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Novel Plant Regeneration and Transient Gene Expression in *Catharanthus roseus*

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Abstract: *Catharanthus roseus* genetic transformation represents a real challenge due, in part, to the lack of regeneration capability and to the recalcitrant nature of this species to genetic transformation. In the present work, we demonstrate the regeneration of *C. roseus* plants from hypocotyls and cotyledons, using specific growth regulator conditions. Plants derived from hypocotyls and cotyledons were successfully acclimated and grown in the greenhouse. Furthermore, *C. roseus* meristem tissues were shown to have high shoot regeneration potential under conditions optimised for cotyledon- and hypocotyl-derived shooting. Meristem tissues were, therefore, investigated as a genetic transformation target using both *Agrobacterium tumefaciens* and *A. rhizogenes*. Although meristem-derived shoots transformed with *A. tumefaciens* harbouring a p35S *GUSPlus* construct revealed transient *GUS* expression and protein accumulation, they were not amenable to selection even after two months on selection medium. Transformation of *C. roseus* meristem tissues with *A. rhizogenes* resulted in a typical hairy root phenotype, in which the adventitious root tissue strongly expressed the p35S *GUSPlus* construct, as revealed by intense GUS staining.

Keywords: *Catharanthusroseus*, regeneration, meristem, transient genetic transformation

Introduction

Medicinal plants represent important sources of drugs and the use of genetic transformation systems to increase the production of these molecules requires efficient plant regeneration and genetic transformation systems. *Catharanthusroseus*(L.) G. Don (Apocynaceae)**,** native to Madagascar, is known to produce many indole alkaloid compounds. Of these, vinblastine and vincristine are effective anti-cancer agents against leukaemia and Hodgkin's diseases[1]. Tissue-based compartmentalization of the last steps of indole alkaloid biosynthesis means that specific cell types, but especially idioblasts and laticifers in leaves and young buds, are required for vinblastine and vincristine biosynthesis *in vivo* [2,3] . These differentiated tissues do not exist in cell cultures (e.g., callus, cell suspension) and the vindoline pathway is blocked in these cultures because the final reactions catalyzed by desacetoxyvindoline 4-hydroxylase (D4H) [4] and acetyl coenzyme A (CoA): deacetylvindoline-4-*O*-acetyltransferase (DAT) are absent [2,5]. There is one report of vindoline production in callus culture transformed by different *Agrobacterium tumefaciens*[6]; however, this has not been further substantiated. Thus, it is necessary to be able to genetically transform and subsequently regenerate *C. roseus* plants to be able to modify and/or analyse the last steps of the indole alkaloid pathway in this species. However, *C. roseus* has proven to be difficult to transform using standard genetic transformation systems, particularly in the steps for plant regeneration.

Hairy roots system has been extensively exploited in plant genetic engineering, gene and promoter and plant molecular pharming studies[2,7-9]. With the exception of one report of the regeneration of *C. roseus* plants from genetically transformed hairy roots under very special conditions [10] and another from hypocotyles[11,12], no regeneration of genetically transformed plants has been reported for this species from apex or primordia tissues; however, these two systems are not reproducible based on our experiments on most organs and tissues explants. Limited success in *C. roseus* plant regeneration has been had with zygotic embryos [11]and petiole explants [13], the latter of which gave rise to shoots with a 40% regeneration efficiency. A few groups have reported the successful regeneration of *C. roseus* using seedlings as explants [14,15]. More recently, an alternative method to generate *C. roseus* shoots *in vitro* through somatic embryos derived from embryogenic calli was described [16]. Yet, another approach made use of nodal explants [17] containing

two auxiliary buds as a target tissue source for microparticle bombardment and plant regeneration [18], albeit with very low transformation rates and the generation of numerous chimeras.

Although meristem shoot regeneration systems have been described for many plant species [19], this system has not been exploited for *C. roseus*. In general, plants are characterized by their post-embryonic organogenesis and have sets of stem cells, localized in shoot apical and root meristems that remain in an undifferentiated state [20]. Shoot apical meristem stem cells are characterized by quick regeneration and competence for genetic transformation and have been successfully used to obtain transgenic plants in maize, wheat, rice, oat, barley, sorghum, and millet [21]. Furthermore, meristematic tissues have been used for shoot apical meristem genetic transformation of several plants such as rice [22], maize [23] ,[24] and yellow lupin[25]. Similarly, axillary shoot meristems have been used for the transformation of pear cultivars recalcitrant to regeneration [26]. Bulk meristematic tissue obtained by removing the apical dome [27]has been used to transform cotton [28]or castor mature seed embryo axes [29].

