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An Protocol for Genetic Transformation of *Catharanthus roseus* by *Agrobacterium rhizogenes* A4

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Abstracts *Catharanthus roseus* (L.) G. Don is a plant species known for its production of a variety of terpenoid indole alkaloids, many of which have pharmacological activities. Catharanthine can be chemically coupled to the abundant leaf alkaloid vindoline to form the valuable anticancer drug vinblastine. To study and extract catharanthine and other metabolites from *C. roseus*, a technique was developed for producing hairy root cultures. In this study, the *Agrobacterium rhizogenes* A4 was induced in the hairy roots from leaf explants, and the concentration of antibiotics (100 mg/L kanamycin) was elucidated for selection after transformation. The polymerase chain reaction amplification of *rol* genes results revealed that transgenic hairy roots contained *rol* genes from the root induced (Ri)-plasmid. Catharanthine from *C. roseus* hairy roots was separated and analyzed using high-performance liquid chromatography. Over-expression of *CrOrca3* (octadecanoid-responsive Catharanthus AP2/ERF domain), and cytohistochemical staining methods were used to validate transgenic hairy roots from *C. roseus*. Hairy root culture of *C. roseus* is a valuable approach for future efforts in the metabolic engineering of terpenoid indole alkaloids in plants.

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Introduction

Catharanthus roseus (L.) G. Don has been extensively investigated because it produces a variety of terpenoid indole alkaloids (TIAs), many of which have physiological effects in humans, and several are therefore used as pharmaceuticals. The dimeric terpenoid indole alkaloids, such as 3′,4′-anhydrovinblastine, vincristine, and vinblastine, have powerful effects as anticancer drugs [23]. These compounds are present in very small amounts and usually have some chiral centers. They are therefore difficult to synthesize chemically, making them expensive to produce commercially. Consequently, extraction from field-grown plants has been the major method used to economically obtain these important secondary metabolites [25]. Hairy roots can be a valuable source of phytochemicals used in pharmaceuticals, cosmetics, and food. The greatest advantage of hairy roots is that they often exhibit about the same or greater biosynthetic capacity for secondary metabolite production as compared to their parent plants [10]. Consequently, these roots can be an economical system for commercial production. Plants transformed by *Agrobacterium rhizogenes* harboring the root induced (Ri) plasmid induce the formation of root structures known as hairy roots. *A. rhizogenes* Ri plasmids have been assigned to three classes depending on the opine synthesized and degraded, mannopine, cucumopine, and agropine. In the first two classes, T-DNA consists of a single region, whereas in the third class two T-DNA regions are present, TL-DNA and TR-DNA. Transformed roots arise as a result of the integration of TL-DNA and TR-DNA into the plant genome by the agropine strains, and its expression induces root differentiation and subsequent growth [3, 4]. These genetically transformed hairy roots are capable of unlimited growth in culture media free of growth hormones. Moreover, transformed roots are often able to regenerate whole viable plants and maintain their genetic stability during continuous subculturing and plant regeneration [25]. Investigation of the molecular regulation mechanisms of TIAs' biosynthetic pathway requires the establishment of protocols for efficient and stable genetic transformation.

In this study, we developed an efficient protocol for transformation of *C. roseus* hairy root cultures by agropine type *A. rhizogenes* A4. We also explore the effect of constitutive overexpression of *CrOrca3* (octadecanoid-responsive *Catharanthus* AP2/ERF domain) to evaluate the transformation efficiency on the differentiated tissue of *C. roseus* hairy root. Catharanthine from *C. roseus* hairy roots was separated and analyzed using high-performance liquid chromatography (HPLC). Our results may serve as a valuable approach for metabolic engineering of TIAs in the medicinal plant *C. roseus*.

Materials and Methods

Plant Material

C. roseus seeds were obtained from the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (Beijing, China). *C. roseus* seeds were surface-sterilized with 75% (*v*/*v*) ethanol for 1 min and 2% (*v*/*v*) sodium hypochlorite solution for 10 min, then rinsed five times in sterilized water and blotted dry on sterile filter paper. The seeds were placed on agar-solidified culture medium, which consisted of half-strength salts and vitamins

of MS medium and solidified with 0.8% (*w*/*v*) agar [14]. The medium was adjusted to pH 5.8 before adding agar and then sterilized by autoclaving at 121°C for 20 min. The seeds were germinated in a growth chamber at 25°C with a dark environment. The germinated seeds were then placed into MS medium which was solidified with 0.8% (*w*/*v*) agar and grown in a standard plant tissue culture room at 25°C under a 16-h photoperiod.

