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Full Length Research Paper

Enhanced accumulation of catharanthine and vindoline in *Catharanthus roseus* hairy roots by overexpression of transcriptional factor ORCA2

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The AP2/ERF-domain transcription factor ORCA2 from *Catharanthus roseus* was demonstrated earlier to regulate the expressions of *Str* gene, an important gene involved in the terpenoid indole alkaloids biosynthetic pathway in *C. roseus* cells. Therefore, the factor was postulated to play an important role in the production of secondary metabolites in plants. To investigate the effect of over expression of ORCA2 on the TIAs biosynthesis in *C. roseus* hair roots, transformation of ORCA2 gene was conducted with the disarmed *Agrobacterium rhizogenes* C58C1 harboring pCAMBIA1304[+], a plasmid that contains the *Orca2* gene, a *Gus* gene and an *Hpt* gene all under the control of the cauliflower mosaic virus 35S promoter (35S-CaMV). Transgenic hairy root cultures expressing *Orca2* gene were obtained and demonstrated by genomic- polymerase chain reaction (PCR) analysis for the integration of the *Orca2* gene in the *C. roseus* genome, by real-time quantitative PCR (RT-QPCR) and β-glucuronidase (GUS) staining for the expression of the foreign genes. Metabolite analysis using high performance liquid chromatography (HPLC) analysis established that the average content of catharanthine and vindoline in the transgenic hairy root extracts was increased up to 2.03 and 3.67-fold in comparison to the control lines, respectively. However, vinblastine could not been detected in the transgenic and control hairy root cultures by HPLC.

Key words: *Catharanthus roseus*, ORCA2, hairy root, overexpression, terpenoid indole alkaloids (TIAs), AP2/ERF-domain transcription factor.

INTRODUCTION

The *Catharanthus roseus* plant synthesizes more than 130 different terpenoid indole alkaloids (TIAs). These TIAs include the dimeric alkaloids vinblastine (VLB) and vincristine (VCR), which are valuable antitumor agents, and the monomeric alkaloid ajmalicine, which is used to reduce hypertension (van der Heijden et al., 2004). Most of these TIAs are produced at low levels in the natural plants and are difficult to be chemically synthesized due to their complex structures. The need for chemotherapy treatment of cancers has prompted extensive efforts to develop inexpensive and efficient approaches for production of these TIAs.

TIAs biosynthetic pathway in *C. roseus* is complex with multiple steps and is under strict molecular regulation (Liu et al., 2007). A variety of TIAs are derived from stricto-

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sidine which is condensed from tryptamine and secologanin and catalyzed by strictosidine synthase (STR). The tryptamine is converted from tryptophan by tryptophan decarboxylase (TDC) through the Shikimate pathway (indole pathway), while the secologanin is derived from geraniol via the terpenoid pathway. Strictosidine can be modified by strictosidine glucosidase (SGD) to form cathenamine, a precursor to various biologically active alkaloids (Collu et al., 2001). It was approved that there is an equilibrium between cathenamine and 4,21-dehydrogeissoschizine (Heinstein et al., 1979). Facchini and St-Pierre (2005) pointed out that many important monoterpenoid indole alkaloids, such as tabersonine and catharanthine, are produced via 4,21-dehydrogeissoschizine, but their enzymology has not been established. The biosynthetic route from 4,21-dehydrogeissoschizine to tabosonine is not completely confirmed. However, it has been established that tabersonine is transformed to vindoline through a sequence of six enzymatic steps. In the final dimerization step of the TIAs biosynthetic pathway, a class III basic peroxidase (CrPRX1) was approved to catalyze the coupling of the monomeric precursors vindoline and catharanthine into α-3',4'-anhydrovinblastine (AVLB), the common precursor of all dimeric alkaloids. AVLB is then transferred into VLB and VCR (Sottomayor et al., 1998).

