmental regulatory genes also play key roles in callus formation and plant regeneration, as these processes are orchestrated by the sequential and spatiotemporal expression of various developmental regulatory genes driving morphogenesis [2–4, 83]. The most well-known developmental regulatory gene is WUSCHEL (WUS), the first gene identified in the WOX gene family and the essential player in both organogenesis [84] and embryogenesis [85]. As a homeodomain transcription factor, WUS is synthesized in the organizing center (OC) of the SAMs and migrates into the central zone (CZ) where it activates CLAVATA3 (CLV3) transcription, which in turn inhibits WUS expression in the OC [86, 87] (Figure 2). The WUS-CLV3 negative feedback circuit regulates cell identity and maintains the existence of the OC and the shoot stem cell niche in the CZ (Figure 2). Su et al. [88] revealed that the activation of WUS transcription by auxin gradients results in the induction of embryogenic callus and somatic embryogenesis in Arabidopsis explants under in vitro culture conditions. Zhang et al. [89] demonstrated that the activation of WUS expression in Arabidopsis explants by the ARR-Bs/HD-ZIP III transcription factor complex in a cytokinin 2-isopentenyladenine (2-IP)-rich environment promotes organogenesis and shoot regeneration. As a result, both auxin and cytokinin activate WUS expression (Figure 1). In addition, various developmental transcription factor genes are involved in organogenesis and/or embryogenesis. These include STM [84,90], MP/ARF5 [91,92], LEC1/2 [93,94], CUC1 [90], ABSCISIC ACID-SENSITIVE3 (ABI3) [94, 95], FUSSA3 (FUSS3) [94, 96], AINTEGUMENTA (ANT) [97], WOX12 [80], and CYCD3 [98].

These regeneration-promoting factors work together to control each step of callus formation and plant regeneration tightly and precisely (Figure 1). However, how
et al. [100, 102] reported that exogenous auxin-induced signaling pathway [32, 100, 101] (Figure 2). Khanday et al. [100, 102] reported that exogenous auxin-induced signaling pathway, which is another developmental regulatory gene involved in somatic embryogenesis and organogenesis via the auxin signaling pathway (ELEMENTBINDINGFACTOR) for the maintenance of the stem cell niche in SAMs, as the most important developmental regulatory gene in plant transformation.

As the most important developmental regulatory gene for the maintenance of the stem cell niche in SAMs, WUS has been used for plant transformation and regeneration in multiple species. Ectopic or estradiol-inducible expression of the A. thaliana WUS (AtWUS) gene (Figure 3A) in transgenic Arabidopsis, tobacco, Gossypium hirsutum, and Coffea canephora induced vegetative-to-embryogenic transition in vegetative tissues, which could differentiate into somatic embryos [23, 24, 26, 85] or organs [84]. However, the resulting transgenic plants exhibited abnormal phenotypes such as coiled root tips, cotton-like root structures, swollen hypocotyls, and distorted leaves [24, 26]. These abnormalities indicate WUS expression needs to be under a tight control since both cytokinin and auxin signaling pathways regulate its expression and its continuous overexpression causes malformations and alterations in transgenic plant growth. WOX genes such as AtWOX2/5/8/9 have also been used individually for tobacco transformation in estradiol-inducible systems, resulting in transgenic plants with abnormal phenotypes such as dwarf plants or bulbous roots [28]. Since CLV1 works as the receptor for the secreted ligand CLV3 that negatively regulates WUS expression in the OC (Figure 2), RNAi-mediated silencing of the Brassica napus CLV1 led to an increase in genetic transformation efficiency in transgenic B. napus and triggered a bushy phenotype [99]. Therefore, strategies are needed to fine-tune WUS/WOXs and CLV1 expression to obtain normal transgenic plant regeneration (see below).

BBM, an APETALA2 (AP2)/ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR (AP2/ERF) transcription factor, is another developmental regulatory gene involved in somatic embryogenesis and organogenesis via the auxin signaling pathway [32, 100, 101] (Figure 2). Khanday et al. [100, 102] reported that exogenous auxin-induced somatic embryogenesis in rice requires the presence of functional rice BBM (OsBBM) genes and the overexpression of OsBBM1 promotes somatic embryogenesis without the use of exogenous auxins, suggesting the OsBBM overexpression increases endogenous auxin production. Moreover, overexpression of BBM genes activates somatic embryo formation and/or regeneration in transgenic tobacco [30], B. napus [32], and Theobroma cacao [33]. However, like the side effects of WUS/WOXs overexpression, plants overexpressing BBMs exhibit pleiotropic phenotypes such as mild-to-severe alterations in leaf and flower morphology.