In this paper, we describe a highly efficient shoot regeneration system using hypocotyl-and cotyledon-derived callus, as well as shoot apical meristems and their surrounding cells. This degree of shoot formation provides an optimal amount of explants for plant regeneration and potentially genetic engineering of this species. We further regenerated whole plants from shoots obtained from hypocotyl-and cotyledon-derived callus, including their acclimation and transfer to the greenhouse. Lastly, genetic transformation using both *A. tumefaciens* and *A. rhizogenes*is was demonstrated.

Results

Plant Regeneration

In a preliminary set of experiments, we applied varying amounts and ratios of NAA and BAP to different organs (e.g., roots, hypocotyls and cotyledons) from cv. Pacifica Pink to determine the optimal conditions for shoot formation. Shoot formation was highest (ca. 70%) from callus derived from hypocotyl explants after 35 days of culture on media supplemented with NAA (1 mg/L) and BAP (1 mg/L) (Figure 1, 2a). Calli from roots and cotyledons did not produce many shoots. Next we tested the shooting ability of hypocotyl explants from six *C. roseus* cultivars using 1 mg/L each of NAA and BAP. We readily obtained shoots from callus derived from the upper part of hypocotyls of all six cultivars, but less so from the lower part (Table1). In this experiment, the highest amount of shoot regeneration was obtained from the cv. Cooler Coconut (16.6 %). Although this was much lower than that obtained with cv. Pacifica Pink in our preliminary experiments, we were able to subsequently obtain whole plants from these shoots (see below).

Table 1. Yield of Shoots from hypocotyl-derived calli segments from six cultivars of *Catharanthusroseus*. Hypocotyl segments from *in vitro* grown seedlings were divided into upper and lower parts prior to plating on MS medium supplemented with 1 mg/L NAA and BAP. The number of explant-derived calli pieces on which shoots were evident was counted after 2 months in culture, and expressed as a fraction of the total number of calli plated. Cultivar name Abbreviations: BP = Blue Pearl, CC = Cooler Coconut, CMI = Cooler Mix Improved, ML = Mediterranean Lilas, PP = Pacifica Pnk, SR = Stardust Rose.

Cultivar	Number of Calli with Shoots	
	Upper Hypocotyls	Lower Hypocotyls
BP	4/40 (10%)	0/40 (0%)
CC	10/60 (16.6%)	0/60 (0%)
CMI	5/80 (6.3%)	0/50 (0%)
ML	2/40 (5%)	1/50 (2%)
PP	9/100 (9%)	0/80 (0%)
SR	9/70 (12.9%)	0/80 (0%)

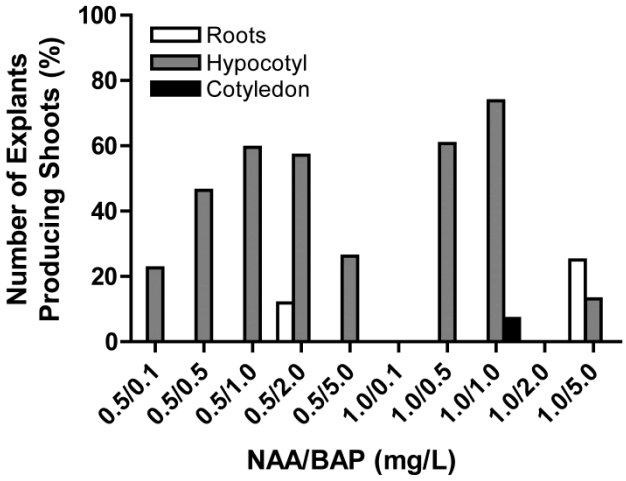

Figure 1. Shoot formation from *Catharanthus roseus* explants. Various explant tissues (cotyledons, hypocotyls, roots) from *in vitro* grown *C. roseus* plantlets (cv. Pacifica Pink) were cultured on MS medium supplemented with different concentrations of NAA and BAP. Categorical values in the x-axis are expressed as ratios of NAA/BAP (both in mg/L). The percentage of explant-derived calli with regenerated shoots (y-axis) was recorded after 35 days in culture. Data are pooled from a minimum of 120-150 explants plated on 4 to 5 independent plates (minimum of 30 explants per plate) for each NAA/BAP combination.

