Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2022) **20**, pp. 1622–1635 doi: 10.1111/pbi.13837

Application of developmental regulators to improve *in planta* or *in vitro* transformation in plants

Zhaoyuan Lian[1], Chi Dinh Nguyen[1], Li Liu[2], Guiluan Wang[1], Jianjun Chen[1], Songhu Wang[3], Ganjun Yi[4], Sandra Wilson[5], Peggy Ozias-Akins[6], Haijun Gong[7,*] and Heqiang Huo[1,*]

[1]*Department of Environmental Horticulture, Mid-Florida Research and Education Center, University of Florida, Apopka, FL, USA*
[2]*State Key Laboratory of Biocatalysis and Enzyme Engineering, School of Life Sciences, Hubei University, Wuhan, China*
[3]*School of Horticulture, Anhui Agricultural University, Hefei, China*
[4]*Guangdong Academy of Agricultural Science, Guangzhou, China*
[5]*Department of Environmental Horticulture, University of Florida, Gainesville, FL, USA*
[6]*Department of Horticulture and Institute of Plant Breeding, Genetics and Genomics, University of Georgia, Tifton, GA, USA*
[7]*Shaanxi Engineering Research Center for Vegetables, College of Horticulture, Northwest Agricultural and Forestry University, Yangling, China*

Received 9 December 2021;
revised 30 March 2022;
accepted 28 April 2022.
**Correspondence* (Tel 011-86-29-87082613; fax 011-86-29-87082613; email gongnavy@163.com (H.G.); Tel +1(407)410-6954; fax +1(407)814-6186; email hhuo@ufl.edu (H.H.))

Keywords: genetic transformation, developmental regulator, *PLT5*, Agrobacterium injection, tissue culture.

Summary

Plant genetic transformation is a crucial step for applying biotechnology such as genome editing to basic and applied plant science research. Its success primarily relies on the efficiency of gene delivery into plant cells and the ability to regenerate transgenic plants. In this study, we have examined the effect of several developmental regulators (DRs), including *PLETHORA (PLT5)*, *WOUND INDUCED DEDIFFERENTIATION 1 (WIND1)*, *ENHANCED SHOOT REGENERATION (ESR1)*, *WUSHEL (WUS)* and a fusion of *WUS* and *BABY-BOOM* (*WUS-P2A-BBM*), on *in planta* transformation through injection of *Agrobacterium tumefaciens* in snapdragons (*Antirrhinum majus*). The results showed that *PLT5*, *WIND1* and *WUS* promoted *in planta* transformation of snapdragons. An additional test of these three DRs on tomato (*Solanum lycopersicum*) further demonstrated that the highest *in planta* transformation efficiency was observed from *PLT5*. *PLT5* promoted calli formation and regeneration of transformed shoots at the wound positions of aerial stems, and the transgene was stably inherited to the next generation in snapdragons. Additionally, *PLT5* significantly improved the shoot regeneration and transformation in two *Brassica* cabbage varieties (*Brassica rapa*) and promoted the formation of transgenic calli and somatic embryos in sweet pepper (*Capsicum annum*) through *in vitro* tissue culture. Despite some morphological alternations, viable seeds were produced from the transgenic Bok choy and snapdragons. Our results have demonstrated that manipulation of *PLT5* could be an effective approach for improving *in planta* and *in vitro* transformation efficiency, and such a transformation system could be used to facilitate the application of genome editing or other plant biotechnology application in modern agriculture.

Introduction

With the advent of genome editing and the advancement of genomic sequencing, genetic transformation is becoming one of the most important biotechnology tools for studying gene functions and modern plant breeding. However, due to the low efficiency in the delivery of exogenous DNA and poor plant regeneration, the success in genetic transformation thus far has been limited to some well-studied plant species (Altpeter *et al.*, 2016). Exogenous DNA is typically delivered to plant cells through *Agrobacterium tumefaciens* infection or biolistic bombardment, both of which require procedures for the regeneration of plants from infected explants *in vitro* (Keshavareddy *et al.*, 2018). Successful *in vitro* plantlet regeneration through tissue culture is highly dependent on genotypes and further complicated by the application of plant hormones combinations (Altpeter *et al.*, 2016; Ikeuchi *et al.*, 2019). Extensive attempts have been made to explore novel transformation methods to bypass the procedures of tissue culture-based transformation. Yet, little progress was made in the most economically important crops, despite the successful application of the floral dip method to Arabidopsis (*Arabidopsis thaliana*) and green foxtail (*Setaria viridis*) (Clough and Bent, 1998; Martins *et al.*, 2015; Saha and Blumwald, 2016). Recently, several studies have shown that gene editing can be achieved by transient transformation of plant organs through plant viral vector delivery systems (Ariga *et al.*, 2020; Ellison *et al.*, 2020; Ma *et al.*, 2020; Wang *et al.*, 2020). However, the small cargo capacities and/or the narrow host range of these viruses, plus the requirement for regenerating plants with heritable edits, limit their potential use to a few crop species (Dinesh-Kumar and Voytas, 2020; Wang *et al.*, 2020). In addition, transgenes delivered by viral vectors are temporarily expressed, making it difficult to regenerate stable transgenic plants for some functional studies. Besides viral delivery, nanomaterials have been considered as reagent delivery carriers; however, nanomaterial-mediated delivery is still less efficient than biolistic delivery approaches and requires further optimization to increase its delivery efficiency (Nasti and Voytas, 2021). Overall, the recalcitrance to genetic transformation and inefficient plant regeneration are still the major barriers for the verification of gene

functions and the improvement of crops through genetic engineering.

Plant cells exhibit remarkable developmental plasticity and totipotency, which leads to plant regeneration from diverse tissues in response to stimuli such as wounding and hormones (Gaillochet and Lohmann, 2015). Two categories of plant hormones, auxin and cytokinin, play an important role in the developmental switch and organ regeneration. Generally, a high ratio of auxin to cytokinin favours *in vitro* callus formation and root regeneration, while a low ratio of auxin to cytokinin stimulates shoot regeneration, so the balance between auxin and cytokinin determines the fate of regenerated organs (Zhao *et al*., 2008). Therefore, a two-step tissue-culture method is routinely used to induce shoot regeneration via an indirect organogenesis pathway. Explants are first incubated on auxin-rich callus-inducing medium for the callus formation, and calli are subsequently cultured on a cytokinin-rich shoot-inducing medium for generating shoots (Valvekens *et al*., 1988). Despite that this method has been proposed for several decades and used for some crop plants, an efficient regeneration system has not been established in most crop species. Over the past decades, accumulating evidence from *in vitro* regeneration in Arabidopsis has shown that the pluripotency of plant somatic cells is governed by a complex regulatory network. The plant cell fate can be determined and switched by a range of developmental regulators (DRs), such as *WUSCHEL (WUS)*, *PLETHORAs (PLT)*, *AUXIN RESPONSE FACTOR (ARF)*, *GROWTH-REGULATING FACTORs (GRF)*, *LEAFY COTYLEDONs (LEC1 and LEC2)*, *BABY-BOOM (BBM)*, *LATERAL ORGAN BOUNDARY DOMAIN (LBDs)*, *CUP-SHAPED COTYLEDON (CUC1 and CUC2)*, *CLAVATA3 (CLV3)*, *SHOOT MERISTEMLESS (STM)* and *ENHANCED SHOOT REGENERATION (ESR)*. The expression of such genes is associated with improving the callus formation and/or plant regeneration, and the callus formation and shoot regeneration were significantly decreased in the deficient mutants of these regulators (Ikeuchi *et al*., 2016, 2019).

In addition to hormone-induced *de novo* shoot regeneration, wound is another primary trigger for tissue repair and organ regeneration. Wound induces the expression of the AP2/ERF transcription factors *WOUND INDUCED DEDIFFERENTIATION 1 (WIND1)* to promote cell dedifferentiation and proliferation for the callus formation at the wound sites (Ikeuchi *et al*., 2017; Iwase *et al*., 2011). Overexpressing *WIND1* and its homologous genes *(WIND2-4)* promotes callus growth in the absence of exogenous hormones (Ikeuchi *et al*., 2017; Iwase *et al*., 2011). A recent study showed that *WIND1* directly up-regulated the expression of *ESR1* to promote *CUC1*-mediated shoot regeneration (Iwase *et al*., 2017). Iwase *et al*. (2013, 2015) also showed that the *WIND1*-dependent regeneration pathway is conserved across diverse plant species, and ectopic expression of Arabidopsis *WIND1* promotes calli formation and shoot regeneration in rapeseed (*Brassica napus*), tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*). More interestingly, *WIND1* was recently reported to play an important role in vascular reformation at wound sites of aerial stems in Arabidopsis, and the vascular reconnection in the *wind1/2/3/4* quadruple mutant was significantly reduced (Iwase *et al*., 2021). In addition to *WIND1*, the AP2/ERF transcription factors *PLT3*, *PLT5* and *PLT7* are also responsive to wound signals. The *plt3/5/7* triple mutant exhibited significant defects in calli induction, demonstrating that these genes participate in callus formation at wound sites (Ikeuchi *et al*., 2017). Like *WIND1*, *PLT3*, *PLT5* and *PLT7* also regulate vascular repair and regeneration from aerial organs after mechanical injuries. The *PLT3*, *PLT5* and *PLT7* are greatly induced by wound signals at the wound sites, which subsequently up-regulate *CUC2* transcription by directly binding its promoter. In addition, both *PLT3/5/7* and *CUC2* up-regulate auxin biosynthesis gene *YUCCA4 (YUC4)* to control local auxin production, which is essential for vascular regeneration (Radhakrishnan *et al*., 2020).

To date, extensive efforts have been made to utilize several DRs for improving the efficiency of plant regeneration and genetic transformation (Boutilier *et al*., 2002; Debernardi *et al*., 2020; Lotan *et al*., 1998; Lowe *et al*., 2016; Maher *et al*., 2020; Nelson-Vasilchik *et al*., 2018; Zuo *et al*., 2002). Lowe *et al*. (2016) improved transformation efficiency in previously non-transformable maize inbred lines through overexpressing the *BABY-BOOM* (*BBM*) and *WUSCHEL2* (*WUS2*) genes. More recently, Debernardi *et al*. (2020) expressed a GRF4-GIF1 chimeric protein to enhance *in vitro* transformation of rice, wheat and citrus. However, these methods still require tedious and complex tissue-culture procedures. In a recent pioneering study, Maher *et al*. (2020) demonstrated that the stable transformation and gene editing could be achieved by the injection of mixtures of *A. tumefaciens* expressing *WUS2*, *STM* and *IPT* from maize in tobacco shoots, but the successful transformation through this injection method was by far limited to tobacco.

This study was intended to improve plant transformation efficiency of four plant species by exploiting the potentials of different DRs, including *PLETHORA (PLT5)*, *WOUND INDUCED DEDIFFERENTIATION 1 (WIND1)*, *ENHANCED SHOOT REGENERATION (ESR1)*, *WUSHEL (WUS)* and a fusion of *WUS* and *BABY-BOOM* (*WUS-P2A-BBM*) for *in planta* transformation in the adult plants or *in vitro* transformation through a tissue-culture method. Our results showed that *PLT5* significantly promoted calli formation and the regeneration of transformed shoots at the wound sites of snapdragon and tomato after *A. tumefaciens* injection, also greatly improved shoot regeneration and genetic transformation two Brassica cabbage varieties (*Brassica rapa*, var 'Bok choy' and 'Pei Tsai'), and somatic embryos formation in sweet pepper (*Capsicum annum*) via *in vitro* tissue culture.