Promoter analysis of the genes that encode STR and TDC reveals that both contain sequences involved in the regulation by stress signals such as UV-irradiation and fungal elicitors (Ouwerkerk et al., 1999; Pasquali et al., 1999). Two transcription factors were isolated by yeast one-hybrid screening with *Str* promoter sequence (Menke et al, 1999). The two proteins were called ORCA1 and ORCA2, for octadecanoid-responsive *Catharanthus* AP2/ERF-domain protein (ORCA). Co-transformation experiments showed that transient overexpression of ORCA2 activated the *Str* promoter, whereas overexpression of ORCA1 had little effect on *Str* promoter activity. Transient expression assays also indicated that ORCA2 transactivated the *Str* promoter via direct binding and its expression was rapidly inducible with jasmonate (JA) and elicitor, whereas ORCA1 was expressed constitutively. Considering that STR is a very important enzyme in TIAs biosynthesis and that STR activity is controlled by expression of ORCA2, therefore, it is necessary to verify the relationship the between function of ORCA2 and TIAs biosynthesis in *C. roseus* cells.

In this study, transcription factor *Orca2* gene was transformed into *C. roseus* hairy root cultures to investigate the transgenic effect of overexpression of ORCA2 on the TIAs biosynthesis in *C. roseus* hairs roots. The results showed that the transgenic hairy root extracts accumulated more catharanthine and vindoline in comparison with the control hair root lines, but VLB could not be detected in the transgenic and non-transgenic hairy root cultures by HPLC analysis. The reasons for the results are discussed.

MATERIALS AND METHODS

Construction of plant expression vector

The β-glucuronidase (GUS) expression cassette was excised from the plasmid pBI121 by *Eco*RI and *Hin*dIII (New England Biolabs, USA) double digestion, and then integrated into plasmid pCAMBIA 1304 (CAMBIA, Canberra, Australia) to form pCAMBIA1304$^+$ (p1304$^+$). The plasmid p1304$^+$ contains two GUS expression cassettes and a hygromycin-resistant gene (*hpt*) expression cassette, which are all driven by CaMV35S promoter.

Total RNA was isolated from the one month old seedlings of *C. roseus* by CTAB method (Chang et al., 1993). Using oligo (dT)18 as template primer and the total RNA as template, the first strand of cDNA was synthesized by AMV reverse transcriptase (Takara Company, China), and then the second strand of the cDNA was replicated by *Escherichia coli* DNA polymerase I after cutting mRNA into oligonucleotides with RNaseH (Takara Company, China).

Based on the CDS sequence of *Orca2* gene (Genbank Accession No. AJ238740), forward primer FO2 5'- GAAGATCTATGTATCAA TCAAATGCCCATAATTCC-3'(*Bgl* II) and reverse primer RO2 5'-GGGTCACCTTATTGAGGACGAAGATGACACG-3'(*Bst*E II) were designed and synthesized for the amplification of *Orca2* gene. The primer sequences contained *Bgl*II or *Bst*EII restriction endonuclease site separately. *C. roseus* cDNAs were used as template and the PCR was performed with PrimeSTAR® HS DNA polymerase kit (Takara Company, China) by denaturing at 94℃ for 1 min, followed by 30 cycles of amplification (98℃ for 10 s, 55℃ for 15 s and 72℃ for 60 s) and then a 10 min final extension at 72℃. The PCR fragment was then cloned into plasmid pMD18-T (Takara Company, China) to form plasmid pMD18-T-*Orca2*. After confirming the sequence, the *Orca2* gene was excised from pMD18-T-*Orca2* by *Bgl*II / *Bst*EII double digestion.

Lastly, plant expression vector p1304$^+$-*Orca2* was constructed by replacing a *gus* gene in the plasmid p1304$^+$ with the *Orca2* gene by *Bgl*II / *Bst*EII double digestion. The vector p1304$^+$-*Orca2* was transformed into the disarmed *Agrobacterium rhizogenes* C58C1 strain carrying the plasmid pRiA4 of *A. rhizogenes*, and the resulting *A. rhizogenes* C58C1 strains was used for the transformation study.