Root Formation:

Shoots obtained from hypocotyl and cotyledon explants were transferred to MS medium containing various growth regulators, and sub-cultured on a 4-week cycle. After four to five subculture cycles on media supplemented with either IAA (10 mg/L) and BAP (0.1 mg/L) or IBA (10 mg/L) and BAP (0.1 mg/L), roots began to appear (Figure 2b, Table 2). Prior to acclimation, these were transferred to MS solidified with gelrite instead of agar to improve rooting and allow plantlet recovery with minimal damage to the roots.

Plant Acclimation:

Plants were successfully acclimated and transferred to the greenhouse (Figure 2c). Out of 40 shoots taken through the rooting steps, only 14 survived through to full plants in the greenhouse (Table 2). The overall process, from initial *in-vitro* shoot formation from hypocotyls or cotyledons through

to the potting of regenerated plants in the greenhouse took approximately 1 year.

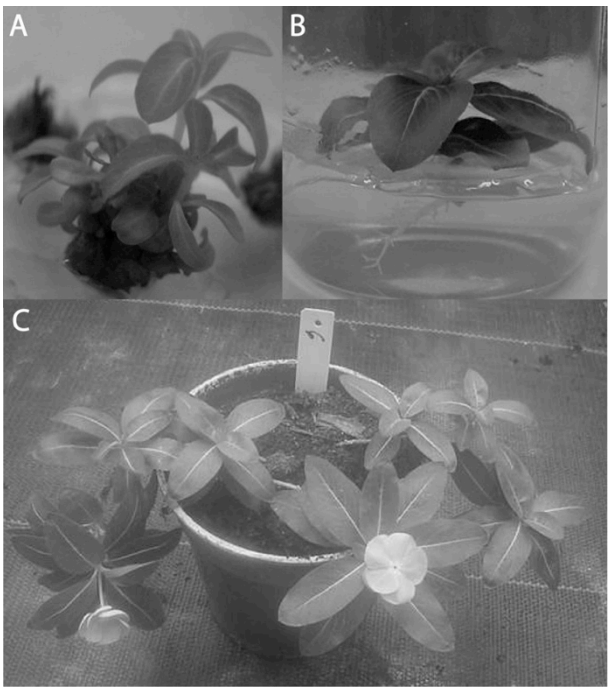

Figure 2. Regeneration of *Catharanthus roseus* plants from callus. (A) Shoots were obtained from hypocotyl-derived callus after several transfers on MS medium supplemented with 1 mg/ml each of NAA and BAP. (B) Shoots were transferred to MS medium supplemented with either IAA (10 mg/L) and BAP (0.1 mg/L) or IBA (10 mg/L) and BAP (0.1 mg/L). (C) Mature plants of *C. roseus* were obtained from in vitro plantlets, after a period of acclimation. Data for cv. Pacifica Pink are shown.

Table 2. Yield of rooted hypocotyl-and cotyledon-derived shoots from *Catharanthusroseus*. Shoots derived from calli obtained from either cotyledons or hypocotyls were rooted on MS media supplemented with either IAA 10 mg/L and BAP 0.1 mg/L or IBA 10 mg/L and BAP 0.1 mg/L and later on the same medium solidified with gelrite instead of agar. After hardening and acclimation, plants placed into pots of vermiculite under 25°C ± 1°C, 18 μE/cm^2/s, (12h/12h) conditions for one month before transferring them to the greenhouse (20°C ± 1°C) under natural light and day length.