Preparation of *A. rhizogenes*

The construction of pCAMBIA 2301-35SCrORCA3-35SGUS was as previously described (Fig. 2c) [24]. This plasmid was electroporated into *A. rhizogenes* A4 (obtained from Agricultural Culture Collection of China, ACCC10060) and selected on YEB media (5 g/L tryptone, 1 g/L yeast extract, 0.5 g/L MgSO4 $7H_2O$, 10 g/L sucrose, and 10 g/L agar, pH 7.4) containing 50 mg/L kanamycin and 50 mg/L rifampicin. The presence of pCAMBIA2301-35SCrORCA3-35SGUS was confirmed by standard PCR amplification. *A. rhizogenes* cultures were inoculated from glycerol stock and grown 48 h at 28°C with shaking (200 rpm) in YEB media containing kanamycin and rifampicin, to OD A_{600} of 0.6–0.8. Cells were collected by centrifugation for 10 min at 4,000 rpm and resuspended in liquid inoculation half-strength B5 medium containing 30 g/L sucrose [6]. Cell density was adjusted to an OD A_{600} of 0.5 for inoculation.

Establishment of Hairy Root Transformation

Excised cotyledon leaves of *C. roseus* from 20-day-old seedlings were used as the explants material for co-cultivation with *A. rhizogenes* A4. The explants were dipped into the *A. rhizogenes* A4 culture in liquid inoculation half-strength B5 medium for 10 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on half-strength B5 agar medium [6]. After 2 days of co-cultivation, the explant tissues were rinsed five times in half-strength B5 liquid medium containing cefotaxime (800 mg/L), and blotted dry on sterile filter paper, and then incubated in the dark at 25°C on half-strength B5 agar medium containing 400 mg/L cefotaxime. Two to three weeks after inoculation, single putative transformed roots were excised from the explants and selected on half-strength B5 agar medium containing 100 mg/L kanamycin and 400 mg/L cefotaxime. Root culture clones were maintained at 25°C in the dark and routinely subcultured every 5 weeks. The rapidly growing kanamycin-resistant lines with no bacterial contamination were used to establish hairy root lines. About 100 mg of fresh roots (3 cm in length) was inoculated into 150-mL conical flasks containing 40 mL of liquid half-strength B5 medium and cultured on an orbital shaker (100 rpm) at 25°C in the dark.

PCR Analysis for *rol* and *Orca3* Genes

Putatively transformed hairy roots were initially analyzed by PCR assays. The genomic DNA was extracted from the hairy roots using the modified acetyl trimethyl ammonium bromide method [19]. In order to show the integration of TL-DNA and TR-DNA of *A. rhizogenes* pRi A4 in the transformed roots, segments from both the TL-DNA and TR-DNA regions were amplified using the gene-specific primers 5′-ATGGAATTAGCCGGAC TAAACG-3′ and 5′-ATGGATCCCAAATTGCT ATTCC-3′, which amplify a segment complimentary to the 5′ coding sequence of *rolA* to the 3′ coding sequence of *rolB* on the TL-DNA region, and 5′-CGGAAATTGTGGCTCGTTGTGGAC-3′ and 5′-AATCGTTCAGA GAGCGTCC GAAGTT-3′, which amplify the agropine synthase (*ags*) gene of the TR-DNA region [2, 3, 24, 26]. To demonstrate that there was no bacterial contamination in the

putative hairy root, segments from the virulence (*Vir*) regions were amplified using the *VirC* specific primers 5′-GGCTTCGCCAACCAATTTGGAGAT-3′ and 5′-TTTTGCTCCTT CAAGGGA GGTGCC-3′. To avoid disturbance of the endogenous *CrOrca3* gene in *C. roseus*, two specific primers were used in the PCR, 5′-CGTTGAAGATGCCTCTGCCG-3′ and 5′-ACCGAGCTCTCTCTCATTTCACAG-3′ [24]. PCR mixtures containing 1 μL of each primer (10 μM), 2 μL of 10 mM dNTPs, 2 μL of 10× PCR buffer (Mg^{2+} plus) and 0.5 U of Taq DNA polymerase (Takara), and 200 ng of genomic DNA in a total volume of 20 μL were heated at 94°C for 5 min followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and a final incubation at 72°C for 10 min. Amplified fragments were analyzed by electrophoretic separation on 1% agarose gels (*w*/*v*) in 1× TAE buffer and staining with ethidium bromide and then visualized with UV light.