Cultures conditions and genetic transformation

Seeds of *C. roseus*, purchased from PanAmerican Seed Company (Cherry Red, USA), were surface sterilized and placed on MS solid medium (1962) in the greenhouse at 25℃ for germination. Young leaves from one month old germinated seedlings of *C. roseus* were broken with sterile surgical knife and pre-incubated on half-strength MS solid medium for 2 to 4 h, and then cultivated with the *A. rhizogenes* C58C1 strain (OD_{600} = 0.7) containing vector p1304$^+$-*Orca2* for co-cultivation. After 48 h co-cultivation, the leaves were maintained on the regulator-free half-strength MS solid medium containing 500 mg/l cefotaxime to eliminate bacterial contamination. Two weeks after the C58C1 strain infection, hairy roots were induced from the wounded edges and surface of the leaf explants. Single transformed roots were excised when they grew over 2 cm in length and were maintained separately as independent clone. The hairy root lines were grown at 25℃ in the dark and were routinely subcultured to fresh regulator-free half-strength MS solid medium every two weeks. After two months of subculture on solid medium, hairy root cultures were obtained and transferred individually into the regulator-free half-strength B5 liquid medium for continuous subculture. All hairy root cultures were kept at 25℃ on a rotary shaker at 100 rpm in the dark. After 30 days of subculture in liquid medium, the hairy root cultures were filtered, washed with 10 ml sterile distilled water and lyophilized immediately in liquid nitrogen for molecular analysis and TIAs extraction. The control hairy root lines were generated by transforming the leaf explants with C58C1

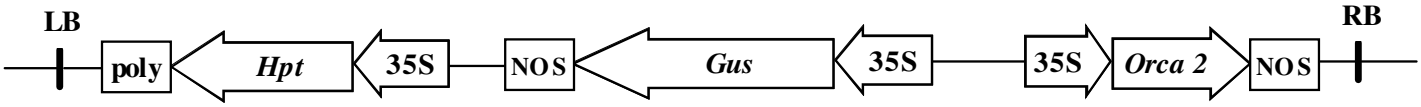

Figure 1. Schematic map of T-DNA region in plant binary expression vector p1304+-Orca2. LB, Left border; RB, right border; 35S, CaMV 35S promoter from cauliflower mosaic virus; NOS, the polyadenosyl signal of the nopaline synthase gene; poly, CaMV 35S poly-A terminator; Hpt, hygromycin-resistant gene; Gus, β-glucuronidase (GUS) gene; Orca2 and Orca2, gene from *C. roseus*.

strain lacking vector p1304$^+$-*Orca2* and were grown for subculture as earlier described.

Polymerase chain reaction (PCR) analysis

The bacterium-free hairy root lines were collected, dried on sterile filter paper and quickly frozen in liquid nitrogen. Total genomic DNA was isolated from putative transgenic and control hairy root lines by using the CTAB DNA isolation method (Woodhead et al., 1998). The DNA samples were then used in PCR analysis for detecting the presence of *Agrobacterium rol* (*rolB*, *rolC*) genes and *hpt* gene in transgenic hairy root cultures. Oligonucleotide primers for the PCR detection of *rol*B, *rol*C and *hpt* gene were designed based on the DNA sequences of these genes described by Fumer et al. (1986). For the amplification of *rol* genes (*rol*B, *rol*C), primers FrolB (5'-GCTCTTGCAGTGCTAGATTT-3'), RrolB (5'-GAAGGTGCAAGCTA CCTCTC-3'), FrolC (5'-CTCCTGACATCAAACTCGTC-3') and RrolC (5'-TGCTTCGAGTTATGGGTACA-3') were used. For the detection of the *hpt* gene, Fhpt (5'-CGATTTGTGTACGCCCGACA GTC-3') and Rhpt (5'-CGA TGTAGGAGGGCGTGGATATG-3') were used. PCR for the detection of all the above genes was carried out by denaturing the template at 94°C for 3 min followed by 35 cycles of amplification (94°C for 40 s, 55°C for 30 s and 72°C for 1 min) and then a 10 min final extension at 72°C. The PCR products were separated by electrophoresis in 0.8% (w /v) agarose gel. Plasmid DNA from *A. rhizoenes* strain containing plasmid p1304$^+$-*Orca2* was used as positive control and genomic DNA from untransformed *C. roseus* root was used as a negative control in PCR analysis.