Results

WUS, *WIND1* and *PLT5* promote *in planta* genetic transformation in *Antirrhinum majus*

To examine the effect of different DRs on *de novo* shoot regeneration and *in planta* genetic transformation from aerial organs, five different plasmids containing Arabidopsis *PLT5*, *ESR1*, *WUS-P2A-BBM*, *WUS* and *WIND1* driven by CaMV35S promoter were used to test whether they could promote shoot regeneration in snapdragon (*Antirrhinum majus*) (Figure 1 and Methods). To facilitate monitoring transgene expression, a green fluorescent protein gene (*eGFP*) fused with *NPTII* (encoding a neomycin phosphotransferase for Kanamycin resistance) and an anthocyanin production regulator *DELIA* (*DEL*) from snapdragon were included in all constructs (Figure 1a,b).

We first tested the effect of DRs on *de novo* shoot regeneration in the soil-grown snapdragon. Snapdragon is one of the top fresh-cut and potted ornamental plants and has long been used as a model plant species for studying plant development due to its substantial genetic diversity and well-established transposon mutagenesis system (Dyer *et al*., 2007; Lian *et al*., 2020;

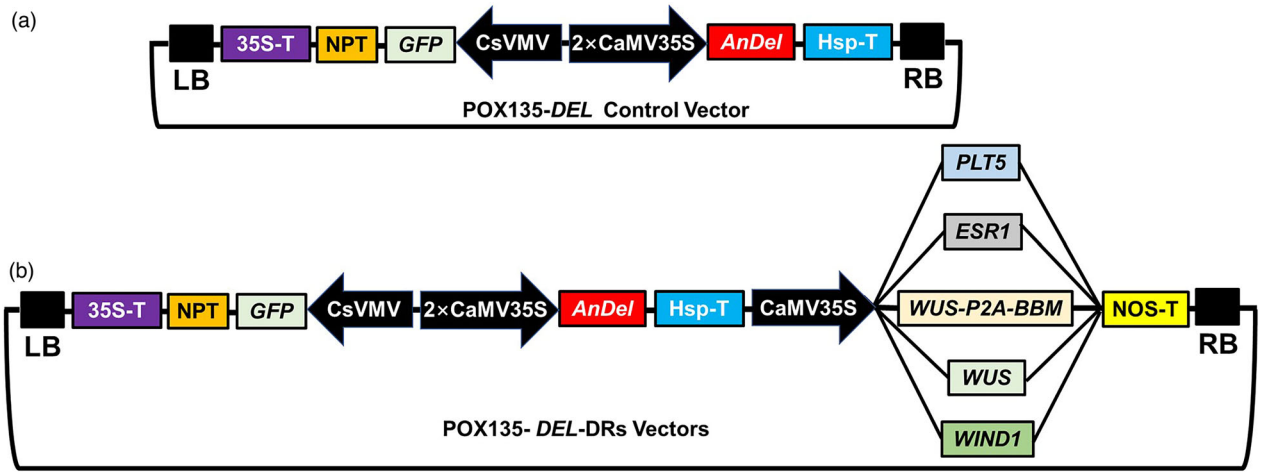
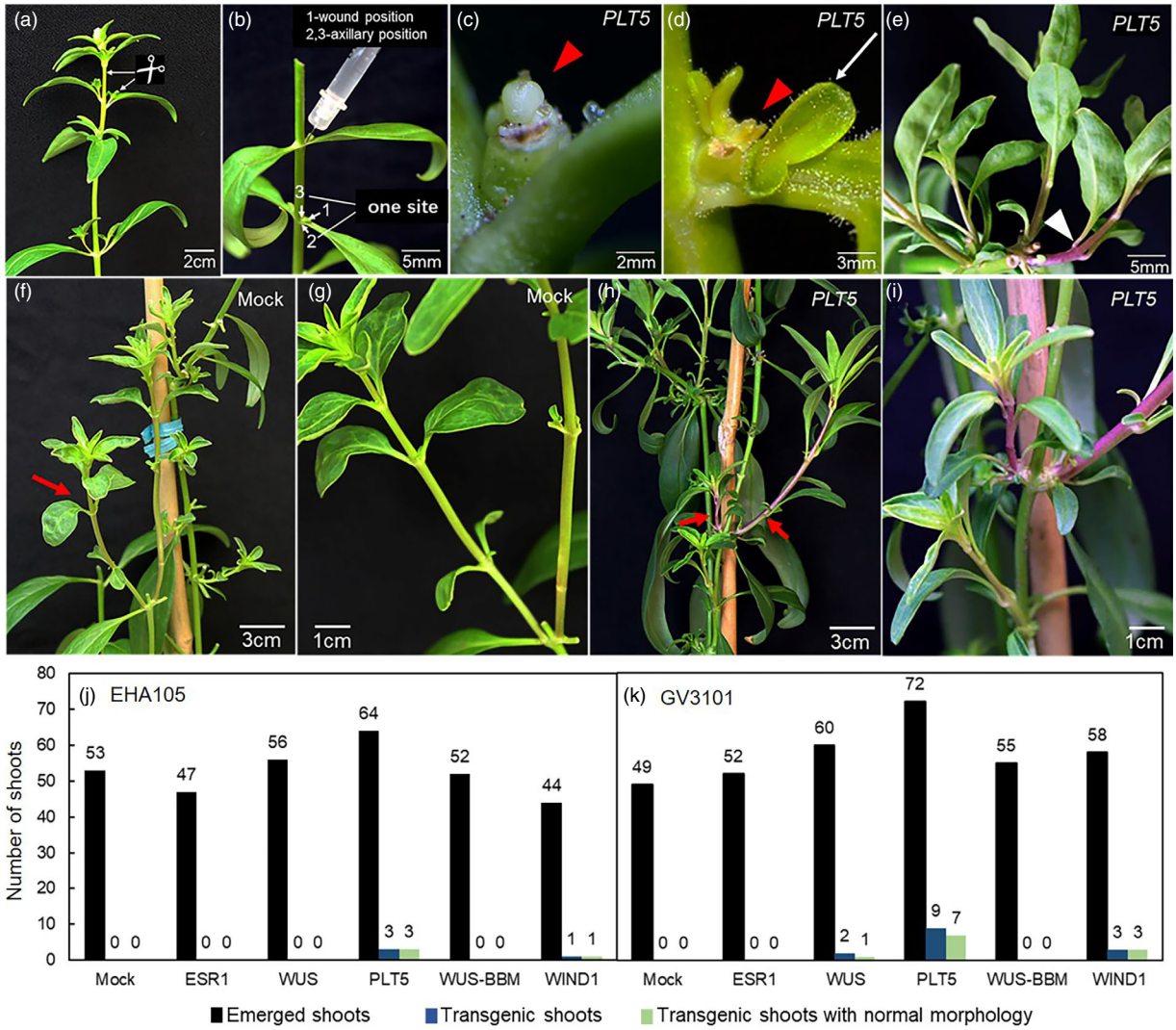


Figure 1 Schematic diagrams for the vectors. (a) The control expression vector POX135-*DEL* contained an *eGFP-NPTII* (*NPTII*: encoding a neomycin phosphotransferase for Kanamycin resistance) fused protein gene driven by a CsVMV (Cassava vein mosaic virus) promoter and an anthocyanin production regulator *DELIA (DEL)* from *A. majus* under 2×CaMV35S promoter. (b) The DRs expression vectors contained different DRs (*PLT5*, *ESR1*, *WUS-P2A-BBM*, *WUS* and *WIND1*) from Arabidopsis driven by a CaMV35S promoter. For *WUS-P2A-BBM*, a short self-cleaving peptide (ATNFSLLKQAGDVEENPGP) from porcine teschovirus was included between *WUS* and *BBM*.

Schwarz-Sommer *et al*., 2003). However, the lack of an efficient genetic transformation pipeline is the primary limiting factor for the complete application of snapdragon in genetic and molecular studies.

To create wounds for *A. tumefaciens* infection, the primary and axillary stems of approximately 70-day-old snapdragon plants were excised at the stage of flower bud initiation (Figure 2a). *A. tumefaciens* solutions containing the plasmid with or without different DRs were injected. One set of injection sites included 1 wound position and 2 axillary meristem positions as indicated in Figure 2b. When the control plasmid without DRs was applied, shoots rapidly emerged from the axillary meristem positions but not from the wound position of axillary stems (Figure 2f,g). Interestingly, none of these shoots was transgenic (Mock, Figure 2j–l). Unlike the DR-free control plasmid, transgenic shoots were produced with the aid of *WUS*, *PLT5* and *WIND1* (Figure 2j–l). We also observed that the strains of *Agrobacterium* influenced transformation efficiency (Transformation efficiency = the number of transgenic shoots/the total number of injection sites). A high frequency of transgenic shoots was obtained for *WUS* (2 transgenic shoots out of total 80 injection sites, 2.50%) *PLT5* (9/80, 11.25%) and *WIND1* (3/80, 3.75%) plasmids when GV3101 strain was applied, whereas the transformation efficiency for *WUS*, *PLT5* and *WIND1* was 0%, 3.75% and 1.25%, respectively, when EHA105 was used (Figure 2j,k). In addition, transformation efficiency was affected by the injection positions. For example, a much higher transformation efficiency was observed for *PLT5* (8.8%) and *WIND1* (2.5%) when GV3101 was injected to the wound positions (Transformation efficiency at the wound position = the number of transgenic lines from the wound position/the number of wound positions), while the transformation efficiency for *PLT5* and *WIND1* was only 1.3% and 0.6%, respectively, when GV3101 was injected to the axillary meristem positions (Figure 2l, Transformation efficiency at the axillary position = the number of transgenic lines from the axillary position/the number of axillary positions). A similar trend was also found when EHA105 was used for delivering *PLT5* or *WIND1* plasmids (Figure 2l). However, no transformed shoots were generated when applying *ESR*1 or *WUS-BBM* in both *A. tumefaciens* stains (GV3101 and EHA105). The detailed information for data collection and data analysis was illustrated in the Appendix S1: spreadsheet (Snapdragons).

In Arabidopsis, *PLT5* plays a role in callus formation in response to wounding signals (Ikeuchi *et al*., 2017; Iwase *et al*., 2021). In our study, we also found that *PLT5* promoted the development of callus tissues at the wound positions (Figure 2c), and shoots were subsequently regenerated from these callus tissues (Figure 2d). Furthermore, the shoot exhibited red-purple pigment, which was caused by the increased anthocyanin production due to the overexpression of the *DEL* gene (Figure 2e,i, see below). Additionally, the detection of fluorescent signals and PCR genotyping for *GFP* presence further suggested the successful integration of T-DNA into the plant genome in these transgenic shoots (Figure S1a,c,e). By contrast, the non-transgenic shoots were lack of red pigments and GFP fluorescence (Figure 2f,g, Figure S1b,d). Strong GFP fluorescence was also detected in the radicles and roots of some segregated T1 germinating seeds (Figure S2a,b,e,f) but not in germinating seeds of non-transgenic plants (Figure S2c,d), suggesting that the transgenes were stably inherited to the next generation. In addition, the red-stem trait due to the *DEL* transgene was also stably transmitted to some T1 progenies (Figure S3a). The PCR genotyping results confirmed that the *GFP* gene was present in these red-stem T1 seedlings but not the green ones (Figure S3b). Consistent with this observation, the anthocyanin content in the T1 transgenic seedlings was 17.6-fold higher than that in the wild-type seedlings (Figure S4a,b). The transcript levels of *DEL* and other downstream biosynthetic genes including chalcone isomerase (*CHI*), chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H2*) and dihydroflavonol 4-reductase (*DFR*) (Naing and Kim, 2018; Xie *et al*., 2016) were 32-, 1.6-, 6.8-, 7.4- and 212-fold higher, respectively, than those in wild-type seedlings (Figure S4c). Collectively, these results demonstrate that *PLT5* promoted stable *in planta* genetic transformation in snapdragon.