GUS Staining Analysis

To assay β-glucuronidase activity, sectioned transformed roots were incubated overnight at 37°C in Eppendorf tubes containing 2 mL staining solution with 100 mM sodium phosphate, pH 7.0, 0.1% Triton X-100, 10 mM EDTA, 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), 0.5 mM $K_3Fe(CN)_6$, and 0.5 mM $K_4Fe(CN)_6$ [7, 26]. After staining, the solution was changed with 70% ethanol until tissue was cleared.

Total RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was isolated from the hairy roots samples stored at −80°C using a Plant RNAout Column (Tiandz, China) according to the manufacturer's protocol and treated with RNase-free DNase (Promega). Reverse transcription was carried out using the M-MuLV Reverse Transcriptase (Revert Aid™ First Strand cDNA Synthesis Kit, Fermentas) according to the manufacturer's protocol using an oligo(dT)18 primer. A negative control (without reverse transcriptase) was performed for each sample. The quantitative PCR (Q-PCR) amplification was carried out in a 96-well plate Bio-Rad iQ5 Multicolor Real-Time PCR Detection System. Each reaction contained 0.5 μL cDNA, 1.6 μL mixed primers, 10 μL SYBR Green PCR Master Mix, and 7.9 μL. nuclease-free water. The primers for the genes (α-subunit of anthranilate synthase- *Asα*, tryptophan decarboxylase-*Tdc*, 1-deoxy-D-xylulose 5-phosphate synthase-*Dxs*, geraniol 10-hydroxylase-*G10h*, cytochrome P450 reductase-*Cpr*, strictosidine synthase-*Str*, strictosidine β-D-glucosidase-*Sgd*, *Orca3*, 40S ribosomal protein S9-*Rsp9*) were previously described [16, 24, 26]. The gene encoding the 40S ribosomal protein S9 (RSP9) was used as control [13]. Reaction mixtures were incubated for 2 min at 50°C, 3 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 60°C, and 20 s at 72°C. Relative gene expression was quantified using the comparative threshold cycle (CT) method [18]. Triplicate quantitative real-time PCR experiments were performed for each sample.

Catharanthine Extraction and HPLC Detection

The hairy roots of *C. roseus* were collected and fresh samples were stored at −80°C until their use. Dried samples were ground into a fine powder using a mortar and pestle, and extracted at room temperature in 1 mL of MeOH for 60 min in a bath sonicator (KQ-500 DB). The extract was centrifuged at 15,000×*g* for 5 min at 25°C, and the supernatant was filtered through a PTFE 0.45 μm filter into an amber glass HPLC vial. The HPLC analysis was as previously described, using a mobile phase consisting of a mixture of 5 mM

NaH_2PO_4-H_3PO_4, pH 6, and acetonitrile [20, 24, 26]. Standards for catharanthine (98%, Sigma) were prepared in methanol at a final concentration of 1 mg/mL.

Results

Establishment of Transgenic Hairy Root Lines

C. roseus hairy roots were induced in leaf explants inoculated with *A. rhizogenes* A4 (pCAMBIA 2301-35SCrORCA3-35SGUS). To determine the appropriate kanamycin concentration for plant selection, explants were initially grown on media without kanamycin and were shifted to selection media containing different kinds of kanamycin concentrations (0, 30, 50, 100, and 150 mg/L) after transformation by *A. rhizogenes* A4 (Fig. 1b). Hairy root growth frequency was approximately 85% on kanamycin-free medium and approximately 50% on medium containing 50 mg/L of kanamycin (data not shown). However, the 100 and 150 mg/L kanamycin media completely inhibited the growth of hairy roots from explants with wild-type *A. rhizogenes* A4. Therefore, we used 100 mg/L kanamycin media to select for transformed hairy roots. After 2–3 weeks of inoculation, putative transformed roots emerged from wound sites on leaves were excised and selected on half-strength B5 agar medium containing 100 mg/L kanamycin and 400 mg/L cefotaxime (Fig. 1c, d). About 5 weeks after co-cultivation with *A. rhizogenes* A4, hairy roots were subcultured on fresh medium only containing 100 mg/L kanamycin (Fig. 1e). The rapidly growing kanamycin-