Real-time quantitative analysis (RT-QPCR)

After 30 days of subculture in liquid medium, total RNA was extracted separately from the putative transgenic and control hairy root cultures with plant RNA mini kit (Watson Company, Shanghai, China), and treated with RNase-free DNase (Takara Company, China) to eliminate the potential contaminating residual DNA. The quality and concentration of RNA samples were tested by agarose gel electrophoresis and spectrophotometer analysis. Total RNA was reversely transcribed by using AMV reserve transcriptase (Takara Company, China) to generate cDNA, which was then subjected to RT-QPCR analysis for the expression of *Orca2* gene. RT-QPCR was performed on a RoterGene 3000 instrument (Corbett Research, Sydney, Australia). The gene-specific primers FOrca2 (5'-GATCAGGATAATTACGAAGACGAAGT-3') and ROrca2 (5'-AGTTCCCAACCATATCCTCGATCCTT-3') were designed according to the conserved sequence of *C. roseus Orca2* gene and were used to amplify the *Orca2* gene. *Ubiqitin* gene (house-keeping gene) was used as an internal calibrator. FUbi (5'-GTGACAA TGGAACTGGAATGG-3') and RUbi (5'-AGACGGAGGATAGCGTG AGG-3') were used as primers to amplify the *Ubiqitin* gene. The RT-QPCR was carried out with SYBR® PrimeScript® RT-PCR kit (Perfect Real Time) according to manufacturer's instructions (Takara Company, China) as follows: 1 min predenaturation at 95°C, 1 cycle; 10 s denaturation at 95°C, 30 s annealing at 56°C and 15 s collection fluorescence at 72°C and 42 cycles. The products of RT-QPCR were run on 1.0% (w/v) agarose gel electrophoresis and it showed an equal-sized band as predicted. Quantification of the gene expression was done with comparative computed tomography (CT) method. The quantitative analysis was repeated three times for each sample.

GUS histochemical assay

GUS assays of the hairy roots were performed by histochemical staining as described by Jefferson et al. (1987) with slight modifications. Both the putative transgenic and the negative control hairy root segments (about 10 mm in length) were incubated at 37°C in the dark in an X-Gluc solution. The X-Gluc solution contained 50 mM Na_3PO_4 (pH 7.0), 10 mM Na_2EDTA, 0.1% (v/v) Triton X-100, 0.1 M $K_3[Fe(CN)_6]$, 0.1 M $K_4[Fe(CN)_6]$, 0.25 mM L-1 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Gluc) and 20% methanol. The roots were subsequently washed in an ethanol gradient at room temperature (30 min in 70% ethanol, 30 min in 40% ethanol and then 30 min in 20% ethanol). After rehydration, the roots were kept in water and then mounted on a slide for observation and photography.

Alkaloids extraction and high performance liquid chromatography (HPLC) analysis

The 30 day old transgenic and control hairy root samples cultured in half-strength B5 liquid medium were harvested and lyophilized overnight, respectively. The resulting dried roots were weighted, ground to very fine powder using a mortar and pestle, and extracted three times at room temperature with 10 ml of MeOH for 1 h in a sonicating bath. The mixture was centrifuged at 13000 g for 15 min at 15°C (Singh et al., 2000). The supernatant was removed and the biomass was re-extracted again prior to HPLC analysis.

The alkaloid analysis of *C. roseus* hairy root samples was performed on a Waters Alliance HPLC system (Alliance model 2690; Waters Corporation, Milford, MA, USA) and separated using a C18 column with binary gradient mobile phase profile (55% 5 mmol/l pH6.0 sodium phosphate buffer, 45% acetonitrile) (Singh et al., 2000; Tikhomiroff and Jolicoeur, 2002). Extracts were analyzed by HPLC with a photodiode array detector (Model 996, Waters) to verify the identity and purity of peaks of interest. HPLC with UV detection at a single wavelength only was employed for quantification of TIAs. An aliquot of 10 µl injection volume provided adequate signal at 220 nm. Authentic standards of catharanthine, vindoline and vinblastine (Sigma, USA) were prepared separately in methanol at a final concentration of 5 g/l and used for the preparation of the calibration graphs. Quantification was repeated three times for each sample.