A dexamethasone (Dex)-inducible expression system has also been used to drive the expression of BBM genes, which were fused in-frame with the hormone-binding domain of the rat glucocorticoid receptor (GR) gene [30, 35]. This resulted in transgenic tobacco [30] and T. cacao [35] with normal phenotypes, but caused thickened roots, pronounced apical hooks, and swollen cotyledons in transgenic Capsicum annuum [36]. Moreover, Deng et al. [37] used an inducible excision system (Figure 3B) to control the B. campestris BBM (BcBBM) overexpression in the calli of Chinese white poplar (Populus tomentosa). The site-directed recombination system containing the recombinase flipase and flipase recognition target (FRT) sites (FLP/FRT) from yeast (Saccharomyces cerevisiae) was under the control of the Arabidopsis heat shock-inducible promoter AThSP18.2. Heat shock treatment on the transgenic stem cuttings caused the removal of the AThSP18.2:FLP and 3SS:BcBBM cassette (Figure 3B) and produced transgenic plants with normal phenotypes.

It was expected that high expression of BBM induces somatic embryogenesis while low expression of BBM promotes organogenesis as reduced cell differentiation was observed in low expression lines in transgenic Arabidopsis [94].

The upstream and downstream genes in the BBM-regulated somatic embryogenesis developmental pathway have also been examined for their roles in plant transformation. SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 (SERK1), a leucine-rich repeat receptor-like kinase (LRR-RLK) gene, regulates somatic embryogenesis by early activation of auxin biosynthesis, leading to the activation of expression of WUS, BBM, and the MADS-box transcription factor AGAMOUS-LIKE15 (AGL15) as well as to the repression of expression of LEC1 [41]. CcSERK1 has been used in C. canephora transformation, but attempts to generate transgenic plants were unsuccessful [41]. Transcription factors LEC1 and LEC2, two downstream proteins in the BBM-regulated somatic embryogenesis development pathway [94], work redundantly with LEC1-LIKE (L1L) and the B3 domain proteins ABI3 and FUS3 in embryogenesis [103]. LEC1 and LEC2 have been used for transformation of Picea abies [42], T. cacao [35], and tobacco [24], and the estradiol-inducible expression of AtLEC2 resulted in the regeneration of transgenic tobacco plants with curved root tips [24]. Since LEC2 directly activates the expression of AGL15, Thakare et al.
Figure 3. Structures and mechanisms of the expression vectors that utilized growth and developmental regulatory genes in plant transformation. (A) Estradiol-inducible AtWUS expression for transformation of Coffea canephora [15]. The XVE fusion gene driven by the constitutive G10–90 promoter contains the DNA-binding domain of the bacterial repressor LexA, the activation domain of the herpes viral protein VP16, and the carboxyl region of the human estrogen receptor. The binding of the estrogen hormone to the estrogen receptor in XVE enables XVE to bind to OLexA, the eight copies of the LexA operator sequence, leading to expression of AtWUS driven by OLexA and a minimal 35S promoter. (B) A heat shock inducible-excision system to control BcBBM expression in transgenic Chinese white poplar [37]. Heat shock treatment on the stem cuttings of transgenic Chinese white poplar activates expression of the yeast FLP recombinase that is driven by the heat shock-inducible promoter ATHSP18.2, leading to the removal of the ATHSP18.2:FLP and 3SS:BcBBM cassette with one footprint (a single FRT recombination site) left in the transgenic genome. (C) Low expression of ZmWUS2 under the control of the weak Agrobacterium nopaline synthase promoter (Nos:ZmWUS2) and high expression of ZmBBM driven by the strong maize Ubiquitin promoter (ZmUbi:ZmBBM) for transformation of maize and sorghum [15]. Desiccation of the embryogenic calli activates the expression of the CRE recombinase driven by the desiscation-inducible ZmRab17 promoter, leading to the removal of Nos:ZmWUS2, ZmUbi:ZmBBM and ZmRab17-CRE, with one footprint (a single LoxP recombination site) left in the transgenic genome. (D) Conditional expression of the 3SS promoter and ZmBBM by the auxin-inducible promoter ZmAux1 and the maize embryo/leaf-specific promoter ZmPLTP, respectively, for maize transformation [38]. (E) A selectable marker-free transformation system in tobacco and hybrid aspen [57]. The 3SS:IPT-containing Ac transposase gene can automatically jump out of the chromosome, leaving no footprint in the transgenic genome. (G) Dexamethasone (DEX)-inducible IPT expression for transformation of tomato and lettuce [58]. The GVG fusion gene driven by the 3SS promoter contains the DNA-binding domain of the yeast transcription factor GAL4, the activation domain of VP16, and the hormone-binding domain of the rat Glucocorticoid Receptor (GR). Exogenous application of the synthetic glucocorticoid DEX releases GVG into the nucleus, where it binds to the 6× UPS binding sites of GAL4 and activates the expression of AtWUS driven by 6× UPS and a minimal 3SS promoter. (H) Low expression of ZmWUS2 (Nos:ZmWUS2) plus high expression of the Agrobacterium IPT (ZmUbi:IPT) for organogenesis in the seedling leaves of Arabidopsis, tobacco, and tomato, and in the mature plants of tobacco, potato and grape [59].