(l)

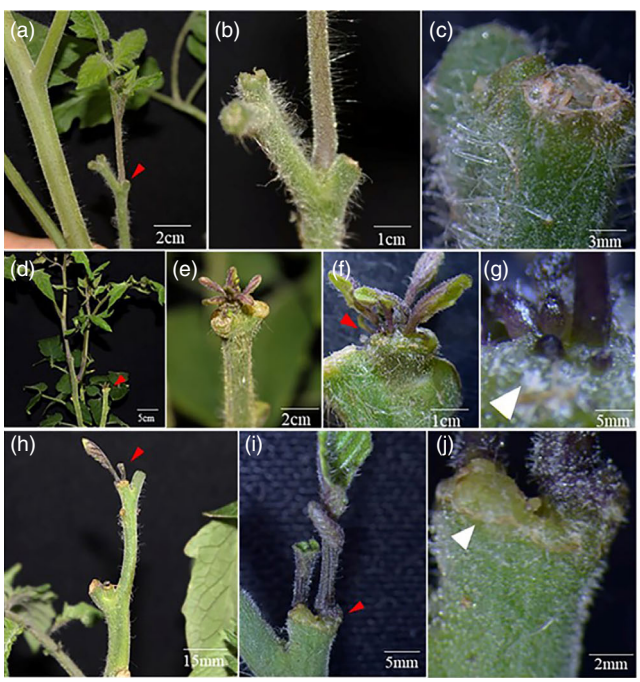
Treatment	EHA105		GV3101	
	Transformation efficiency in the wound position (%)	Transformation efficiency in the axillary position (%)	Transformation efficiency in the wound position (%)	Transformation efficiency in the axillary position (%)
Mock	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
ESR1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
WUS	0.0±0.0	0.0±0.0	0.0±0.0	1.3±0.7
PLT5	3.8±2.4	0.0±0.0	8.8±0.5	1.3±0.7
WUS-BBM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
WIND1	1.3±1.3	0.0±0.0	2.5±1.4	0.6±0.6

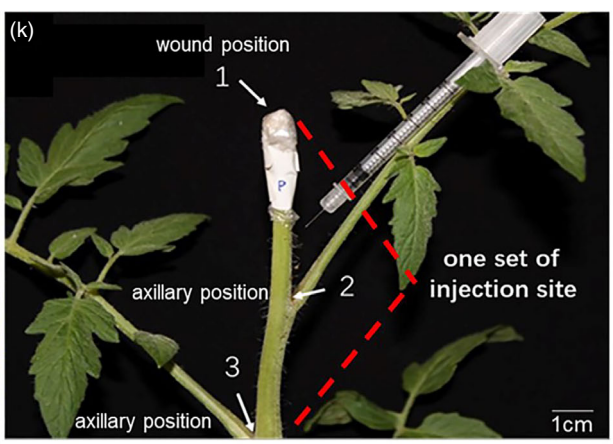
Figure 2 Promoting shoot regeneration and transformation by overexpression of *DR* genes in soil-grown *A. majus*. (a) Primary and axillary shoots were cut off from the about 70-day-old snapdragons, but a roughly 0.3 mm stem base of the axillary shoot remained for *A. tumefaciens* injection. (b) Injection of *A. tumefaciens* (GV3101) containing different DRs at injected sites (one set of injection site contains one wound position and two axillary positions indicated by white arrows). (c) Callus tissues (red arrowhead) were developed after injection at the wound position. (d) *De novo* shoots (red arrowhead) were regenerated at wound position after injections, while the shoot indicated by the white arrow emerged from the axillary position. (e) The bright red pigments (white arrowhead) due to overexpression of *DEL* gene in the stems of shoots after injection. (f) The representative image of a plant with the emerged shoot (red arrow) after injection of GV3101 containing the DR-free plasmid as a negative control. (g) The close-up image of the shoot indicated by the red arrow in (f); (h) The representative image of a plant with transgenic shoots (red arrows) after injection of GV3101 containing the *PLT5* plasmid. (i) The close-up image of the transgenic shoots with the red stems indicated by the red arrows in (h). (j, k) The effect of different DRs on the total number of emerged shoots, transgenic shoots and transgenic shoots with normal morphology after injected with *A. tumefaciens* strains EHA105 (j) or GV3101 (k). (l) The effect of different DRs on transformation efficiency when injected to the wound position or axillary injection positions. For each plasmid, data were collected from 20 injection sets of sites including 20 at wound position and 40 at axillary injection position. Four independent injection experiments were applied to each plasmid. Transformation efficiency in the wound position = the number of transgenic lines from the wound position/the number of wound positions; transformation efficiency in the axillary position = the number of transgenic lines from the axillary position/the number of axillary positions. Data were collected when the new shoots fully developed at ~6 weeks after injection. The Mean ± SE data were presented in M. The detailed information for data collection and data analysis was illustrated in the Appendix S1: spreadsheet (Snapdragons).

PLT5 and *WUS* promotes *in planta* genetic transformation in tomatoes

Based on the positive results of snapdragons, we extended the injection transformation study to soil-grown tomatoes (*S. lycopersicum*) to examine whether *WUS*, *WIND1* and *PLT5* could also promote regeneration of transgenic shoots by *A. tumefaciens* injection (GV3101). The tomato plants were decapitated, and the pre-existent axillary branches were removed; then, *A. tumefaciens* strain GV3101 containing the plasmids without DRs or with *PLT5*, *WUS* and *WIND1* were injected. To be consistent with the snapdragon, one set of tomato injection sites also included one wound site at the top of primary stems and two axillary meristem positions (Figure 3k). When the plasmid without DRs was applied, a single green shoot typically emerged from axillary positions after injection (Figure 3a,b), and no visible callus tissue developed at the wound sites (Figure 3c). By contrast, callus tissues were formed at the wound sites (Figure 3g,j), and the multiple purple shoots emerged from these calli after injections of the *PLT5* plasmid (Figure 3d–f,h,i). Surprisingly, all of the transgenic tomato shoots were initiated from the wound positions, and none of these shoots from axillary meristem positions were transgenic (Figure 3l). The highest transformation efficiency of 13.3% was observed for *PLT5*, followed by *WUS* with a transformation efficiency of 3.3% (Figure 3l), and no transgenic shoots were obtained after injection of plasmids with *WIND1* or without DRs (Figure 3l). Unlike snapdragons, in which only one shoot was generated from each injection position, multiple shoots were able to regenerate from the wound injection position in tomatoes. In this scenario, all the transgenic shoots initiated from the same wound position were considered as one transgenic line. The detailed information for data collection and data analysis was illustrated in the Appendix S1: spreadsheet (Tomatoes).

Similar to the snapdragon, the transgenic tomato shoots were also confirmed by the GFP fluorescence and improved anthocyanin accumulation (Figure 4). Microscopic examination showed that GFP fluorescence was not detected in the shoots directly emerged from axillary meristem positions (Figure 4a,b), while both calli and newly regenerated shoots from the wound position displayed GFP fluorescence (Figure 4c–g), indicating that these *de novo* regenerated shoots from the wound positions were transgenic. The transformation success was corroborated by the notable anthocyanin accumulation in the mature shoot, the abaxial side of leaves and flower sepals (Figure 4k–m), while there was no such pigment in the counterparts of non-transgenic shoots (Figure 4h–j). PCR genotyping results also suggested the





(l)

Treatment	GV3101	
	Transformation efficiency in the wound position (%)	Transformation efficiency in the axillary position (%)
Mock	0.0±0.0	0.0±0.0
WUS	3.3±3.3	0.0±0.0
PLT5	13.3±3.3	0.0±0.0
WIND1	0.0±0.0	0.0±0.0

Figure 3 Promoting *de novo* shoot regeneration by the overexpression of *PLT5* in the soil-grown tomatoes. (a) A single green shoot emerged from axillary position after injection of GV3101 containing the DR-free plasmid. (b) A close-up image of the emerged shoot from A to show no notable anthocyanin accumulation in its stem. (c) No callus tissue was formed and no shoots were regenerated from the wound site of stems after injection of GV3101 containing the DR-free plasmid. (d–g) Multiple shoots emerged from the wound site with anthocyanin accumulation after injection of GV3101 containing the *PLT5* plasmid. The section indicated by red arrowheads in (d) and (f) were magnified in (e) and (g), respectively, to show the shoots that emerged from calli at the wound site. (h–j) Two shoots were initiated from calli at the wound sites after injection of GV3101 containing the *PLT5* plasmid. (i) and (j) magnified details for the section indicated by red arrowheads in (h) and (j). The visible callus tissues were highlighted by the white arrowheads in (g) and (j). (k–l) The effect of different DRs on transformation efficiency when injected to the wound site or axillary injection positions. Data were collected from three independent experiments with 5 injection set of sites for Experiment 1, and 10 injection sites for Experiments 2 and 3, respectively (each injection set includes 1 at wound position and 2 at axillary injection position). Transformation efficiency in the wound position = the number of transgenic lines from the wound position/the number of wound positions; Transformation efficiency in the axillary position = the number of transgenic lines from the axillary position/the number of axillary positions. All the transgenic shoots from the same position were counted as one transgenic line. The data were presented in Mean ± SE. The detailed information for data collection and data analysis was illustrated in the Appendix S1: spreadsheet (Tomatoes).