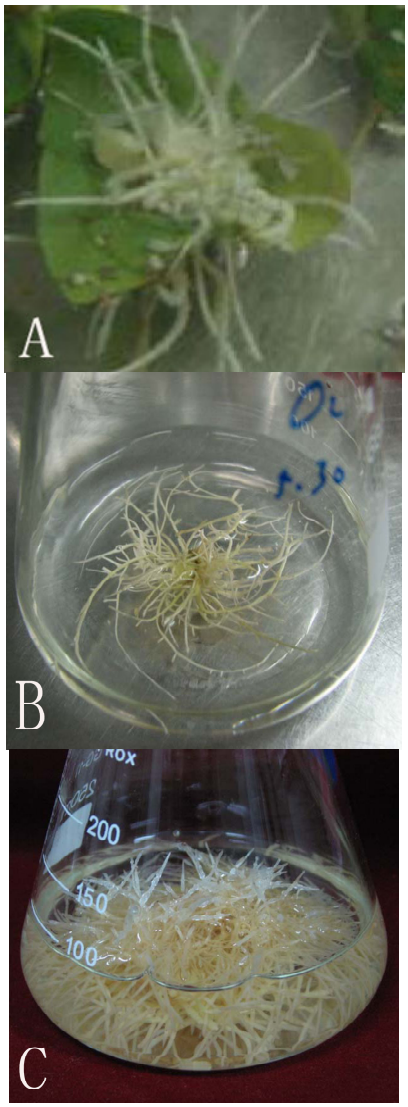

Figure 2. The induction and subculture of transgenic hairy root cultures; (A), Hairy roots were induced from the leaf explants *C. roseus* and were cultured on MS solid medium; (B), hairy roots growing in half strength B5 liquid medium in a 250 ml Erlenmeyer flask for ten days; (C), hairy roots were cultivated in the liquid medium for one month.

RESULTS

Establishment and subculture of *C. roseus* hairy root cultures

The plant expression vector p1304$^+$-*Orca2*, harboring the coding region of the wild-type *C. roseus Orca2* gene and two selectable marker genes (*hpt* and *Gus*) on the same T-DNA fragment, was constructed (Figure 1) and transformed into disarmed *Agrobacterium tumefaciens* C58C1 strain carrying the plasmid pRiA4 of *A. rhizogenes*. Integration of *Orca2* gene into the vector p1304$^+$ and transformation of vector p1304$^+$-*Orca2* into C58C1 strain was confirmed separately by PCR analysis and sequencing. Two weeks after infecting the leaf explants, hairy roots were induced from the wounded edges and surface of the leaves. Ten days later, the percentage of leaf explants to form hairy root lines was about 70%.

50 putative transgenic hairy root lines and 30 control hairy root lines were excised separately when they grew over 2 cm in length and transferred to the fresh regulator-free half-strength MS solid medium that contained 500 mg/l cefotaxime to eliminate bacterial contamination. These hairy root lines were cultured in the dark at 25°C and were then routinely subcultured to the same MS solid medium every two weeks (Figure 2A). After two months of subculture, 38 independent putative transgenic hairy root cultures and 16 control hairy root cultures were obtained and transferred individually into the regulator-free half-strength B5 liquid medium for subculture. In comparison with growth on the solid medium, hairy roots cultured in the liquid medium grew more rapidly and had higher lateral branching (Figures 2B and C). However, the hairy root cultures changed gradually from white to red-brown during the subculture, and it could be observed that a few of red-brown substance was secreted from hairy roots into the culture medium after 3 to 4 weeks. As a control, adventitious roots excised from *C. roseus* sterile seedlings were cultured on the growth regulator-free half-strength MS solid medium, but these roots grew very slowly, did not branch and perished after 2 or 3-week subculture period.