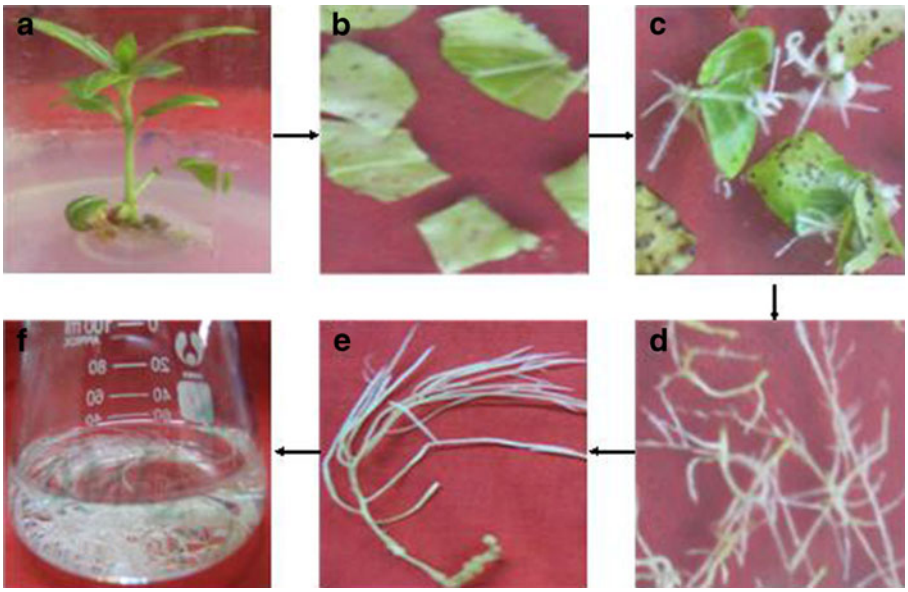

Fig. 1 Establishment of hairy root lines. **a** Aseptic cultivation of plants. **b** Pre-culture wounded plant tissues are inoculated with *A. rhizogenes* A4. **c** After 2–3 weeks, roots emerge at the wounding site. **d** Roots are excised to be cultured individually on selective solid culture medium containing 100 mg/L kanamycin and 400 mg/L cefotaxime. **e** Kanamycin resistant lines with no bacterial contamination were used to establish hairy root lines. **f** Each putative positive clone is then transferred to liquid culture medium for obtaining established hairy root lines

resistant lines with no bacterial contamination were used to establish hairy root lines (Fig. 1f). After repeated transfer to fresh medium, rapidly growing hairy roots were transferred to liquid half-strength B5 medium and routinely subcultured every 5 weeks. The mature hairy root phenotype is characterized by fast hormone-independent growth, lack of geotropism, and lateral branching (Fig. 5a).

The abbreviations A and O refer to the transgenic hairy root lines generated from transformations with strains *A. rhizogenes* A4 (blank transformation) and *A. rhizogenes* A4/pCAMBIA2301-35SCrORCA3-35SGUS, respectively. Ten kanamycin-resistant O hairy root lines and ten A hairy root lines were generated, of which seven O and nine A lines survived the successive subculture steps (Fig. 2a, b). Some O hairy root lines turned brown and aged considerably faster than A hairy root lines (including O5, O8, and O9). These lines were discarded, and the remaining hairy root lines were subcultured for 5 weeks in hormone-free, half-strength B5 liquid medium. Both types of hairy root lines reached the highest growth rate at the third week and accumulated maximum fresh weight after 5 weeks of culture.