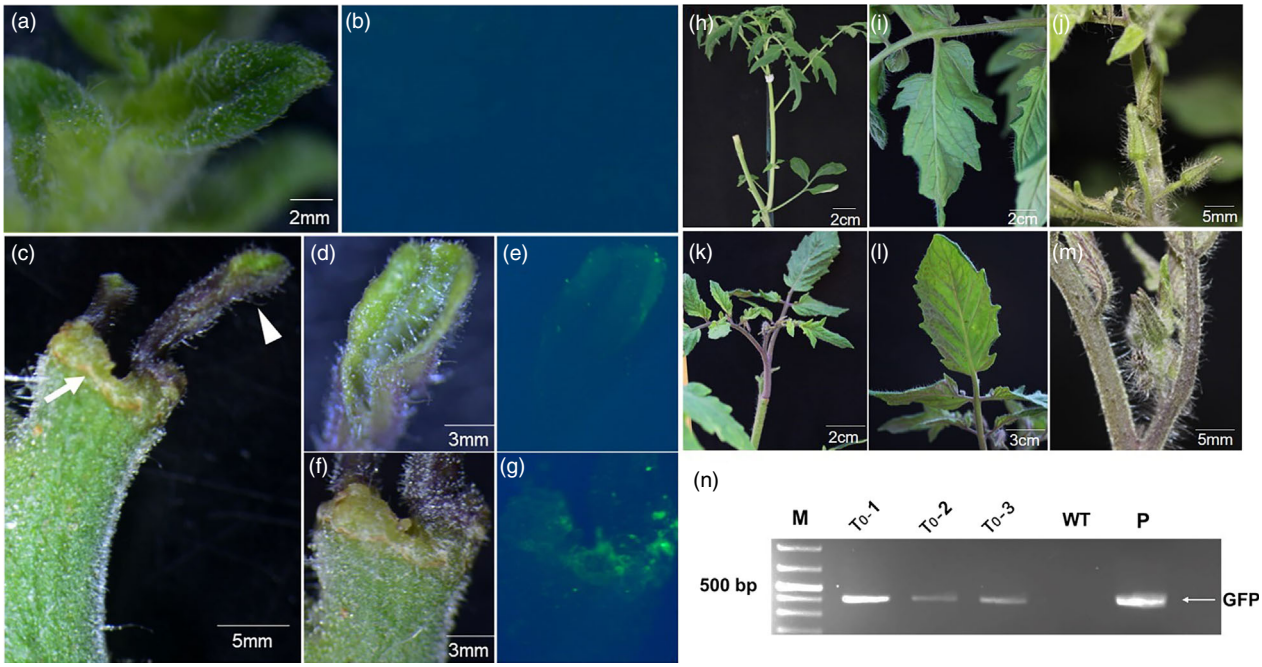


Figure 4 Transformation confirmation of regenerated shoots from the wound sites of the soil-grown tomatoes with the aid of *PLT5*. (a) The emerged shoots form the axillary position without DRs as the control. (b) No GFP was detected in the emerged shoots in the (a). (c) Regenerated tomato shoots from the callus at the wound site. (d) A close-up image of regenerated shoot indicated with an arrowhead in (c). (e) The GFP fluorescence in the regenerated shoot. (f) A close-up image of the callus tissues from wound sites indicated with an arrow in (c). (g) GFP fluorescence in the callus tissues from wound sites. (h–j) No anthocyanin accumulated in the in the stem (h), abaxial side of leaves (i) and flower sepals (j) of the non-transgenic shoot. (k–m) The enhanced anthocyanin in the stem (k), abaxial side of leaves (l) and flower sepals (m) of the transgenic shoot. (n) PCR detection of GFP in the purple transgenic shoots from the wound position.

presence of the transgene in these shoots (Figure 4n). All these results suggested that *PLT5* could promote *in planta* transformation in tomatoes.

PLT5 promotes *in vitro* shoot regeneration and genetic transformation in *Brassica rapa* and embryonic calli regeneration in sweet pepper

In addition to snapdragons and tomatoes, we tested our *in planta* transformation method in sweet pepper (*Capsicum annuum var.' California Wonder'*) and Bok choy (*B. rapa* ssp *chinensis*), which are highly recalcitrant to genetic transformation. The Bok choy injection tests were repeated three times, included 18 wound positions in total for control or *PLT5* injection, respectively. The sweet pepper injection tests were repeated three times as well, included 25 wound positions in total for control or *PLT5* injection, respectively (Appendix S1: Brassica and pepper injection). Injection of GV3101 with *PLT5* plasmid was able to promote calli or embryo-like tissues formation at wound positions of primary stems, but no shoots regenerated from these tissues in Brassica cabbage and sweet pepper (Figures S5 and S6), possibly due to the rapid deposition of suberin and lignin in response to wounding to prevent water loss and pathogen infection (Ginzberg, 2008; Graça, 2010). Therefore, we next tested whether *PLT5* could promote *in vitro* shoot regeneration and genetic transformation through tissue culture in these two species.

When the *PLT5* plasmid was applied to Bok choy transformation, adventitious roots were directly induced from cotyledon petioles, which were cultured on hormone-free MS media within 2 weeks (Figure S7a); but no adventitious root appeared from the explants infected with the DR-free plasmid (Figure S7b). Subsequently, calli formed from these regenerated roots after a continuous culture (Figure S7c,d). GFP fluorescence was detected in the roots and in the callus (Figure S7e). Although no shoots were directly regenerated from these callus on hormone-free MS media, embryo-like tissues could be induced from detached root tips when cultured on MS medium with 3 mg/L 6-BA for 3 weeks (Figure S8a–e). After another 2 weeks of culture, multiple shoots with GFP fluorescence regenerated from these embryo structures on the same medium containing 3 mg/L 6-BA (Figure S8f–h). In sum, with the aid of *PLT5*, the Bok choy transformation efficiency reached as high as 6.7% when the *de novo* transformed roots were used for shoots induction, and no *de novo* roots and transgenic shoots were obtained for the DR-free control plasmid (Table S1).

Furthermore, when the explants (cotyledons with petiole) of Bok choy were infected with the *PLT5* plasmid and directly cultured on the MS medium supplemented with 3 mg/L 6-BA, the formation of adventitious roots was significantly inhibited. However, the improved calli regenerated from the wound sites of petioles (Figure 5c). Some areas of these calli turned green after 2 weeks of culture, and strong GFP fluorescence was also observed in this green zone of the callus (Figure 5c and Figure S9a,b). Shoots with GFP fluorescence could subsequently regenerate from these green zones of the callus on the same medium (Figure 5d,e, Figure S9c–h). By contrast, no shoots regenerated from the explants infected with the DR-free plasmid despite that calli were formed and exhibited no or weak GFP fluorescence (Figure 5a,b). With closer observation, somatic embryos were found in the greenish callus (Figures S9c,d and

Table 1 Effects of *PLT5* on shoot regeneration and genetic transformation in Bok choy and Pei-Tsai

Varieties	Treatments	Experiments	No. of the explants	No. of the transgenic embryonic calli	Total numbers of the transgenic shoots	Transformation efficiency (%)*
Bok choy		No. 1	25	0	0	0.0
		No. 2	25	0	0	0.0
	Control	No. 3	25	0	0	0.0
		No. 4	25	0	0	0.0
		Total	100	0	0	0.0
		No. 1	25	2	7	8.0
		No. 2	25	1	3	4.0
	PLT5	No. 3	25	2	5	8.0
		No. 4	25	3	10	12.0
		Total	100	8	25	Mean ± SE = 8.0 ± 1.6
Pai-tsai		No. 1	25	0	0	0.0
		No. 2	25	0	0	0.0
	Control	No. 3	25	0	0	0.0
		No. 4	25	0	0	0.0
		Total	100	0	0	0.0
		No. 1	25	0	0	0.0
		No. 2	25	2	5	8.0
	PLT5	No. 3	25	3	8	12.0
		No. 4	25	1	3	4.0
		Total	100	6	16	Mean ± SE = 6.0 ± 2.6

Twenty-five explants (cotyledons) were directly placed on shoot-induction MS medium with 3 mg/L 6-BA addition. The experiment was repeated four times. Values = Mean ± SE.

*Transformation efficiency = The number of transgenic lines (equal to the number of transgenic embryonic calli)/the number of explants. All the transgenic shoots from the same callus were counted as one independent transgenic event.

S10a,b). Sudan Red 7B is an indicator stain for triacylglycerol that is enriched in embryos; staining with Sudan Red 7B revealed that the callus from the infection with the *PLT5* plasmid was embryogenic (Figure S10e,i). The dark-red staining of these embryogenic calli resembled the staining of Bok choy seed embryo (Figure S10c,g). By contrast, the light staining colour of calli from the infection with the DR-free plasmid indicated that these calli are non-embryogenic (Figure S10d,h), which resembled the staining of the expanded cotyledon of Bok choy seedling (Figure S10f,j). These results indicate that *PLT5* might promote embryogenesis, as reported previously in Arabidopsis (Radoeva and Weijers, 2014).

To determine whether overexpression of *PLT5* resulted in any alteration of its downstream genes regulating shoot regeneration, the greenish calli (with GFP) from explants infected with the *PLT5* plasmid and white calli (with GFP) from explants infected with DR-free plasmid were collected at the same stage to examine the transcript levels of several key regulator genes for shoot regeneration (Figure S11a,b). The qRT-PCR results showed that *PLT1*, *PLT2*, *CUC1*, *CUC2*, *STM* and the auxin biosynthetic gene *YUC4* in the callus transformed with the *PLT5* plasmid were 7.6-, 4.1-, 3.3-, 26.8-, 15.5- and 1.8-fold, respectively, higher than those in the callus transformed with the DR-free plasmid (Figure S11c). These results suggest that overexpression of *PLT5* could alter the transcription of these key regulators to promote cellular differentiation and shoot meristem formation in Bok choy.

To test whether our method was genotype-independent, we performed genetic transformation in *B. rapa cv* Pei-Tsai. Similarly, plant regeneration and genetic transformation were significantly improved by the overexpression of *PLT5*. Transgenic shoots with strong GFP signal were able to regenerate rapidly from the cotyledon explants after being infected with the *PLT5* plasmid (Figure S12c–f), but only calli with weak or no GFP fluorescence were formed from explants infected with DR-free plasmid, and no shoots developed (Figure S12a,b). GFP fluorescence detection suggested the presence of transgenes in the regenerated shoots (Figure S12d,f). Additionally, GFP was also clearly detected in the stems of transgenic plants (Figure S13d,e). PCR genotyping results further confirmed the success of genetic transformation in both *B. rapa* genotypes (Figure S13f). With the aid of *PLT5*, the average transformation efficiency was up to 8% for Bok choy and 6% for Pei-Tsai, compared to 0% in the control groups that did not regenerate shoots (Table 1).

Lastly, we tested whether *PLT5* promoted plant regeneration and genetic transformation in sweet pepper. Cotyledons or hypocotyls were infected with *A. tumefaciens* containing *PLT5* plasmid or DR-free plasmid. Results showed that leaves were directly regenerated from the hypocotyls without callus formation regardless of the plasmid types (Figure S14a). No pigment or GFP fluorescence was observed in these regenerated leaves (Figure S14b–d, Figure S16a,b). Therefore, hypocotyls are not suitable explants for sweet pepper transformation. When cotyledons were used as explants for the DR-free plasmid infection, calli with pigment formed, but no shoots were regenerated from these calli (Figure S15a–c). By contrast, somatic embryos could develop from the calli derived from cotyledon transformed with *PLT5* plasmid about 45 days after infection (Figure S15d,e); and leaf-like organs with various degrees of anthocyanin slowly appeared after further culturing these somatic embryos (Figure S15f–h). Furthermore, the GFP fluorescence was observed in the regenerated callus and leaf-like organs when the *PLT5* plasmid was applied (Figure S16c–g). The transgene in the

