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

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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 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 annuum) 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; Ikuchi 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, transgenens 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 (Gaillloch et al., 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 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 medium for callus formation and shoot regeneration, 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 (LBs), CUP-SHAPED COTYLEDON (CUC1 and CUC2), CLAVAT3 (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; Lwase et al., 2011). Overexpressing WIND1 and its homologous genes (WIND2-4) promotes callus growth in the absence of exogenous hormones (Ikeuchi et al., 2017; Lwase et al., 2011). A recent study showed that WIND1 directly up-regulated the expression of ESR1 to promote CUC1-mediated shoot regeneration (Lwase et al., 2017). Lwase 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 (Lwase 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/SI7 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-Vasilikha 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 IFT 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), WUSCHEL (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 annuum) 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 DELA (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;
and WUS porcine teschovirus was included between studies.

The complete application of snapdragon in genetic and molecular genetic transformation pipeline is the primary limiting factor for were excised at the stage of flower bud initiation (Figure 2a).

et al. Schwarz-Sommer, 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–i). 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 ESR1 or WUS-BBM in both A. tumefaciens strains (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, 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 S3a). 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) (Nair 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.
Figure 2  Promoting shoot regeneration and transformation by overexpression of DR genes in soil-grown *A. majus*. (a) Primary and auxiliary shoots were cut off from the about 70-day-old snapdragons, but a roughly 0.3 mm stem base of the auxiliary 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).
PLTS 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 PLTS 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 PLTS, 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 3c). 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 PLTS 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 PLTS, followed by WUS with a transformation efficiency of 3.3% (Figure 3i), and no transgenic shoots were obtained after injection of plasmids with WIND1 or without DRs (Figure 3j). 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
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). Inoculation 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

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**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.
from the same callus were counted as one independent transgenic event.

| Varieties   | Treatments | Experiments | No. of the explants | No. of the transgenic embryonic calli | Total numbers of the transgenic shoots | Transformation efficiency (%)* |
|-------------|------------|-------------|---------------------|---------------------------------------|----------------------------------------|-----------------------------|
| Bok choy    | Control    | No. 1       | 25                  | 0                                     | 0                                      | 0.0                         |
|             |            | No. 2       | 25                  | 0                                     | 0                                      | 0.0                         |
|             |            | No. 3       | 25                  | 0                                     | 0                                      | 0.0                         |
|             |            | No. 4       | 25                  | 0                                     | 0                                      | 0.0                         |
|             |            | Total       | 100                 | 0                                     | 0                                      | 0.0                         |
|             | 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    | Control    | No. 1       | 25                  | 0                                     | 0                                      | 0.0                         |
|             |            | No. 2       | 25                  | 0                                     | 0                                      | 0.0                         |
|             |            | No. 3       | 25                  | 0                                     | 0                                      | 0.0                         |
|             |            | No. 4       | 25                  | 0                                     | 0                                      | 0.0                         |
|             |            | Total       | 100                 | 0                                     | 0                                      | 0.0                         |
|             | 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 embryonic (Figure S10e,i). The dark-red staining of these embryogenic calli resembled the staining of Bok choy seed (Figure S10f,j). These results indicate that the staining of the expanded cotyledon of Bok choy seedling (Figure S10f,j). These results suggest 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). However, 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
PLT5 improves genetic transformation

(a) CK
(b) CK
(c) OE-PLT5
(d) OE-PLT5
(e) OE-PLT5

10 d
10 d
10 d
21 d
25 d
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 phylloysis (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, i). Interestingly, the transformation efficiencies for the wound position were usually much higher compared with the ones for the axillary position (Figures 2i and 3i). 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 pl3/5/7, suggesting the indispensable function of PLT3/5/7 in wound-induced callus formation (Iwase et al., 2021; Melnyk et al., 2015). Although
PLT5 improves genetic transformation

(a) CK
(b) CK
(c) CK
(d) OE-PLT5
(e) OE-PLT5
(f) OE-PLT5
(g) WT
(h) T1 seed
(i) Cotyledon
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 2). 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/Si7 controlled plant regeneration via a two-step mechanism, in which the root stem cell regulators PLT1/2 were first activated by PLT3/Si7 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/Si7 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/Si7 is required for both types of organ regeneration, which could be explained by the response of PLT3/Si7 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 × Bsal-GFP) containing a fused eGFP-NPTII gene under a double-enhanced CsVMV (dCsVMV) was modified through the following steps (Nguyen et al., 2021). The NPTh is a gene encoding a neomycin phosphotransferase for kanamycin resistance, which is used as a selectable marker for transformant screening. The AtU6 promoter cassette of the PHN-SpCas9-4 × Bsal-GFP vector was removed through digestion with AvrII; two oligos for an omega enhancer were designed in the process at the Asct 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 Bsal 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 AtESRAscF3/AtESRBamHR3 and AtWIND1AscF3/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 (ATNFSLLKQAGDVENPGP)
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 PGSA1165 (www.arabidopsis.org) was replaced by the NOS short cassette of CaMV35S::DRs::NOS, the OCS terminator in by Gene Universal (Gene Universal Inc., Newark, DE). To make a 
carried out with Q5 Hot Start High-Fidelity DNA polymerase (New 
autoclaved medium dropped to  
with POX135-DEL-DRs 
method (Del 
Genomic DNA was extracted from leaf tissues according to the 
with an imaging system (Nikon D800 Digital Camera, USA, 
and GFP fluorescence 
DNA was amplified with the primer set of 35S-AatII-F/ NOSterMluI-R and 
BamHI 
and subsequent ligation. The CaMV35S::DRs::NOS were 
AmDEL (MB84913.1), AmCHS (X03710), AmChl (AB861648), AmF3H (LC194907), AmDFR (P14721) and AmUBQ (ubiquitin) (X67957) 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. 

**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 N2 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 H2O 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 H2O 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 H2O 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 iso-propanol (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, Cincinnati, 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 (MB84913.1), AmCHS (X03710), AmCHl (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. 

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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.

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Conflict of interest
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