A recent groundbreaking study was published for monocot transformation through somatic embryogenesis by fine-tuning the expression of WUS and BBM [15]. Low expression of the maize WUS2 gene by the Agrobacterium nopaline synthase promoter (Nos:ZmWUS2), a weak promoter for monocots, and high expression of the maize BBM by the strong maize Ubiquitin promoter (ZmUbi:ZmBBM) induced somatic embryogenesis and regeneration of fertile transgenic plants in immature embryos and/or callus of maize, sorghum, sugarcane and rice (Figure 3C). This approach significantly increased callus transformation efficiency, shortened regeneration time, and made various non-transformable genotypes transformable [15, 16]. For example, combined expression of Nos:ZmWUS2 and ZmUbi:ZmBBM dramatically increased transformation efficiency from 0.0–2.0% to 25.3–51.7% in four transformation-recalcitrant maize inbred lines and made another 33 out of 50 commercially important Pioneer maize inbred lines transformable [15]. However, the continuous expression of both regulatory
genes caused aberrant phenotypes such as stunted, twisted, sterile plants with thick, short roots. Lowe et al. [15] designed an inducible excision strategy to remove Nos:ZmWUS2 and ZmUbi:ZmBBM in the transformed embryogenic calli by using the tyrosine recombinase CRE from the P1 bacteriophage and its recognition site LoxP (Figure 3D). Drying the embryogenic calli on filter paper for three days activated CRE expression driven by the desiccation-inducible maize promoter ZmRab17, leading to the removal of the transgenes (WUS2, BBM, and CRE) located between the two LoxP sites on the T-DNA (Figure 3D). The removal resulted in healthy, fertile T0 transgenic sorghum plants expressing the selectable marker gene but did not contain ZmWUS2 and ZmBBM. This approach shortened sorghum transformation time by nearly half and made several previously untransformable genotypes transformable.

STMS, a KNOX homeodomain transcription factor, is expressed in SAMs and prevents the differentiation of the meristic cells [105]. STMS induces expression of IPT7, a cytokinin biosynthesis gene, leading to an increased cytokinin level [79, 106] (Figure 1). Overexpression of the maize STMS homolog Knotted1 (ZmKn1) in transgenic citrus significantly increased citrus transformation efficiency 3~15 times [48]. ZmKn1 overexpression in transgenic tobacco significantly increased transformation efficiency by 3 times via organogenesis on a hormone-free medium without antibiotic selection [47]. However, overexpression of STMS homologs resulted in the regeneration of transgenic tobacco plants with a bushy phenotype [46, 47]. In addition, overexpression of CUC1 and CUC2 that positively regulate SAM formation via STMS-dependent (and STMS-independent) pathways resulted in enhanced transformation efficiency of Arabidopsis by 10 times via a tissue culture method, and the resulting transgenic plants showed phenotypic abnormalities [49]. The estradiol-inducible expression of ESR2, an AP2-domain transcription factor, also enhanced Arabidopsis tissue culture transformation by directly regulating CUC1 transcription [52].