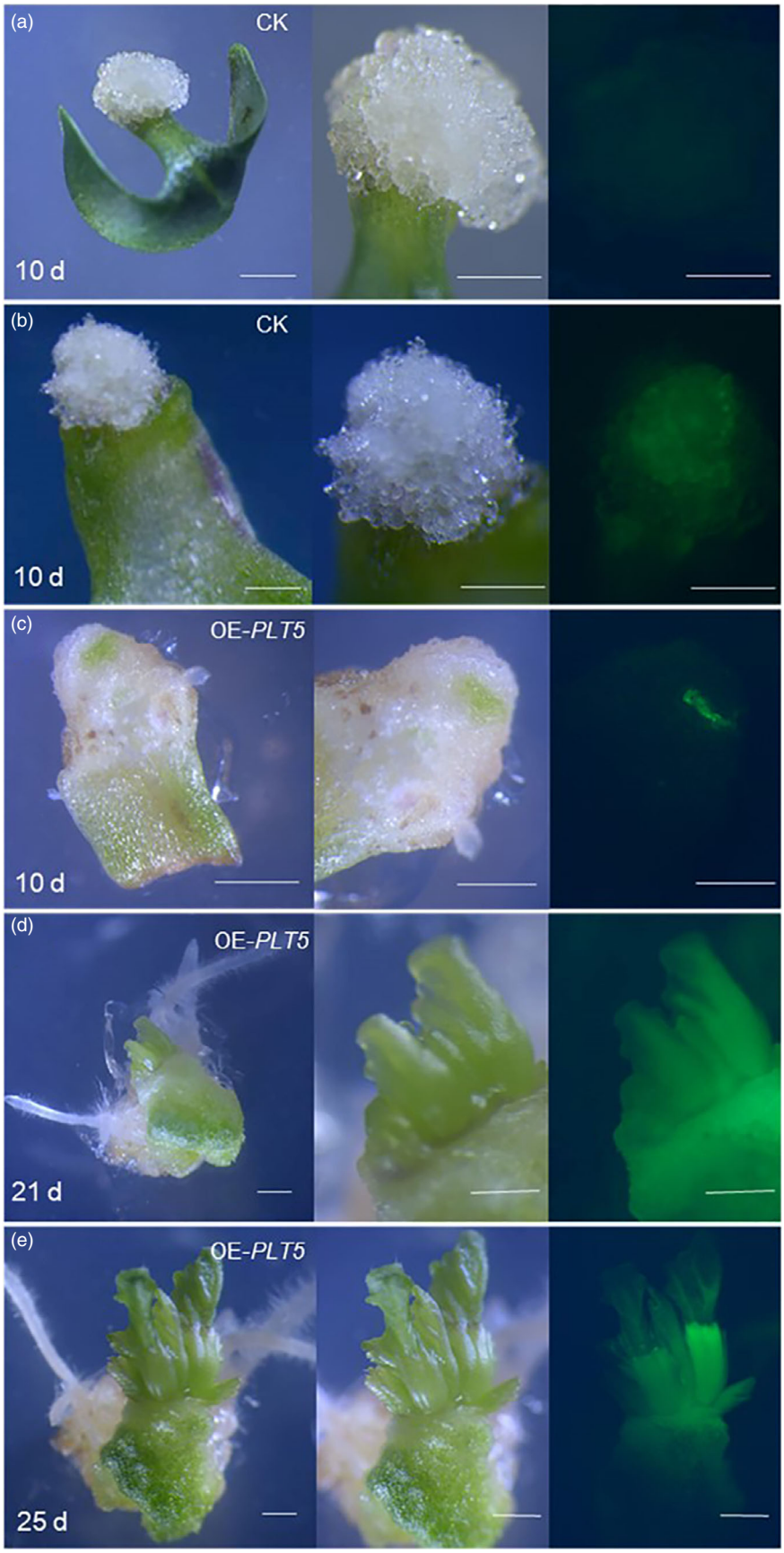


Figure 5 Promoting shoots regeneration from embryogenic calli of Bok choy with the aid of *PLT5*. (a) The callus with no GFP fluorescence was formed from cotyledon explants after infections with the DR-free plasmid. (b) The callus with GFP fluorescence was formed from cotyledon explants after infections with the DR-free plasmid, but no shoots could be induced from these calli even after further culture. (c) Embryogenic callus with strong GFP fluorescence at the green zone was formed from cotyledon explants infected with the *PLT5* plasmid. (d) Shoot buds with strong GFP fluorescence was initiated from embryogenic callus due to the expression of *PLT5*. (e) Shoots with strong GFP fluorescence were regenerated from shoot meristems due to the expression of *PLT5*. All cotyledon explants (with petiole) were cultured on the MS medium with 3 mg/L 6-BA. Images in (c–e) were taken from the same explant at the different developmental stages. The numbers in the left bottom corner of images are days after inoculation of explants. CK: the explants infected with the DR-free plasmid (without *PLT5*). Scale bars: 3 mm.

transformed leaf-like organs was also confirmed by the PCR genotyping result (Figure S16h). However, these leaf-like organs could not develop normal shoot meristem and roots, so the overexpression of *PLT5* could only improve the formation of embryogenic callus in sweet pepper. In terms of transformed leaf-like organs, the average transformation efficiency was 3.8% for the *PLT5* plasmid and 0% for the DR-free plasmid (Table S2).

The effect of *PLT5* on plant morphology and seed development

Arabidopsis *PLT5* is an AP2/ERF5 transcription factor and has been reported to regulate embryogenesis, flower development, seed germination and shoot phyllotaxis (Krizek, 2015; Prasad *et al*., 2011; Yano *et al*., 2009). Despite that overexpression of *PLT5* promoted shoot regeneration and genetic transformation through tissue culture and injection methods, we did observe mild developmental changes in some but not all regenerated plants, including curled leaves in snapdragons (Figure S17b), the twisted leaf growth in tomato (Figure S18c,d). In snapdragon, 9 transgenic lines were generated from *PLT5*/GV3101 injection, and only 2 lines showed mild developmental changes (Figure 2K). In tomato, 4 transgenic lines were generated from *PLT5* injection, and only 1 line showed developmental changes, which did not develop into normal shoot, and not counted when calculating the transformation efficiency (Figure S18c,d, Appendix S1: Spreadsheet-Tomatoes). Notably, the developmental changes were a little severer in *Brassica* cabbages. For example, various degrees of curled leaves were commonly observed during vegetative growth in the transgenic Bok choy plants (Figure S19c–f). More strikingly, compared with normal flower morphology of wild-type plants (Figure 6a–c), clustered inflorescence (Figure 6d), continuous development of flowers from the ovary position (Figure 6e) and the overgrown flower branches (Figure 6f) were observed in the transgenic Bok choy plants during reproductive development. The severer developmental changes in *Brassica* cabbages could be explained by the stronger function of Arabidopsis *PLT5* due to conserved pathways in plant species closely related to Arabidopsis. Despite these developmental changes due to *PLT5* overexpression, viable seeds were produced in Bok choy (Figure 6g). GFP fluorescence was detected in the germinating T1 seeds and cotyledons of their seedlings, which was absent in the segregated non-transgenic ones (Figure 6h), indicating the stable inheritance of the transgene to the next generation. The production of viable transgenic seeds in snapdragon and Bok choy suggested the high potential of *PLT5* in promoting *in planta* or *in vitro* transformation across different plant species.

Discussion

Plant regeneration and genetic transformation are fundamental and essential for the genetic engineering of plants. Until today, only a limited number of plant species are amenable to the available transformation process, and successful transformation is highly genotype-dependent. Achieving stably transformed plants is still a challenge for applications of biotechnological tools to improve crops. Recently, DRs have been used in tissue culture and injection of *A. tumefaciens* to aboveground meristems for improving plant regeneration and genetic transformation (Lowe *et al*., 2016; Maher *et al*., 2020). Among them, *WUS* and *BBM* have been extensively used in monocotyledonous species (Hoerster *et al*., 2020; Jones *et al*., 2019; Lowe *et al*., 2016), but little success has been reported in dicotyledonous species (Heidmann *et al*., 2011; Zhang *et al*., 2021). *PLT5* was reported to be a master regulator for stem vascular repair after wounding in Arabidopsis (Radhakrishnan *et al*., 2020). Wound signals induced and enhanced transcription of *PLT5* at wound sites, leading to calli formation at first and subsequent differentiation into vascular tissues (Radhakrishnan *et al*., 2020). In the present study, we observed that overexpression of *PLT5* could promote calli formation and shoot regeneration at the wound position after injection in snapdragons and tomatoes (Figures 2c and 3g,j). Interestingly, the transformation efficiencies for the wound position were usually much higher compared with the ones for the axillary position (Figures 2l and 3l). This result could be explained by the following two reasons: Firstly, the emerged shoots were probably axillary shoots instead of *de novo* regenerated shoots from calli, and these axillary shoots directly initiated from the intact pre-existent shoot meristems, leading to the inefficiency in Agrobacterium infection. Secondly, no visible calli were developed after Agrobacterium injections at the axillary positions, but callus formation seems to be beneficial to the higher transformation efficiency. It has been reported in many plant species that the plant transformation efficiency was significantly improved when plant regeneration acts through an indirect organogenesis pathway instead of a direct organogenesis pathway during tissue culture, such as snapdragon (*A. majus*) (Lian *et al*., 2020), trifoliate orange (*Poncirus trifoliata* L.) and common bean (*Phaseolus vulgaris* L.) (Collado *et al*., 2016; Mukeshimana *et al*., 2013).

In parallel to *PLT3/5/7*, *WIND1* was also responsive to wounding signals to control cell dedifferentiation and promote callus formation at wound sites in Arabidopsis (Iwase *et al*., 2011). *WIND1* also promotes shoot regeneration through direct transcriptional activation of *ESR1* at wound sites of *in vitro* explants (Iwase *et al*., 2017). Recently, Iwase *et al*. (2021) showed that similar to *PLT3/5/7*, *WIND1* was strongly up-regulated in the vasculature and epidermis of the scion's hypocotyls and promoted vascular reconnection in Arabidopsis. However, the *wind1/2/3/4* quadruple mutant was not defective in the wound-induced callus, which is different from the significant reduction in callus formation observed in triple mutant *plt3/5/7*, suggesting the indispensable function of *PLT3/5/7* in wound-induced callus formation (Iwase *et al*., 2021; Melnyk *et al*., 2015). Although