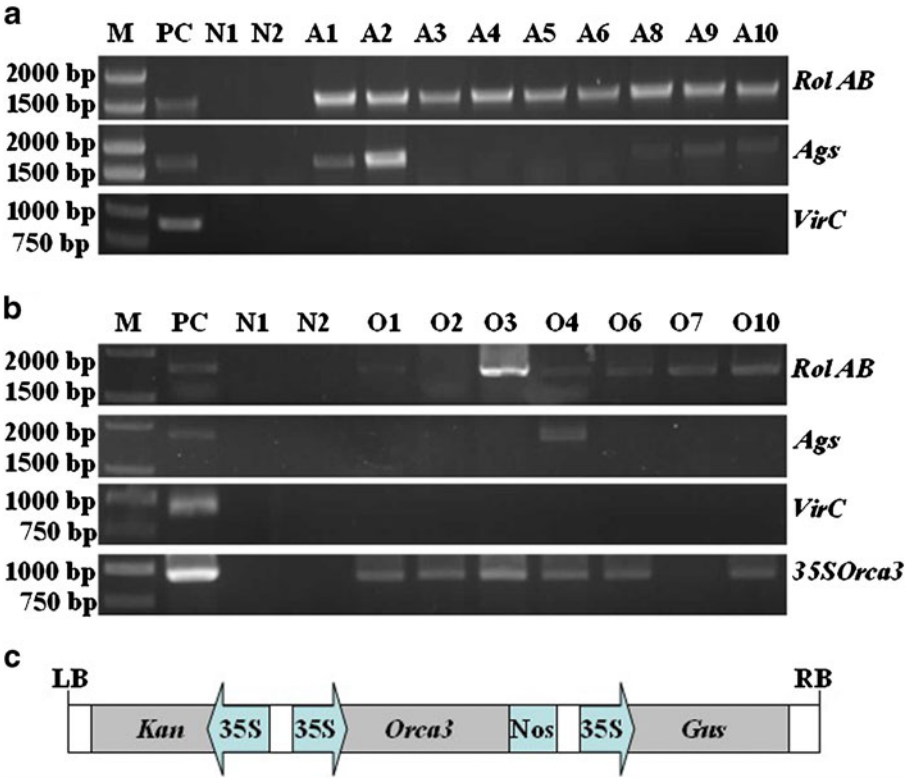

Fig. 2 Molecular analysis of transgenic hairy root lines. **a** Representative PCR analysis for the presence of *rolA*, *rolB*, *ags*, and *virC* genes in A hairy root lines. **b** Representative PCR analysis for the presence of *rolA*, *rolB*, *ags*, *virC*, and *CrOrca3* genes in O hairy root lines. **c** Schematic representation of pCAMBIA-2301-35SCrORCA3-35SGUS. *M* DNA marker, *PC* pRiA4 (positive control), *N1* A hairy root line, *N2* wild-type *C. roseus* root. The abbreviations *A* and *O* refer to the transgenic hairy root lines generated from transformations with strains *A. rhizogenes* A4 (blank transformation) and *A. rhizogenes* A4/pCAMBIA2301-35SCrORCA3-35SGUS, respectively

Molecular Analysis of Transgenic Hairy Roots

To demonstrate the presence of Ri T-DNA in nine AR and seven O transformed hairy root clones, PCR analysis was performed on the integration of the *rolA* and *rolB* genes (1.5 kb) from TL-DNA segments and/or the agropine synthase (*ags*) gene (1.5 kb) from TR-DNA segments into the genome (Fig. 2a, b). In all nine A and seven O transformed hairy root cultures, *rolA* and *rolB* specific PCR yielded the expected products (1.5 kb), identical to that of the positive control (pRi A4 plasmid), whereas no PCR products were obtained from wild-type *C. roseus* roots (negative control) and from all the tested transformed roots when using the *virC* primers (Fig. 2a, b). However, not all transformed hairy root lines contain the *ags* gene: four AR (A3, A4, A5, and A6) and six O (O1, O2, O3, O6, O7, and O10) hairy root lines did not show the amplified products (Fig. 2a, b). These results indicated that the hairy roots contained the *rolA* and *rolB* genes in their genomes and that they were free of bacteria as shown by the inability to detect the *virC* gene. These results also revealed that *rol* genes of the Ri-plasmid in *A. rhizogenes* are responsible for the induction of hairy roots from plant species.