Of the 38 putative transgenic hairy root cultures, only 12 cultures could be maintained in liquid media after one month of subculture. At the same time, 8 control hairy root cultures were obtained. These cefotaxime-resistant hairy root lines (12 putative transgenic and 5 control hairy root cultures) were evaluated for growth, integration and expression of Ri plasmid T-DNA genes, and alkaloid contents in dry hairy root samples.

Molecular analysis of the transgenic hairy root cultures

By using the genomic DNA from the putative transgenic and the control hairy root cultures as template, integration of the *rol* genes (*rol*B, *rol*C) and *hpt* gene into the genome of *C. roseus* hairy root cultures was confirmed by PCR analysis (Figure 3). As expected, it was demonstrated that three fragments, with lengths of 423, 622 and 812 bp corresponding to *rolB*, *rolC* and *hpt* gene, respectively, were amplified only from the putative transgenic hairy root cultures but not from the control hairy root samples. These results indicated that the *rolB* and *rolC* genes from the Ri plasmid of *A. rhizogenes* C58C1 and the *hpt* gene from the plant expression vector p1304$^+$-*Orca2* were all integrated into the genome of transgenic *C. roseus* hairy root cultures.

Real time-PCR is the most sensitive method for quanti-

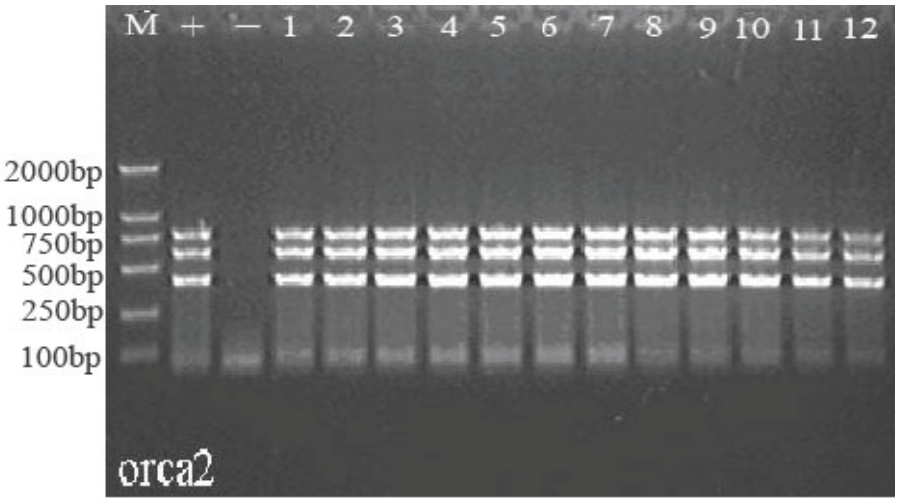

Figure 3. PCR analysis for the presence of *rolB*, *rolC* and *hpt* gene in independently transgenic hairy root cultures. M, DL2000 marker; lane +, plasmid p1304^{+}-*Orca2* was used as positive control; lane -, untransformed *C. roseus* root DNA was used as a negative control; lanes 1 to 12, 12 individual hairy root cultures transformed with *Orca2* gene.