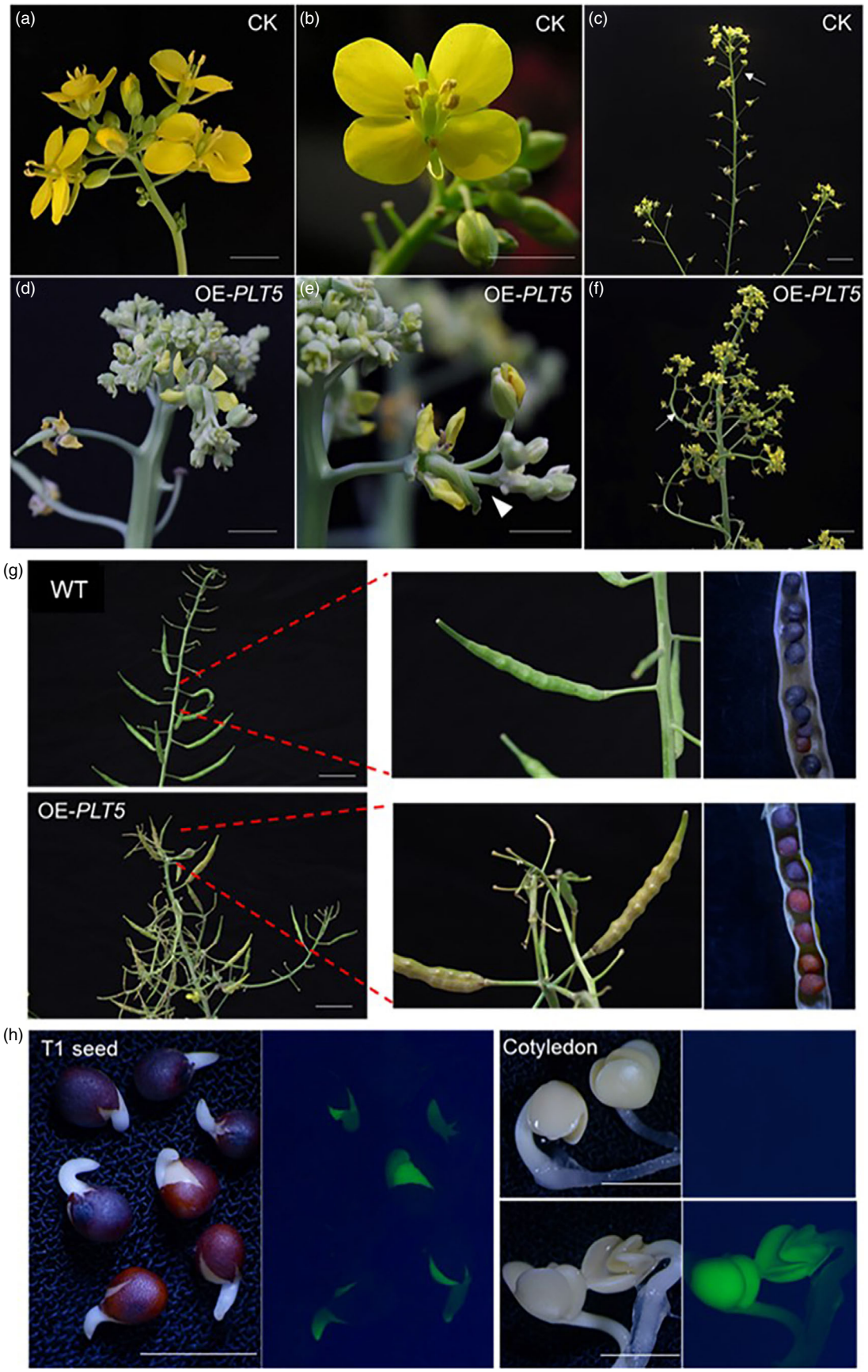


Figure 6 Viable seeds produced in *PLT5*-expressing Bok choy in spite of some morphological changes. (a–c) Normal flowers and inflorescence in the wild-type Bok choy plant, CK: Wildtype Bok choy. (d–f) Abnormal flowers and inflorescence due to the overexpression of *PLT5* in the transgenic Bok choy plant. The arrowhead in (e) indicated flowers formed from the ovary position; the white arrow in (f) indicated the overgrowth of flower branches. (g) Normal seed production in the transgenic Bok choy. (h) The GFP fluorescence detection in segregated T1 germinating seeds and cotyledons. Scale bars = 1 cm.

WIND1 could also promote *de novo* shoot regeneration and *in planta* transformation in snapdragons, the efficiency was lower compared with that promoted by *PLT5* (Figure 2l). Additionally, *PLT5* is known to function in the acquisition of cellular pluripotency that precedes the establishment of shoot progenitors by *CUC1/2*, *WIND1* and *WUS* (Shin *et al*., 2020), which may also explain its stronger effect on promoting callus formation and genetic transformation in the wound sites of adult plants. Therefore, we postulate that cellular dedifferentiation reflected by calli formation and re-differentiation reflected by *de novo* shoot regeneration from calli were important for the success of gene delivery into plant cells in the planta injection process with the aid of *PLT5*.

Our results showed that the overexpression of *PLT5* could also promote plant or embryos regeneration and genetic transformation through the *in vitro* tissue culture in *B. rapa* and sweet pepper since no shoots or embryos were regenerated from the callus without the aid of *PLT5* (Figure 5, Figures S12 and S15). It was reported that *PLT3/5/7* controlled plant regeneration *via* a two-step mechanism, in which the root stem cell regulators *PLT1/2* were first activated by *PLT3/5/7* to establish pluripotency, and the *CUC2* gene was required for completion of shoot regeneration (Kareem *et al*., 2015). Our qPCR results showed that the *CUC2* and *PLT1/2* were substantially increased in the *PLT5*-expressing calli of Bok choy, while the increase in *YUC4* transcript was not remarkable (Figure S11c). However, Radhakrishnan *et al*. (2020) found that *PLT3/5/7* require *YUC4* and *CUC2* but not *PLT1/2* for vascular regeneration in damaged aerial organs. This may indicate that the mechanism for shoot regeneration from *in vitro* explants is different from the one regulating shoot regeneration from the damaged aerial tissues. However, *PLT3/5/7* is required for both types of organ regeneration, which could be explained by the response of *PLT3/5/7* to wound signals in both scenarios (i.e. wound of excised explants and wound of mechanical injury in aerial tissues) (Kareem *et al*., 2015; Radhakrishnan *et al*., 2020).

Despite the success of *in planta* transformation promoted by *PLT5* in snapdragon and tomato, no success was made in Bok choy and sweet pepper. One of the possible explanations is that wounding triggers the defence response of plants, resulting in rapid accumulation of lignin, deposition of suberin and cell death around wounded tissues to form a barrier for restricting pathogen infection at the wound sites (Ginzberg, 2008; Graça, 2010). Our results also showed that *PLT5* was able to promote sweet pepper to acquire cell pluripotency during *in vitro* culture, yet only transgenic leaf-structure organs but no shoots regenerated from the somatic embryos (Figure S15). In Arabidopsis, overexpression of *PLT5* promotes *de novo* shoot regeneration from calli on the cytokinin-free medium; nevertheless, expression of *PLT5* resulted in a lower frequency of shoot regeneration when compared to cytokinin-induced shoot regeneration (Kareem *et al*., 2015). As discussed above, *PLT5* expression precedes the expression of *WUS*, which is induced by the high cytokinin for shoot progenitor establishment (Shin *et al*., 2020). This raises the question of whether co-expression of *PLT5* and *WUS* will have a synergistic effect in promoting embryogenic calli formation and completing reconstitution of programming for *de novo* shoot regeneration in some recalcitrant species like pepper.

To date, *in vitro* transformation is the predominant method used for most plant species. However, several advantages should be considered for *in planta* transformation. Firstly, compared with the *in vitro* transformation, procedures for *in planta* transformation are relatively simple and less time-consuming. The *in planta* transformation could circumvent the barrier for functional studies and genetic improvement in plant species with a long juvenile phase, such as some fruit trees, because a lengthy process is required for harvesting progenies (e.g. seeds or fruits) from these species. Secondly, grafting genomics is gaining popularity for studying the long-distance communication between the scion and rootstock (Thomas and Frank, 2019). However, the development of transgenic plants as scions is generally required before these scions are grafted to rootstocks. Thus, the development of successful *in planta* transformation may facilitate the discovery of new long-distance signalling molecules in a wide range of plant species.

Methods

Plant materials

Snapdragon (*A. majus*) stock line 'JI2' and 'Sippe50' were kindly provided by John Innes Center, UK. Inbred snapdragon seeds were derived from the 6th round of 'JI2×Sippe50'. Seedlings of tomato hybrid (*S. lycopersicum var* 'Big Beef'), sweet pepper (*C. annuum*, *var* 'California Wonder') and the seeds of Bok choy and Pai-Tsai (long white stalk) (*B. rapa*) were purchased from the local market.

Vector construction

A previous binary expression vector (PHN-SpCas9-4 × BsaI-GFP) containing a fused eGFP-*NPTII* gene under a double-enhanced CsVMV (dCsVMV) was modified through the following steps (Nguyen *et al*., 2021). The *NPTII* is a gene encoding a neomycin phosphotransferase for Kanamycin resistance, which is used as a selective marker for transformant screening. The AtU6 promoter cassette of the PHN-SpCas9-4 × BsaI-GFP vector was removed through digestion with *AvrII*; two oligos for an omega enhancer with two *BsaI* cloning sites were synthesized, annealed and inserted into the plasmid at the *AscI* digestion sites to replace the SpCas9 gene; the 2×CaMV35S promoter was amplified from the pGWB402 (Nakagawa *et al*., 2007) and inserted into the aforementioned construct through *HindIII* digestion and ligation to form an overexpression vector POX135 as shown in Figure 1.

A *DELILA* gene (*DEL*) (M84913.1) for anthocyanin biosynthesis in *A. majus* was synthesized (Gene Universal Inc.) and ligated into the POX135 at two *BsaI* cloning sites to form POX135-DEL (Figure 1a). In addition, two genes for *ESR1* (AT1G12980) and *WIND1* (AT1G78080) were PCR cloned from Arabidopsis Columbia ecotype with primers AtESRAscIF3/AtESRBamHR3 and AtWIND1AscIF3/AtWIND1BamHR3, respectively (Table S3). The coding sequences of three genes of *WUS* (AT2G17950), *PLT5* (AT5G57390) and a fused *WUS-P2A-BBM* (AT5G17430) gene linked with a self-cleaving peptide (ATNFSLLKQAGDVEENPGP)

from porcine teschovirus (P2A) (Sun *et al*., 2017) were synthesized by Gene Universal (Gene Universal Inc., Newark, DE). To make a short cassette of CaMV35S::DRs::NOS, the OCS terminator in PGSA1165 (www.arabidopsis.org) was replaced by the NOS terminator amplified with the primers NOSterSpeI-F and NOSterHindIII-R from pGWB402 through digestions with *SpeI* and *HindIII* and subsequent ligation. Each DR was ligated to the resultant vector CaMV35S::NOS through digestions with *AscI* and *BamHI* and subsequent ligation. The CaMV35S::DRs::NOS were amplified with the primer set of 35S-AatII-F/ NOSterMluI-R and ligated to POX135-DEL at the *AatII* and *MluI* restriction sites and to form the final expression plasmids without or with different DRs (Figure 1b). All plasmid DNAs were transformed into *A. tumefaciens* strain EHA105 or GV3101 through freeze and thaw methods.

Transformation through injection of *A. tumefaciens* with POX135-DEL-DRs

Seeds of the aforementioned plants were germinated in Petri dishes with moistened blot papers, then transferred to Pro-Mix soil and grown in the growth chamber with 16 h light/8 h dark photoperiod at 25 °C. The 20 μL *A. tumefaciens* stock solution was first cultured in the 5 mL liquid LB (LB Broth, Thermo Scientific) at 28 °C, 180 rpm overnight, then re-growing 1 mL fresh *A. tumefaciens* solution to the 10 mL new LB for the second round culture until the O.D. 600 is up to 0.4-0.5, and *A. tumefaciens* cells were collected by a centrifugation at 4415 *g* for 15 min and resuspended to an O.D.600 of 0.8 with 1 x MS liquid consisting of 4.6 g/L MS salts and vitamins (PhytoTechnology Laboratories), 2% sucrose and 50 μM acetosyringone with an adjusted pH of 5.8. Injection of *A. tumefaciens* was performed as illustrated in Figure 2, and two rounds of injections were applied to each set of site. The injection sites were covered with a cotton ball, which was pre-soaked with *A. tumefaciens* injection solution (i.e. liquid MS+50 μM acetosyringone) and then wrapped with plastic Saran wraps to keep humidity for 2 days. The plants were placed in the dark at 25 °C for 2 days after injections before being transferred to the greenhouse.