Transcription factor MP, a mediator of auxin responses and regulator of cytokinin signaling and biosynthesis, can also be leveraged to increase transformation efficiency [107, 108]. When the regulatory domain of MP is removed, the resultant MAP becomes irrepresible but maintains the normal MP function. Overexpression of AtMPA increased transformation efficiency in transgenic Arabidopsis with abnormal phenotypes via the upregulation of expression of AtWUS, AtSTM, AtESR1, AtCUC1 and AtCUC2 [54, 107] and repression of type-A ARR5 and ARR7 [81].

In contrast to the negative pleiotropic effects of overexpression of the aforementioned genes (i.e. WUS, WOXs, BBM, SERK1, LEC1/2, AGL15, STM/Kn1, CUC1/2, ESR2, and MAPA) and silencing/knockout of CLV1 and SAUR15, constitutive expression of a small family of transcription factor genes GROWTH-REGULATING FACTOR (GRF) and its transcriptional cofactor GRF-INTERACTING FACTOR1 (GIF) does not cause observed undesirable phenotypes in transgenic plants [55, 56], making conditional expression or excision of the transgenes unnecessary. During callus induction and plant regeneration, the miR396-regulated GRF-GIF duo can recruit SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeling complexes to regulate expression of their target genes and specify meristematic identity for organogenesis [109–111]. For example, poplar PpnGRF5–1 forms a complex with PpnGIFs and then inhibits expression of cytokinin oxidase/dehydrogenase1 (PpnCKX1), which is a membrane-bound protein catalyzing the degradation of cytokinins
[112], leading to the accumulation of cytokinins and meristematic induction [113] (Figure 2). Kong et al. [55] found that the overexpression of AtGRF5 or its homologs from various plant species enhanced shoot organogenesis and transformation efficiency in transformation-recalcitrant sugar beet, canola, soybean, and sunflower, and promoted somatic embryogenesis and transformation efficiency in maize. When compared to the control plants, a 4.5 ~ 11.5×, 1.9 ~ 2.3× and 1.1 ~ 1.4-fold increase in transformation efficiency was achieved in sugar beet, canola, and soybean, respectively. The resultant transgenic plants showed normal phenotypes. Debernardi et al. [56] demonstrated that overexpression of the wheat GRF4-GIF1 chimeric gene dramatically increases transformation efficiency and regenerates fertile transgenic plants with normal phenotypes in multiple plant species without the use of exogenous cytokinins. These species included wheat, rice, citrus as well as some difficult-or recalcitrant-to-transform species/genotypes such as commercial durum, bread wheat and a triticale line [56]. Most importantly, it was shown that overexpression of GRF4-GIF1 chimera increased the regeneration efficiency by an average of 7.8-fold and shortened the transformation process time from 91 days to 56 days in the wheat genotypes, permitting transgenic shoot selection in auxin media without using antibiotic selectable marker genes. Since there are multiple members in the GRF-GIF families, and not all GRFs or GRF-GIF pairs work equally effectively in plant transformation, research needs to be conducted to identify the GRFs or GRF-GIF pairs that have high transformation efficiency in a given economically important crop.

The use of plant growth regulatory genes in plant transformation

The IPT gene from the Ti-plasmids of A. tumefaciens catalyzes the formation of isopentenyl-adenosine-5-monophosphate (isopentenyl-AMP), the first intermediate in the cytokinin biosynthesis pathway, resulting in elevated cytokinin levels [114, 115] (Figure 2). Overexpression of an Agrobacterium IPT gene enhanced cytokinin level 23 ~ 300 times, resulting in a 24 ~ 2,000-fold increase in the cytokinin-to-auxin ratios in tobacco and cucumber [116, 117]. The abnormal phenotypes of the transgenic plants included loss of apical dominance and a poor ability to root, reduced internode elongation, and altered leaf morphology [116–118]. To overcome the negative phenotypic effects, Ebinuma et al. [57] developed a selectable marker-free transformation system by inserting the 35S:IPT cassette into an Ac-element from maize (Figure 3F) for transformation of tobacco and hybrid aspen (Populus sieboldii × P. grandidentata). Following the somatic self-excision of the IPT-containing Ac-element in the hemizygous transgenic lines where the retroelement failed to integrate into the sister chromatin, normal marker-free shoots were obtained at a frequency of 0.5 ~ 1.0%. Kunkel et al. [58] used the IPT gene for antibiotic marker-free tobacco and lettuce transformation by using a Dex-inducible IPT expression system (Figure 3G). The induced IPT expression improved transformation efficiency by 24.3 and 6.6 times in tobacco and lettuce, respectively, and produced transgenic plants without observed morphological defects.