Original Explant	Number of Explants	Number of Acclimated plants	Time to Acclimation (months)
Cotyledon	16	7 (43%)	12-15
Upper hypocotyl	9	3 (33%)	14-15
Lower hypocotyl	15	4 (26%)	15

Meristem Culture

Meristem explants obtained from all six cultivars were able to form shoots with similar efficiency (Table 3) in as little as 30 days, when cultured with NAA (1 mg/L) and BAP (1 mg/L). The highest efficiency achieved (97%) was with the cultivar Mediterranean Lilas. Adding high concentrations (e.g., higher than 5 mg/L of BAP) caused shoot malformation (data not shown).

Table 3. Yield of Shoots from meristem explants from six cultivars of *Catharanthusroseus*. Meristem segments from 9-to 10-day old in vitro grown seedlings were plated on MS medium supplemented with 1 mg/mL NAA and BAP. The number of explants on which shoots were evident was counted after 35 days in culture, and expressed as a fraction of the total number of explants plated.

Cultivar	Number of Meristems with Shoots
BP	35/50 (70%)
CC	130/138 (94.2%)
CMI	122/127 (96.1%)
ML	77/79 (97.5%)
PP	122/129 (94.5%)
SR	136/151 (90.1%)

Transformation of meristems with *Agrobacterium tumefaciens*

Amplification of a band of the correct size (750 bp) using an exon-exon junction primer confirmed the presence of the β-glucuronidase (*GUSPlus*) transgene in meristem cells (Figure 3a). Further proof of the incorporation of *GUSPlus* into meristem tissues, as well as its expression, was obtained using a dot-blot assay to detect His-tagged proteins. Thus, proteins extracted from meristems co-cultivated with *A. tumefaciens* were blotted onto nitrocellulose and probed with anti-His antibody [30]. Only those extracts obtained from transformed meristems revealed the presence of His-tagged protein (i.e., GUS); untransformed meristems did not contain any His-tagged proteins (Figure 3b).

Transient reporter gene expression was further observed in *C. roseus* apices co-cultured with *A. tumefaciens* harbouring the pCAMBIA *GUSPlus* construct using a colourimetric assay for GUS enzyme activity (Figure 4a-f). In an attempt to optimize *GUS* expression levels, we tested a variety of parameters, including the age of apices at the time of inoculation, the number of days of co-cultivation, the optical density of the cultures used, and wounding the tissue prior to *Agrobacterium* treatment. The duration of the co-cultivation period had an effect on gene expression since those co-cultured for 4 days showed a higher level of GUS activity than for those co-cultivated for only 2 days (Figure 4a,b). Even though a wounding treatment resulted in an enhanced level of (transient) GUS activity, there were no significant

differences in GUS activity observed between apices that were wounded with either a scalpel or needle, regardless of whether the wounding was longitudinal or transversal (Figure 4c-f). No difference in transient gene expression levels were noted for apices with or without first leaves or old (9 or 15 days) apices (data not shown). By contrast, bacterial colony density had an impact on gene expression, with cultures adjusted to an OD_{600} = 0.5 resulting in higher GUS activity than with cultures used at a higher optical density (data not shown).

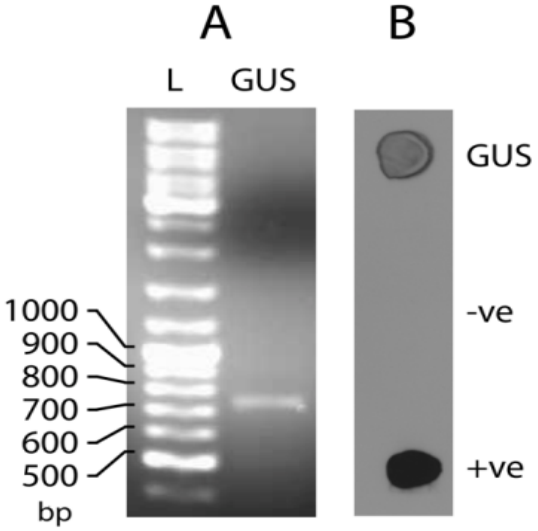

Figure 3.GUS Gene expression and protein accumulation in *Agrobacterium tumefaciens*-transformed *Catharanthus roseus* meristem tissue. (A) RT-PCR using an exon-exon bridging primer was used to detect *C. roseus* derived GUS transcripts (see Materials and Methods for details) from mRNA isolated from *A. tumefaciens* transformed meristems. (B) His-tagged GUS protein was detected in crude protein extracts obtained from *A. tumefaciens* transformed meristems (GUS). The negative control (-ve) is a crude protein extract from non-transformed *C. roseus* meristem cultures. The positive control (+ve) is a sample of His-tagged recombinant potato fatty acid ω-hydroxylase expressed in *E. coli*. Crude protein extract (10 µL) was spotted onto nitrocellulose and probed with anti-His antibody conjugated with horseradish peroxidase and detected using the chemiluminescence (ECL) assay system (GE Healthcare).