In all seven kanamycin-resistant hairy root lines, *Orca3*-specific PCR yielded the expected products, whereas the wild-type roots and O7 yielded no amplification products (Fig. 2b). Cytohistochemical staining for GUS activity was used to determine whether the transformation resulted in completely transgenic hairy roots or in chimeras that were composed of transgenic and wild-type tissues [15]. The cauliflower mosaic virus 35S promoter-GUS fusion sequence contained in the pCAMBIA2301 binary vector should result in constitutive GUS activity in all cells of kanamycin-resistant tissues. In six of seven kanamycin-resistant hairy root lines, strong GUS staining was observed. The O1 hairy root line and wild-type root had no GUS staining (Fig. 3).

Quantitative real-time PCR (QPCR) was used to quantify *Orca3* and OCRA3 regulated TIA pathway gene's transcription levels in hairy roots. The expression levels of O hairy root lines were calculated relative to the expression level of A hairy root lines. This means that for each gene, the relative level of A hairy root lines is always one. After using PCR to verify the insertion, *Orca3* transcription in the hairy roots was assessed by QPCR, which revealed that *Orca3* over-expressed hairy root cultures had fourfold increases in *Orca3* transcription. In response to *Orca3* overexpression, an increase was found in the levels of *Asα*, *Tdc*, *Dxs*, *Str*, *Cpr*, and *Sgd* transcripts and constant levels of *G10h* transcripts (Fig. 4).

Catharanthine Contents in *C. roseus* Hairy Roots

The catharanthine contents of wild-type *C. roseus* roots, A hairy root lines, and O hairy root lines were measured as shown in Fig. 5b. In wild-type roots, catharanthine was undetectable. The average catharanthine contents in A hairy root lines were about 0.17 mg/g fresh weight (FW). This demonstrates that the T-DNA genes derived from the pRi A4 plasmid positively affected the catharanthine biosynthesis pathway. The average catharanthine levels in O hairy root lines were lower compared to those of the A lines, around 0.12 mg/g FW.

Discussion

The natural DNA-transferring property of the soil pathogen *A. rhizogenes* provides one of the most widely used approaches for introducing DNA into plant cells. It has been reported that more than 450 species of many different genera and families are known to

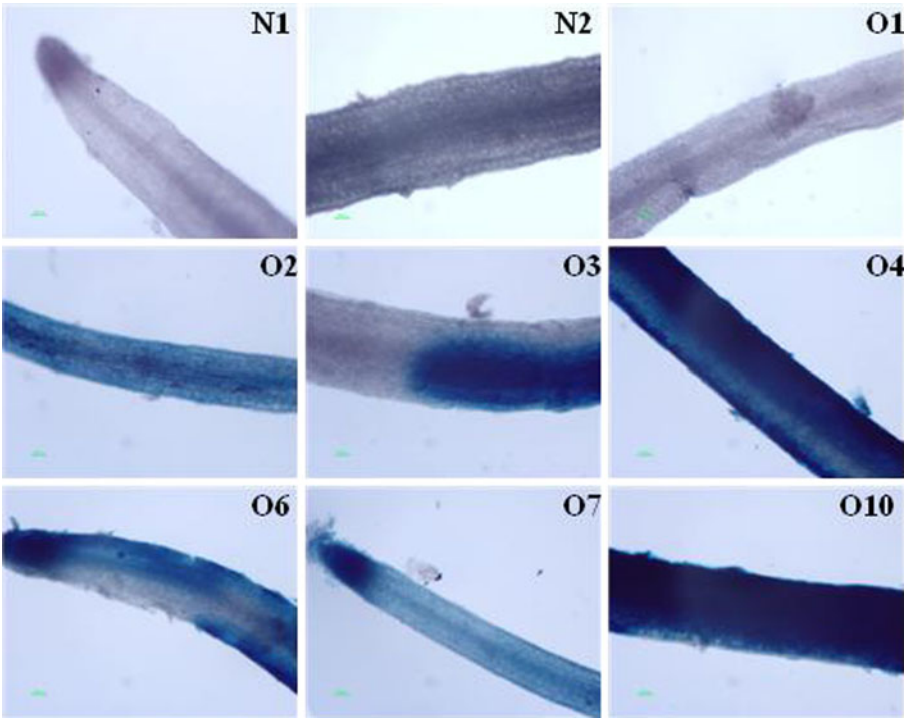

Fig. 3 GUS staining of transgenic hairy root lines. *N1* A hairy root line, *N2* wild-type *C. roseus* root, *O* different transgenic lines