Using Agrobacterium-mediated transient expression, a recent breakthrough in callus-free plant regeneration of multiple species was published [59]. This work revealed that low expression of ZmWUS2 (Nos:ZmWUS2) plus high expression of the Agrobacterium IPT (ZmUbi:IPT) or ZmUbi:AtSTM (Figure 3H) promoted organogenesis in aseptically grown seedling leaves of Arabidopsis, tobacco, and tomato, and in mature plants of tobacco, potato and grape [59]. When used together with a Cas9/gRNA plasmid, this novel approach produced gene-edited shoots without the use of tissue culture, offering great potential to speed up the breeding cycles for many plant species [59]. This tissue culture-free approach provides a good tool to test the effects of different candidate genes and regulatory elements on plant transformation.

Conclusion and future perspectives

With the demonstration of the dramatic effects of growth and developmental regulatory genes in plant tissue culture and transformation, regulatory genes have emerged as innovative, game-changing tools in plant transformation. These regulatory genes have been demonstrated to work efficiently in dicots, monocots and gymnosperms (Table 1) and are expected to work in various plants including specialty crops such as potato, sweetpotato, tomato and ornamentals. They offer excellent opportunities to develop genotype-independent genetic transformation methods and gene editing approaches in specialty crops.

In addition to the genes discussed above, many other upstream and downstream interacting factors promote meristem formation, shoot regeneration, or somatic embryogenesis, but have yet to be developed for use in plant transformation. These include PLTs [21], WINDs [65,119], ARRs [120], ABI3 [121], Fus3 [121], LILs [122], AGL18 [123], Pollen ole e 1 (POE1) [124], EMBRYO SAC DEVELOPMENT ARREST (EDA40) [124], SUPERMAN (SUF) [124, 125], and AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED 15 (AHL15) [126]. Research needs to be conducted to fine-tune the expression of each of these genes to examine their effects on plant regeneration and transformation.

Future research to identify additional growth and developmental regulatory genes and novel regulatory network looks promising. Various combinations of different growth and developmental regulatory genes need to be tested rationally for their synergistic and additive effects on plant transformation. Fine-tuning the expression of these genes is critical for the regeneration of normal, fertile plants in different plant species since their constitutive/ectopic expression typically interferes
with normal plant growth and development and causes undesirable pleiotropic effects. Strategies to counter these pleiotropic effects include tissue- or growth stage-specific expression, inducible or conditional expression, or removal of the transgenes. Synthetic promoters or devices could be used to conditionally express the regulatory genes [127, 128]. T-DNA read through-based transient expression [129] is also worth further testing. In addition, protein or DNA-free delivery of the regulatory proteins could be explored for their effects on plant transformation and regeneration, which could be conducted in explants cultured on callus induction media, in protoplasts [130], or suspension cells [131]. Transient or protein delivery of these regulatory genes could enable DNA-free approaches for the generation of engineered crop cultivars, which will minimize regulations and public opposition. No matter the genes or the delivery and regeneration systems, the success of next-generation agriculture will greatly depend on our ability to expand transgenic capabilities to previously recalcitrant species and elite genotypes. The recent progress in this exciting area of plant science provides an optimistic outlook for the future of crop transformation.

Acknowledgements

The authors thank Drs. Yi Li, Qiudeng Que, Guo-Qing Song, Mitra Mazarei, Anna Stepanova, and the anonymous reviewers for their constructive comments and suggestions. This work was financially supported by the United States Department of Agriculture (USDA) – Agriculture Research Service (ARS) Base funds to D. H., and the USDA Floriculture and Nursery Research Initiative (FNRI) grant # 8020–21000-071-23S and the USDA National Institute of Food and Agriculture (NIFA) Hatch project 02685 to W. L.

Conflict of interest statement

None.

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