Transformation with *A. rhizogenes*

Normally, plant tissue infected with *A. rhizogenes* generates a hairy root phenotype, and this is what we observed (Figure 4G). That the tissue harboured the *GUS* gene was evident from intense blue staining in the root tissue when assayed for glucuronidase activity. At this stage, we have not been successful in regenerating whole plants from hairy roots obtained from meristem cultures transformed with *A. rhizogenes*. Nevertheless, it is clear that *C. roseus* is readily transformed with *A. rhizogenes*, and this may be a good alternative to *A. tumefaciens*.

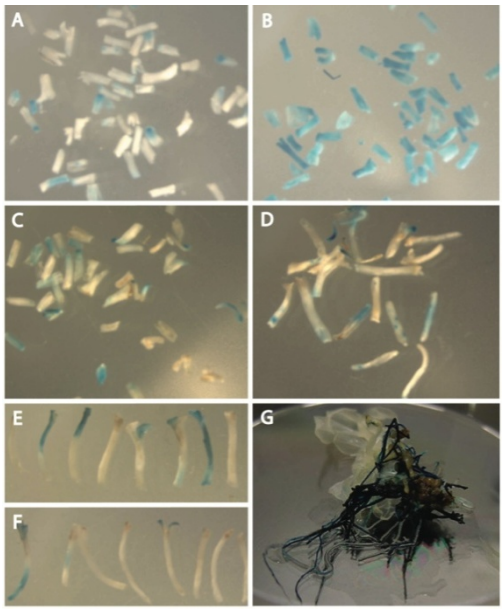

Figure 4. Transient expression of GUS in apical meristem and hairy root cultures of *Catharanthusroseus*. (A-F) Apical meristems (20 to 30 per plate) were subjected to different conditions to optimize *GUS* transformation using *Agrobacterium tumefaciens*, including (A) 2-or (B) 4-day of co-culture, (C) transverse or (D) longitudinal wounding (with 2-day co-culture), and wounding by (E) needle or (F) scalpel (with 2-day co-culture). *In situ* GUS activity is evident by dark staining, especially at the meristem ends of the explants. Two replicate plates were analysed; a representative plate form each treatment is shown. (G) *In vitro* cultured *C. roseus* meristems were infected with *A. rhizogenes* harbouring a p35S::*GUSPlus* construct. After approx. 1 month on non-selective media, the tissue was subjected to GUS staining (see Materials and Methods). The intense dark staining of the adventitious (hairy) roots is indicative of active GUS protein.

Discussion

Catharanthus roseus produces medicinally valuable indole alkaloids, including vinblastine and vincristine, which are used in the treatment of leukemia and Hodgkin's disease. Since the amounts of indole alkaloids produced in this species is relatively low (< 1% of dry weight), and over 500 kg of *C. roseus* leaves is required to produce 1 g of vincristine, [31] it is desirable to apply biotechnological approaches to enhance production. While there have been many attempts to genetically engineer *C. rosues* to enhance indole alkaloid production, most have been unsuccessful. This is in part due to (1) compartmentation of indole alkaloid biosynthesis *in planta*, (which largely rules out the use of tissue cultures for indole alkaloid production), (2) difficulty with regenerating *C. roseus* plants from *in vitro* cultures (i.e., as part of an *Agrobacterium*-mediated gene transfer system), and (3) the lack of a suitable, stable genetic transformation protocol for this species. Therefore, the development of a genetic transformation protocol for *C. roseus* requires progress in both plant regeneration and plant genetic transformation.