be susceptible to the infection by *A. rhizogenes*, and since then many more additions have been made to the list in the least reports [15]. Various *A. rhizogenes* strains can be used in this technique, the efficiency of these strains is highly dependent on the plant species and must be determined in order to develop optimal transformation methods [15]. In agropine Ri plasmids the T-DNA region consists of two parts, TL-DNA and TR-DNA, which are physically separated by a non-transferred DNA sequence of approximately 18 kb [8]. *A. rhizogenes* is able to transfer the T-DNA of binary vectors, thereby enabling the production of transformed roots bearing other foreign genes on a second T-DNA (cotransformation).

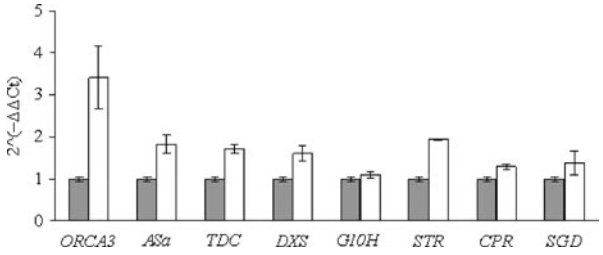

Fig. 4 Expression levels of genes involved in the catharanthine biosynthesis pathway. *Dark gray* and *white bars* refer to the A and O hairy root lines, respectively. 2^(−△△Ct) values represent the relative expression levels. The data were normalized to A hairy root lines (set at 1) using the comparative threshold cycle method. *Error bars* represent the standard deviation of triplicate runs for QRT-PCR

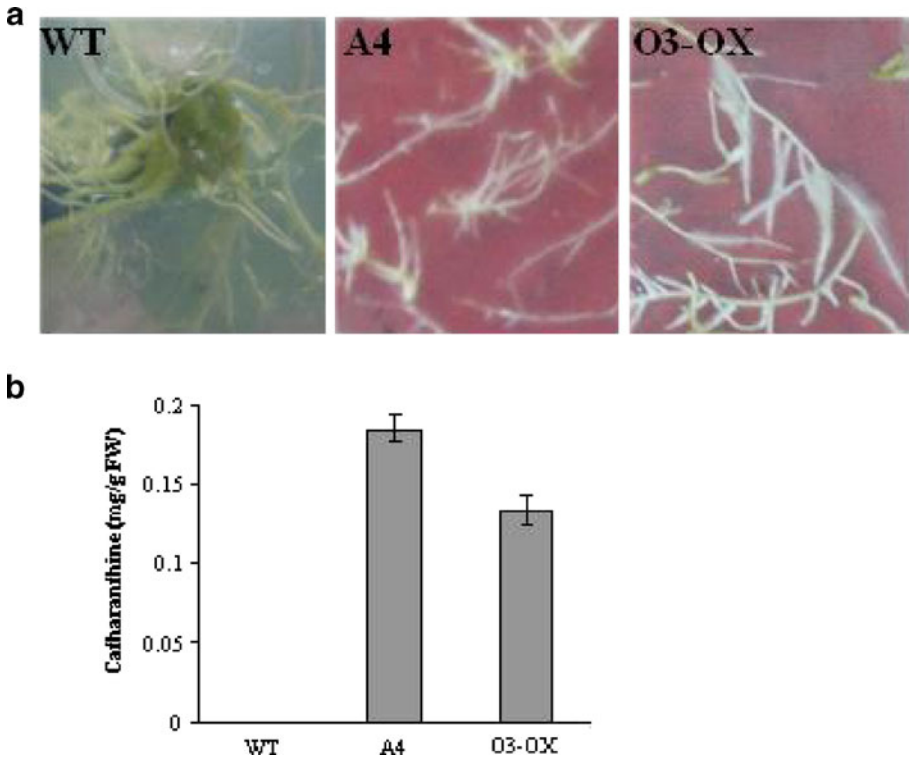

Fig. 5 Phenotype and catharanthine content of three different roots. **a** Phenotype of three different roots. **b** Catharanthine content of three different roots. *WT* wild-type *C. roseus* roots; *A4* hairy root lines generated from transformations with strains *A. rhizogenes* A4 (blank transformation); *O3-OX CrOrca3* over-expressed hairy root lines. *Error bars* represent the standard deviation of triplicate detection