Tissue-culture media

The basic MS medium was used for tissue culture, which was consisted of 4.6 g/L MS salts and vitamins (PhytoTechnology Laboratories, Lenexa, KS), 30 g/L sucrose and 8 g/L agar. The MS medium was autoclaved at 121 °C for 25 min after pH was adjusted to 5.8. The stock solutions of hormones were filtered and added into the medium as needed when the temperature of autoclaved medium dropped to ~50 °C. Callus and shoot-induction medium of MS + 3 mg/L 6-BA + 100 mg/L kanamycin + 100 mg/L Timentin, and root induction medium of 1/2 MS + 50 mg/L Kanamycin + 100 mg/L Timentin were applied to all *in vitro* culture experiments in this study.

Detection of GFP fluorescence

GFP fluorescence was detected with a fluorescent microscope (Leica, Wetzlar, Germany). The fluorescent signals were captured with an imaging system (Nikon D800 Digital Camera, USA, Melville, NY) attached to the microscope.

PCR analysis

Genomic DNA was extracted from leaf tissues according to the CTAB method (Del *et al*., 1989). PCR with primers for amplifying GFP was performed to detect presence of transgene. PCR were carried out with Q5 Hot Start High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA) according to its manual instruction. Amplified DNA fragments were separated by electrophoresis on 1.2% (wt/v) agarose gels, which were stained with SYBR green (Thermo Scientific, Waltham, MA) and visualized with the Omega LumTM Imaging system (Gel Company, San Francisco, CA). The primers used for GFP amplification are listed in Table S4.

Anthocyanin determination

Anthocyanin was extracted from the shoots of T1 snapdragon seedlings. The extraction and determination methods of anthocyanin were modified according to the one described by Neff and Chory (1998). In brief, five seedling stems were collected from T1 transgenic and wild-type snapdragons, respectively, and then ground to powder in the liquid N_2 after weighing. Ground samples were incubated in 300 μL extraction solution (297 μL of Methanol and 3 μL HCl) overnight; then, 200 μL Milli-Q H_2O and 500 μL of chloroform were added to each sample prior to spinning down the extract. 400 μL of supernatant was used and mixed with 237.6 μL of methanol and 2.4 μL HCl +160 μL Milli-Q H_2O to bring the volume up to 800 μL. The absorbance of each sample was read at 530 and 657 nm using the spectrophotometer for anthocyanin determination. The blank was 432 μL Methanol 48 μL HCl and 320 μL Milli-Q H_2O for a total of 800 μL.

Sudan-7B staining

The callus regenerated from Bok choy explants after about 16-day of culture on the MS medium containing 3mg/L 6-BA was used for Sudan Red 7B staining according to a previous method (Thermo Scientific) (Kadokura *et al*., 2018). In brief, the callus and seedling samples were dehydrated through a series of isopropanol (20%, 40% and 60%) for 20 min per treatment, then incubated in 60% isopropanol containing 0.5% Sudan Red 7B for 1 h. All samples were subsequently rehydrated through a reverse process and washed three times with distilled water before imaging. For seed staining, seed samples were first incubated in 15% commercial bleach containing 6% NaClO, and rotated in a shaker with 200 rpm at room temperature until the seed embryo was isolated for staining as described above.

RNA extraction and RT-PCR analysis

To determine transcript levels of anthocyanin biosynthetic genes in snapdragon T1 seedlings and downstream genes of *PLT5* in the transgenic calli of Bok choy, total RNA was isolated from 100 mg of samples using RNAzol® RT RN190 (Molecular Research Center, Cinicinnati, OH). The synthesis of cDNA was performed using QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) and then diluted 25-fold. The qRT-PCR reaction was composed of 4.5 μL cDNA, 0.5 μL 10 μmol/L primers and 5 μL Power SYBR® Green Master Mix (Thermo Fisher), and detected on a CFX96 real-time PCR system (BIO-RAD, Hercules, CA). The primers used for examining the transcripts of anthocyanin biosynthetic genes including *AmDEL* (M84913.1), *AmCHS* (X03710), *AmCHI* (AB861648), *AmF3H* (LC194907), *AmDFR* (P14721) and *AmUBQ* (ubiquitin) (X67957) were designed according to the previous report (Fujino *et al*., 2018); the primers used for examining the transcripts of *PLT5* downstream genes including *PLT1* (XM_009111838.2), *PLT2* (XM_009149529.2), *CUC1* (XM_009118219.3), *CUC2* (XM_033282328.1), *STM* (GU480585.1), *YUC4* (XM_009127551.3) and the reference gene Tubulin (*Tub*) (D78496) were designed according to the previous studies (Kareem *et al*., 2015; Qi *et al*., 2010; Radhakrishnan *et al*., 2020), and all primers for RT-PCR are listed in Table S4.

Acknowledgements

We thank Dr. Kent Bradford and Dr. Balasubramani Rathinasabapathi for the critical reading and comments.

Funding

This project was supported by the University of Florida-Early Scientist Award, USDA-HATCH program (FLA-MFC-005713) and USDA-NIFA GRANT12683186.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

H.H., H.G. and L.Z. conceived and designed experiments. C.N. and G.W. created plasmid DNAs. L.L., J.C., S.W., J.Y., S.W. and P.O.A provided critical suggestion in design and implementation of experiments. L.Z. and H.H. wrote the manuscripts. All authors read and revised the manuscript.

References

Altpeter, F., Springer, N.M., Bartley, L.E., Blechl, A.E., Brutnell, T.P., Citovsky, V., Conrad, L.J. *et al*. (2016) Advancing crop transformation in the Era of genome editing. *Plant Cell*, **28**, 1510–1520. https://doi.org/10.1105/tpc.16.00196

Ariga, H., Toki, S. and Ishibashi, K. (2020) Potato virus X vector-mediated DNA-free genome editing in plants. *Plant Cell Physiol.* **61**, 1946–1953. https://doi.org/10.1093/pcp/pcaa123

Boutilier, K., Offringa, R., Sharma, V.K., Kieft, H., Ouellet, T., Zhang, L.M., Hattori, J. *et al*. (2002) Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *Plant Cell*, **14**, 1737–1749. https://doi.org/10.1105/tpc.001941

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x

Collado, R., Bermúdez-Caraballoso, I., García, L., Veitía, N., Torres, D., Romero, C. and Angenon, G. (2016) Epicotyl sections as targets for plant regeneration and transient transformation of common bean using *Agrobacterium tumefaciens*. *In Vitro Cell. Dev. Biol. Plant*, **52**, 500–511.

Debernardi, J.M., Tricoli, D.M., Ercoli, M.F., Hayta, S., Ronald, P., Palatnik, J.F. and Dubcovsky, J. (2020) A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants. *Nat. Biotechnol.* **38**, 1274–1279.

Del, G.S., Manfioletti, G. and Schneider, C.(1989) The CTAB-DNA precipitation method: a common mini-scale preparation of template DNA from phagemids, phages or plasmids suitable for sequencing. *Biotechniques*, **7**, 514–520.

Dinesh-Kumar, S.P. and Voytas, D.F. (2020) Editing through infection. *Nat Plants*, **6**, 738–739. https://doi.org/10.1038/s41477-020-0716-1

Dyer, A.G., Whitney, H.M., Arnold, S.E., Glover, B.J. and Chittka, L. (2007) Mutations perturbing petal cell shape and anthocyanin synthesis influence bumblebee perception of *Antirrhinum majus* flower colour. *Arthropod Plant Interact.* **1**, 45–55.

Ellison, E.E., Nagalakshmi, U., Gamo, M.E., Huang, P.J., Dinesh-Kumar, S. and Voytas, D.F. (2020) Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nat Plants*, **6**, 620–624. https://doi.org/10.1038/s41477-020-0670-y.

Fujino, N., Tenma, N., Waki, T., Ito, K., Komatsuzaki, Y., Sugiyama, K., Yamazaki, T. *et al*. (2018) Physical interactions among flavonoid enzymes in snapdragon and torenia reveal the diversity in the flavonoid metabolon organization of different plant species. *Plant J.* **94**, 372–392.

Gaillochet, C. and Lohmann, J.U. (2015) The never-ending story: from pluripotency to plant developmental plasticity. *Development*, **142**, 2237–2249.

Ginzberg, I. (2008) Wound-periderm formation. In A. Schaller (Ed.), *Induced Plant Resistance to Herbivory*. pp 131–146. Dordrecht: Springer.

Graça, J. (2010) Hydroxycinnamates in suberin formation. *Phytochem. Rev.* **9**, 85–91.

Heidmann, I., De Lange, B., Lambalk, J., Angenent, G.C. and Boutilier, K. (2011) Efficient sweet pepper transformation mediated by the BABY BOOM transcription factor. *Plant Cell Rep.* **30**, 1107–1115.

Hoerster, G., Wang, N., Ryan, L., Wu, E., Anand, A., McBride, K., Lowe, K. *et al*. (2020) Use of non-integrating Zm-Wus2 vectors to enhance maize transformation. *In Vitro Cell. Dev. Biol. Plant*, **56**, 265–279.

Ikeuchi, M., Favero, D.S., Sakamoto, Y., Iwase, A., Coleman, D., Rymen, B. and Sugimoto, K. (2019) Molecular mechanisms of plant regeneration. *Annu. Rev. Plant Biol.* **70**, 377–406.

Ikeuchi, M., Iwase, A., Rymen, B., Lambolez, A., Kojima, M., Takebayashi, Y., Heyman, J. *et al*. (2017) Wounding triggers callus formation via dynamic hormonal and transcriptional changes. *Plant Physiol.* **175**, 1158–1174. https://doi.org/10.1104/pp.17.01035

Ikeuchi, M., Ogawa, Y., Iwase, A. and Sugimoto, K. (2016) Plant regeneration: cellular origins and molecular mechanisms. *Development*, **143**, 1442–1451.

Iwase, A., Harashima, H., Ikeuchi, M., Rymen, B., Ohnuma, M., Komaki, S., Morohashi, K. *et al*. (2017) WIND1 promotes shoot regeneration through transcriptional activation of ENHANCER OF SHOOT REGENERATION1 in Arabidopsis. *Plant Cell*, **29**, 54–69.

Iwase, A., Kondo, Y., Laohavisit, A., Takebayashi, A., Ikeuchi, M., Matsuoka, K., Asahina, M. *et al*. (2021) WIND transcription factors orchestrate wound-induced callus formation, vascular reconnection and defense response in Arabidopsis. *New Phytol.* **232**, 734–752. https://doi.org/10.1111/nph.17594

Iwase, A., Mita, K., Nonaka, S., Ikeuchi, M., Koizuka, C., Ohnuma, M., Ezura, H. *et al*. (2015) WIND1-based acquisition of regeneration competency in Arabidopsis and rapeseed. *J Plant Res.* **128**, 389–397. https://doi.org/10.1007/s10265-015-0714-y

Iwase, A., Mitsuda, N., Koyama, T., Hiratsu, K., Kojima, M., Arai, T., Inoue, Y. *et al*. (2011) The AP2/ERF transcription factor WIND1 controls cell dedifferentiation in Arabidopsis. *Curr. Biol.* **21**, 508–514.