Herein we report that *C. roseus* cv. Pacifica pink plants have been regenerated from hypocotyl- and cotyledon-derived

callus tissue. Shoots were obtained after one to two months on MS medium supplemented with NAA and BAP (both at 1.0 mg/L). Shoots were subsequently rooted on medium supplemented with either IAA (10 mg/L) and BAP (0.1 mg/L) or IBA (10 mg/L) and BAP (0.1 mg/L), acclimated and transferred to the greenhouse, where they developed and produced flowers. Thus, we were able to generate whole, mature plants starting with hypocotyls and cotyledons obtained from *in vitro* germinated seeds. This process required up to one year, with an overall efficiency of 35%. In addition, shoots were readily obtained in high yield from apical meristems cultured on MS medium supplemented with NAA and BAP (both at 1.0 mg/L). Consequently, meristem explants represent an excellent means whereby shoots can be readily obtained, and are superior to somatic embryos, which require more than nine months, many subculture cycles and a few combinatorial hormones [16]. The formation of shoots from meristems is important because we were also able to demonstrate transient gene expression in *Agrobacterium*-infected meristems (see below) using both *A. tumefaciens* and *A. rhizogenes*.

Previously, transient gene expression systems for *C. roseus* have been attempted using protoplasts [32], biolistic bombardment [33]agroinfiltration[34] or vacuum infiltration [35]. We recently expressed a promoter-GUS construct using the *C. roseus DAT* gene (which encodes the enzyme acetyl-CoA:deacetylvindoline-4-*O*-acetyltransferase involved in the last step of vindoline biosynthesis) promoter in agroinfiltrated *C. roseus* leaves and *A. rhizogenes*-derived *C. roseus* hairy roots, albeit only transiently [2]. Successful transformation of *C. roseus* by *A. Tumefaciens* was performed on suspension cultures [36]. However, given the difficulty in regenerating plants from suspension culture and the need for intact plant tissue to study some biochemical processes, an alternative transformation system is necessary. For this we proceeded with *Agrobacterium*-mediated transformation of meristem cultures, with the goal of ultimately re-generating transformed plants from them. As proof of concept, we initially worked with a p35S::*GUSPlus* construct. We tested many factors to find the best transformation conditions, including the *Agrobacterium* strain, preculture period, bacterial suspension density, methods of co-cultivation (i.e., with or without wounding and type of wounding), and the duration of the co-cultivation period (2 and 4 days). While wounding may facilitate attachment or the release of virulence gene inducers [37], we did not observe a significant enhancement of transformation (inferred using *in situ* GUS enzyme activity as a proxy for gene expression) when meristems were wounded. Instead, the highest levels of GUS enzyme activity were observed when suspension cultures of *A. tumefaciens* (e.g., OD_{600} = 0.5) were used, and co-cultivation was allowed to continue for four days. Other conditions resulted in less apparent transformation, as evident by less intense *in situ* GUS activity. To ensure that meristems treated with *A. tumefaciens* were indeed transformed and contained the *GUS* gene, exon-exon junction primers were used to avoid the amplification of bacterial DNA and to only amplify cDNA generated from properly spliced mRNA.

While we have only demonstrated transient GUS gene expression (i.e., the *A. tumefaciens* transformed tissue was not subjected to selection), the process provides a potential means to obtain stable genetic transformation of *C. roseus*. Even though meristems have been shown to overcome *Agrobacterium* infections, giving rise to healthy plants [38], we have now demonstrated, through the transient expression of GUS, that these explants can be an *Agrobacterium*-mediated transformation target for *C. roseus*. The use of meristem cultures allows the regeneration of *C. roseus* shoots *in vitro* without a callus stage. Such an approach increases genotype fidelity and avoids the complications of somaclonal variation [39].

Agrobacterium rhizogenes can also be a useful tool to generate transgenic plants with high genetic stability and growth rate [8] ,[40] , even for species that are difficult to transform using *A. tumefaciens*[41] ,[42]. However, plant regeneration from hairy roots, derived from *A. rhizogenes* transformation, is difficult, and has only been reported once for *C. roseus*[7]. Nevertheless, we obtained a typical hairy root phenotype when we treated *C. roseus* meristems with *A. rhizogenes* harbouring the same p35S::*GUS* construct as with *A. tumefaciens*. Adventitious roots obtained in this manner demonstrated intense *in situ* GUS activity, especially when these were derived from tissue below the main apical meristem.