The presence of the *rolA*, *rolB*, and *CrOrca3* genes in O lines demonstrates that the Ri T-DNA and the binary plasmid T-DNA were successfully transferred to *C. roseus* hairy roots. Based on the combination of integrated Ri and Ti T-DNA genes, we identified four different categories of independent hairy root clones: H1, presence of TL-DNA (*rolA* and *rolB*), TR-DNA (*ags*), *CrOrca3* and *Gus* segments (*rolAB*+/*ags*+/*CrOrca3*+/*Gus*+, O4 line); H2, presence of TL-DNA (*rolA* and *rolB*), *CrOrca3* and *Gus* segments (*rolAB*+/*ags*–/*CrOrca3*+/*Gus*+, O2, O3, O6 and O10 lines); H3, presence of TL-DNA (*rolA* and *rolB*), and *CrOrca3* segments (*rolAB*+/*ags*–/*CrOrca3*+/*Gus*–, O1 line); H4, presence of TL-DNA (*rolA* and *rolB*), and *Gus* segments (*rolAB*+/*ags*–/*CrOrca3*–/*Gus*+, O7 line) (Figs. 2 and 3). The absence of *rolA* and *rolB* gene amplification belonged to the callusing and slow-growing morphological category [3]. Due to their extremely slow growth, these root clones did not increase in mass compared to other clones and were discarded. This result indicates the importance of *rolA* and *rolB* in both the induction and sustained growth of hairy roots. Results from the PCR analysis indicated that the transformed hairy root clones of *C. roseus* had both complete and partial insertion of Ri T-DNA. White et al. [22] identified the *rol* loci on TL-DNA to be the most important virulent factors and indicated that *rolB* has a role in pathogenicity. The more frequent occurrence of hairy root clones did not possess *ags* gene integration, indicating that the loss of the *ags* gene from the TR-DNA did not affect the developmental fate of the infected cell and further growth of the transformed roots in *C. roseus* [3]. It has been reported that the integration of the Ri-

plasmid T-DNA and the T-DNA from the binary vector occur independently [17]. More recently, Kumar et al. [12] have shown that during transformation, co-transfer of T-DNA from both Ri plasmid and binary plasmid is not obligatory in *Coffea canephora*, which is consistent with the H3 and H4 categories of hairy root lines. We found variability in the distribution of GUS expression among three O hairy root lines (O3, O6, and O7 lines). The strongest GUS expression was always seen in the root tip and in the central cylinder. This wide variability in the spatial distribution of GUS expression has often been reported in hairy roots [1, 26]. It might result from differences in the number of T-DNA copies or in T-DNA rearrangements ([11, 26]).

The hairy roots from both the A and O lines grew slowly, were thin and straight, and had slender, dense, and white branches with thin tips (Fig. 5a). Generally, a high content of secondary metabolites in plant tissues is associated with poor growth [9]. Catharanthine levels in WT roots were undetectable, in agreement with a previous report [5]. Catharanthine levels in the O lines were lower compared with those of the A lines. These results are consistent with *Orca3* overexpression in *C. roseus* cell suspensions and inducible *Orca3* expression in *C. roseus* hairy roots [16, 21]; no increase in catharanthine was observed. *Orca3* overexpression in *C. roseus* hairy roots exhibited an increase in the levels of *Orca3* transcripts, which then enhance the transcription levels of *Asα*, *Tdc*, *Dxs*, *Str*, *Cpr*, and *Sgd*. This is in agreement with the results observed in *C. roseus* cell cultures [21]. In this study, we find that the constitutive overexpression of *Orca3* in *C. roseus* hairy roots did not lead to a significant increase in the accumulation of catharanthine but instead to a slight decrease. This was due to the fact that *G10h* was not up-regulated under *Orca3* overexpression in *C. roseus* hairy root [24].

Genetically stable transformation of root cultures has been used as a model system to study functional genomics and metabolic regulation of several natural product pathways. The protocol for the development of rapidly growing transgenic hairy root cultures of *C. roseus* provides a powerful and versatile model system to study the molecular regulation of the TIAs pathway.

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