Iwase, A., Mitsuda, N., Ikeuchi, M., Ohnuma, M., Koizuka, C., Kawamoto, K., Imamura, J. *et al*. (2013) Arabidopsis WIND1 induces callus formation in rapeseed, tomato, and tobacco. *Plant Signal. Behav.* **8**, e27432. https://doi.org/10.4161/psb.27432

Jones, T., Lowe, K., Hoerster, G., Anand, A., Wu, E., Wang, N., Arling, M. *et al*. (2019) Maize transformation using the morphogenic genes Baby Boom and Wuschel2. In S. Kumar, P. Barone and M. Smith (Eds.), *Transgenic Plants. Methods in Molecular Biology*. vol 1864, pp 81–93. New York: Springer.

Kadokura, S., Sugimoto, K., Tarr, P., Suzuki, T. and Matsunaga, S. (2018) Characterization of somatic embryogenesis initiated from the Arabidopsis shoot apex. *Dev. Biol.* **442**, 13–27.

Kareem, A., Durgaprasad, K., Sugimoto, K., Du, Y., Pulianmackal, A., Trivedi, Z., Abhayadev, P. *et al*. (2015) PLETHORA genes control regeneration by a two-step mechanism. *Curr. Biol.* **25**, 1017–1030.

Keshavareddy, G., Kumar, A. and Ramu, V.S. (2018) Methods of plant transformation – a review. *Int. J. Curr. Microbiol. Appl. Sci.* **7**, 2656–2668.

Krizek, B.A. (2015) AINTEGUMENTA-LIKE genes have partly overlapping functions with AINTEGUMENTA but make distinct contributions to *Arabidopsis thaliana* flower development. *J. Exp. Bot.* **66**, 4537–4549.

Lian, Z., Nguyen, C.D., Wilson, S., Chen, J., Gong, H. and Huo, H. (2020) An efficient protocol for *Agrobacterium*-mediated genetic transformation of *Antirrhinum majus*. *Plant Cell Tissue Organ Cult.* **142**, 527–536.

Lotan, T., Ohto, M., Yee, K.M., West, M.A.L., Lo, R., Kwong, R.W., Yamagishi, K. *et al*. (1998) Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell*, **93**, 1195–1205. https://doi.org/10.1016/S0092-8674(00)81463-4

Lowe, K., Wu, E., Wang, N., Hoerster, G., Hastings, C., Lenderts, B., Chamberlin, M., Arling, M., Anand, A. and Annalura, V. *Use of the maize morphogenic genes BBM and WUS2 to improve monocot transformation*. In IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY-ANIMAL, 2016. SPRINGER 233 SPRING ST, NEW YORK, NY 10013 USA, pp S5-S5

Ma, X.N., Zhang, X.Y., Liu, H.M. and Li, Z.H. (2020) Highly efficient DNA-free plant genome editing using virally delivered CRISPR-Cas9. *Nat Plants*, **6**, 773–779. https://doi.org/10.1038/s41477-020-0704-5

Maher, M.F., Nasti, R.A., Vollbrecht, M., Starker, C.G., Clark, M.D. and Voytas, D.F. (2020) Plant gene editing through de novo induction of meristems. *Nat. Biotechnol.* **38**, 84–89.

Martins, P.K., Nakayama, T.J., Ribeiro, A.P., Cunha, B., Nepomuceno, A.L., Harmon, F.G., Kobayashi, A.K. *et al*. (2015) *Setaria viridis* floral-dip: a simple and rapid *Agrobacterium*-mediated transformation method. *Biotechnol Rep (Amst)*, **6**, 61–63. https://doi.org/10.1016/j.btre.2015.02.006

Melnyk, C.W., Schuster, C., Leyser, O. and Meyerowitz, E.M. (2015) A developmental framework for graft formation and vascular reconnection in *Arabidopsis thaliana*. *Curr. Biol.* **25**, 1306–1318.

Mukeshimana, G., Ma, Y., Walworth, A.E., Song, G.-Q. and Kelly, J.D. (2013) Factors influencing regeneration and *Agrobacterium tumefaciens*-mediated transformation of common bean (*Phaseolus vulgaris* L.). *Plant Biotechnol. Rep.* **7**, 59–70.

Naing, A.H. and Kim, C.K. (2018) Roles of R2R3-MYB transcription factors in transcriptional regulation of anthocyanin biosynthesis in horticultural plants. *Plant Mol. Biol.* **98**, 1–18.

Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maeo, K., Tabata, R. *et al*. (2007) Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci. Biotechnol. Biochem.* **71**, 2095–2100.

Nasti, R.A. and Voytas, D.F. (2021) Attaining the promise of plant gene editing at scale. *Proc. Natl Acad. Sci. USA*, **118**(22), e2004846117.

Neff, M.M. and Chory, J. (1998) Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. *Plant Physiol.* **118**, 27–35.

Nelson-Vasilchik, K., Hague, J., Mookkan, M., Zhang, Z.J. and Kausch, A. (2018) Transformation of recalcitrant sorghum varieties facilitated by baby boom and Wuschel2. *Curr Protoc Plant Biol.* **3**, e20076. https://doi.org/10.1002/cppb.20076

Nguyen, C.D., Li, J., Mou, B., Gong, H. and Huo, H. (2021) A case study of using an efficient CRISPR/Cas9 system to develop variegated lettuce. *Vegetable Res.* **1**, 1–10.

Prasad, K., Grigg, S., Barkoulas, M., Yadav, R., Sanchez-Perez, G., Pinon, V., Blilou, I. *et al*. (2011) Arabidopsis PLETHORA transcription factors control phyllotaxis. *Curr. Biol.* **21**, 1123–1128.

Qi, J., Yu, S., Zhang, F., Shen, X., Zhao, X., Yu, Y. and Zhang, D. (2010) Reference gene selection for real-time quantitative polymerase chain reaction of mRNA transcript levels in Chinese cabbage (*Brassica rapa* L. ssp. pekinensis). *Plant Mol. Biol. Rep.* **28**, 597–604.

Radhakrishnan, D., Shanmukhan, A.P., Kareem, A., Aiyaz, M., Varapparambathu, V., Toms, A., Kerstens, M. *et al*. (2020) A coherent feed-forward loop drives vascular regeneration in damaged aerial organs of plants growing in a normal developmental context. *Development*, **147**, dev185710.

Radoeva, T. and Weijers, D. (2014) A roadmap to embryo identity in plants. *Trends Plant Sci.* **19**, 709–716.

Saha, P. and Blumwald, E. (2016) Spike-dip transformation of *Setaria viridis*. *Plant J.* **86**, 89–101. https://doi.org/10.1111/tpj.13148

Schwarz-Sommer, Z., Davies, B. and Hudson, A. (2003) An everlasting pioneer: the story of Antirrhinum research. *Nat. Rev. Genet.* **4**, 655–664.

Shin, J., Bae, S. and Seo, P.J. (2020) De novo shoot organogenesis during plant regeneration. *J. Exp. Bot.* **71**, 63–72.

Sun, H., Zhou, N., Wang, H., Huang, D. and Lang, Z. (2017) Processing and targeting of proteins derived from polyprotein with 2A and LP4/2A as peptide linkers in a maize expression system. *PLoS One*, **12**, e0174804.

Thomas, H.R. and Frank, M.H. (2019) Connecting the pieces: uncovering the molecular basis for long-distance communication through plant grafting. *New Phytol.* **223**, 582–589.

Valvekens, D., Van Montagu, M. and Van Lijsebettens, M. (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl Acad. Sci. USA*, **85**, 5536–5540.

Wang, M., Gao, S.L., Zeng, W.Z., Yang, Y.Q., Ma, J.F. and Wang, Y. (2020) Plant virology delivers diverse toolsets for biotechnology. *Viruses*, **12**, 1338. https://doi.org/10.3390/v12111338

Xie, Y., Tan, H., Ma, Z. and Huang, J. (2016) DELLA proteins promote anthocyanin biosynthesis via sequestering MYBL2 and JAZ suppressors of the MYB/bHLH/WD40 complex in *Arabidopsis thaliana*. *Mol. Plant*, **9**, 711–721.

Yano, R., Kanno, Y., Jikumaru, Y., Nakabayashi, K., Kamiya, Y. and Nambara, E. (2009) CHOTTO1, a putative double APETALA2 repeat transcription factor, is involved in abscisic acid-mediated repression of gibberellin biosynthesis during seed germination in Arabidopsis. *Plant Physiol.* **151**, 641–654.

Zhang, X., Xu, G., Cheng, C., Lei, L., Sun, J., Xu, Y., Deng, C. *et al*. (2021) Establishment of an *Agrobacterium*-mediated genetic transformation and CRISPR/Cas9-mediated targeted mutagenesis in Hemp (*Cannabis sativa* L.). *Plant Biotechnol. J.*, 19(10), 1979–1987.

Zhao, X.Y., Su, Y.H., Cheng, Z.J. and Zhang, X.S. (2008) Cell fate switch during in vitro plant organogenesis. *J. Integr. Plant Biol.* **50**, 816–824.

Zuo, J.R., Niu, Q.W., Frugis, G. and Chua, N.H. (2002) The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. *Plant J.* **30**, 349–359. https://doi.org/10.1046/j.1365-313X.2002.01289.x

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Genetic transformation confirmation of snapdragon by GFP fluorescence and PCR genotyping.

Figure S2 Detection of GFP fluorescence in the radicles and roots of germinated T1 seeds of transgenic snapdragons.

Figure S3 Stable inheritance of transgenes to snapdragon T1 seedlings.

Figure S4 Enriched anthocyanin due to the upregulation of anthocyanin biosynthetic genes.

Figure S5 Induction of callus-like tissues by *PLT5* at wound sites in soil-grown Bok choy plants.

Figure S6 Induction of the callus-like tissues formation by *PLT5* at the wound site in soil-grown sweet pepper.

Figure S7 Promoting root regeneration and genetic transformation in the Bok Choy with the aid of *PLT5*.

Figure S8 Shoots regeneration from *PLT5*-expressing transgenic root tips of the Bok choy.

Figure S9 Promoting somatic embryo development, shoot regeneration, and genetic transformation with the aid of *PLT5* in Bok choy.

Figure S10 Induction of somatic embryogenesis by *PLT5* in Bok choy.

Figure S11 The relative expression level of key regulator genes for shoot regeneration in the callus of Bok choy.

Figure S12 The effect of *PLT5* on shoot regeneration and genetic transformation in Pei-Tsai.

Figure S13 Transformation confirmation in Bok choy and Pei-Tsai.

Figure S14 Genetic transformation in sweet pepper using hypocotyls as the explants.

Figure S15 Promoting regeneration of leaf-like organs from the calli in sweet pepper with the aid of *PLT5*.

Figure S16 Confirmation of genetic transformation by GFP fluorescence detection and PCR genotyping in sweet pepper.

Figure S17 The morphology of regenerated shoots in snapdragon due to overexpression of *PLT5*.

Figure S18 The morphology of regenerated tomato shoots due to overexpression of *PLT5*.

Figure S19 The abnormal vegetative morphology of the Bok choy due to the overexpression of *PLT5*.

Table S1 Effects of *PLT5* on shoot regeneration and transformation from transgenic root tips in Bok choy.

Table S2 Effects of *PLT5* on embryogenic calli regeneration and transformation in sweet pepper.

Table S3 The primers used for constructing POX135-DEL-DRs vector.

Table S4 The primers used for qRT-PCR analysis and transformation genotyping.

Appendix S1. Illustration for data collection and statistical analysis.