Conclusions

The establishment of a *C. roseus* genetic transformation system is a challenge in part because of the difficulty in regenerating shoots from primary transformed tissues; however, it is also a challenge because of difficulty in generating primary transformants. In the present paper, we address two of these issues and demonstrate the regeneration of plantlets from hypocotyl-and cotyledon-derived tissue as well as *Agrobacterium*-mediated (both *A. tumefaciens* and *A. rhizogenes*) transformation of *C. roseus* meristems. We are still not at a stage where we can combine these two results into a routine genetic transformation system because it remains technically challenging to generate genetically modified *C. roseus* tissue that is also amenable to plant regeneration. It is beyond the scope of the parameters considered herein to recommend a strategy for *C. roseus* transformation and plant regeneration. Nevertheless, our data provide the basis for further studies on shoot apical meristem genetic transformation as a tool to overcome the intrinsic difficulties in *C. roseus* genetic transformation, as well as the basis for plantlet regeneration.

Material and methods

Plant material

Explants were obtained from seedlings grown in sterile culture from seed. For whole plant regeneration from various organs (hypocotyls, cotyledons and roots) and for genetic transformation assays, only the Pacifica Pink cultivar was employed. To estimate shooting efficiency and for meristem experiments, six different cultivars (Blue Pearl, Cooler Coconut, Cooler Mix Improved, Mediterranean Lilas, Pacifica Pink and Stardust Rose) were examined.

Bacterial strains and plasmid construct

Agrobacterium tumefaciens AGL1 and *A. Rhizogenes* 15834 were transformed, using electroporation, with the Binary vector pCAMBIA1305.1 in which the T-DNA region harboured the reporter gene *GUSPlus*™ encoding a 6 x His-tagged β-glucuronidase (*GUS*) and hygromycin phosphotransferase (*HPTII*). Both genes are driven by separate 35S promoters.

Sterilization and germination of seeds

Sterilization of *C. roseus* seeds was carried out by immersion in ethanol for 2 minutes followed by Na-hypochlorite (3%) for 20 minutes, and finally washing three times in sterile distilled water. Later, seeds were soaked in sterile distilled water in the dark for one day, then sown on Petri dishes containing hormone-free MS medium [43] (0.8% agar), covered by aluminium foil and left in the dark to germinate. After three days, the foil was removed and the seedlings were grown at 24°C with a 16 hour light, 8 hour dark, photoperiod (18μE/cm^2/s). Meristems from nine- and ten-day old plantlets were used for *Agrobacterium*-mediated transformation.

Plant culture

Explants were excised from 9-or 10-day old seedlings and sub-cultured on MS medium supplemented with different NAA and BAP ratios (see below), under the same conditions noted previously. To study the influence of organs and hormones on regeneration, various organs (cotyledons, roots and hypocotyls) from c.v. Pacifica Pink were used with different NAA/BAP combinations (values in mg/L) including: 1.0/5.0; 1.0/2.0; 1.0/1.0; 1.0/0.5; 1.0/0.1; 0.5/2.0; 0.5/1.0; 0.5/0.5; 0.5/0.1. Calli arising from explants were sub-cultured onto new medium every 3 weeks. For regeneration of whole plants and acclimation, shoots derived from callus were first rooted on MS media supplemented with either IAA 10 mg/L and BAP 0.1 mg/L or IBA 10 mg/L and BAP 0.1 mg/L for several sub-culture cycles. Once roots appeared, plantlets were subcultured on the same medium solidified with gelrite instead of agar. For hardening and acclimation, plants were removed from gelrite cultures and the roots washed with water. The plants were then placed into pots of vermiculite wetted with Knop solution [44], under 25 °C ± 1°C, 18 μE/cm^2/s, (12h/12h) conditions for one month. Later, plants were transferred to the greenhouse (20 °C ± 1°C) under natural light and day length.

The appearance of shoots on hypocotyls was established separately for the upper and lower segments of hypocotyls from 6 *C. roseus* cultivars on MS supplemented with NAA 1 mg/L and BAP 1 mg/L. For meristem shoot regeneration, meristems from all 6 cultivars were used on MS containing NAA 1 mg/L and BAP 1 mg/L. Meristems of the Pacifica Pink cultivar were used to optimize growth regulator concentrations by placing them on MS media supplemented with different concentrations of BAP (1 mg/L, 2 mg/L, 5 mg/L, 10 mg/L, 15 mg/L and 20 mg/L) in combination with 1 mg/L NAA.

Meristem genetic transformation assays

Agrobacterium tumefaciens transformation: Meristems from 9-to 10-day old plantlets were isolated and plated on MS basal medium as above. *Agrobacterium tumefaciens* suspensions (strain AGL1), harbouring the binary vector pCAMBIA 1305.1, containing the *GUSPlus*™ gene (modified with a 6 x His C-terminal tag) encoding β-glucuronidase were prepared in LB medium supplemented with kanamycin monosulphate (50 mg/L). The optical density of overnight cultures was measured and adjusted (to as low as 0.5; [45] and meristems, with or without pre-inoculation wounding (stabbing or cutting) were immersed in bacterial suspension for 10 minutes prior to transfer to Petri dishes containing hormone free MS medium. After 2 or 4 days of co-culture, the meristem explants were washed twice with sterile, distilled water and once with MS (hormone free) containing 500 mg/L cefotaxime, and subjected to GUS assay (see below).

Agrobacterium rhizogenes transformation: Transformation of apical meristems by *A. rhizogenes* was carried out with a needle dipped in solid colonies, using a dissecting microscope to target tissue (e.g., meristem or apical meristem zones, or the main veins of young leaves from *in vitro*-grown plantlets). Finally, explants were placed in Petri dishes on MS medium with BAP (1 mg/mL) and NAA (1 mg/mL) supplemented with 500 mg/L cefotaxime and incubated at room temperature with 16 hours light and 8 hours dark.

RT-PCR

To demonstrate transient transformation of meristems, we amplified GUS cDNA using an exon- exon junction-based primer [46,47]. Total RNA was extracted from 100 mg infected and washed meristems using the RNeasy plant mini kit (Qiagen), according to manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) primed by oligo(dT). PCR was performed using first-strand cDNA as the transformed meristems template and the over-intron (e.g., exon-exon primer) pintr3f primer (gpint3f TGGTAGATCTGAGGAACCGA) as forward primer with gp3r (AATCTCCACGTTACCGCTCA as the reverse primer. The PCR conditions were as follows: initial conditions: 94°C for 4 min, followed by 35 cycles at 94°C for 30 seconds, 54°C for 45 seconds, 72°C for 2 min and a final cycle at 72°C for 7 min. The PCR reactions were carried out on a Techne thermal cycler (TC-3000G).

Dot blot assay

In order to test for GUS accumulation in meristems transformed with *A. tumefaciens*, total protein was isolated from 500 mg of transformed or untransformed (negative control) meristems, and probed for His-tagged protein. Briefly, total protein was extracted using QB extraction buffer (100 mM KPO_4 (pH 7.8), 1 mM EDTA, 1% Triton X-100, 10% Glycerol, dH_2O and 1 mM DTT) [48] (2 mL of QB buffer/g of ground tissue) and subsequently centrifuged for 15 minutes at 4°C at 13,000 rpm. The concentration of the total protein in the supernatant was determined using a BCA kit, according to manufacturer's instructions (Pierce). Sixty micrograms of total protein (in 10 μL) isolated from transformed and untransformed meristems was spotted onto nitrocellulose membrane. After blocking for 1h in 5% skim milk, the membrane was probed with Anti-His HRP

conjugated antibody (1:5000) (Invitrogen). Protein presence was revealed using ECL (GE Healthcare).

Histochemical GUS staining

Transient and stable genetic transformation of explants was assessed by assay for GUS enzyme activity *in situ* as described [49]. Explants were immersed in staining solution (50 mMNa-phosphate, (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1% Triton X-100, 1 mg/mL 5-bromo-4-chloro-3-indolylglucuronide) in the dark at 37°C for 2 or 3 days. Next, chlorophyll was removed from explants by immersion in 70% ethanol. GUS staining was observed under a microscope (Olympus BX51) and photographed.

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