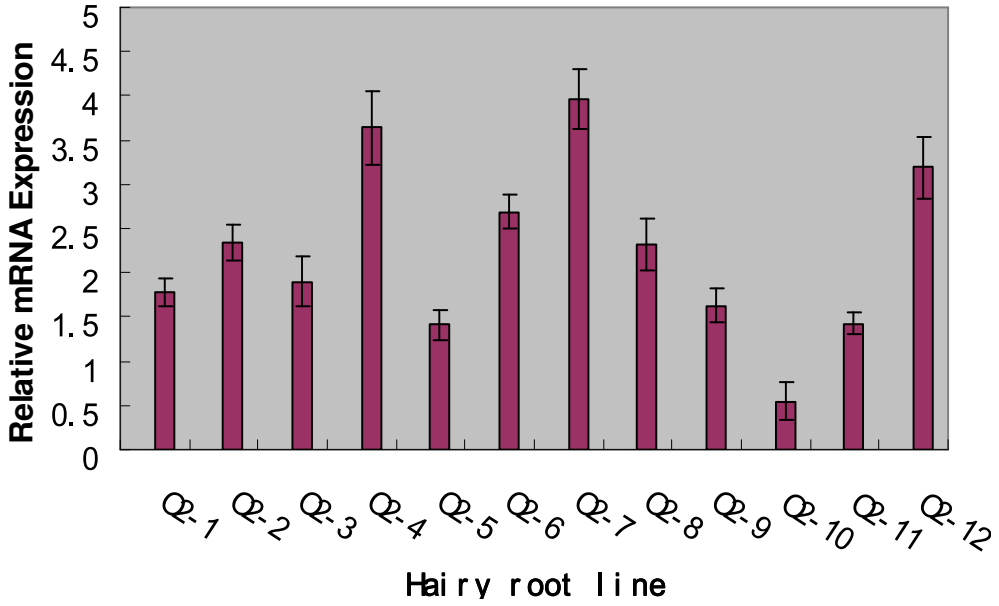

Figure 4. Relative mRNA expression levels of *Orca2* gene in transgenic hairy root cultures of *C. roseus* checked by real-time PCR method. The mRNA expression value in untransformed control sample was 1.0; O2-1 to O2-12, 12 individual hairy root cultures transformed with *Orca2* gene; Data shown are means ± standard deviation of three replicate measurements.

tation of gene expression levels. SYBR green I-based quantitative real-time PCR method was used to characterize the *Orca2* gene relative expression status in transgenic *C. roseus* hairy root cultures. The results indicated that the expression levels of *Orca2* gene in 12 putative transgenic hairy root samples were different. Among them, the samples No.4, No.7 and No.12 expressed the maximum level of *Orca2* gene (3.646-, 3.96- and 3.19-fold) in comparison with the control samples, respectively, while the sample No.10 expressed the lowest level of *Orca2* gene in all hairy root samples (the value is about half as much expression as in the control samples) (Figure 4). The fact indicated that expression of *Orca2* gene was inhibited in the transgenic hairy root sample

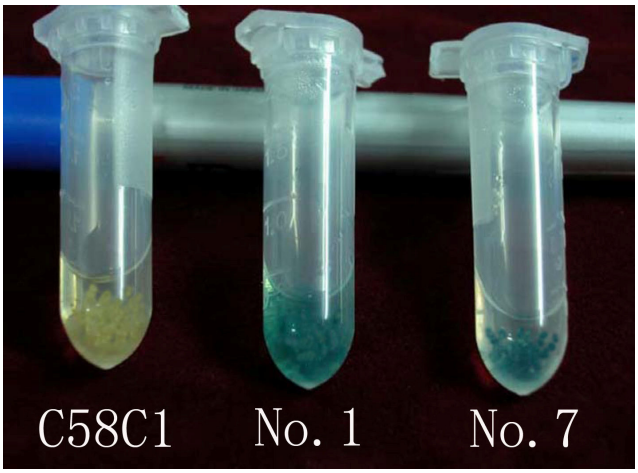

Figure 5. GUS staining results of *C. roseus* hairy root cultures. C58C1, non-transformed (negative control) hairy root cultures induced from C58C1; No.1, No.7, Number 1 and Number 7 of hairy root cultures transformed with *Orca2* gene, respectively.

No.10. After detecting the gene relative expression of *Orca2* gene, the seven hairy root cultures with high expression level were used for HPLC analysis.

In conclusion, molecular analysis experiments for the hairy root cultures proved that the T-DNA from the disarmed *A. rhizogenes* C58C1 strain containing vector p1304^{+}-*Orca2* had been integrated into all the putative transgenic hairy root cultures.

GUS histochemical assay

To monitor transgenic expression in the hairy root cultures, histochemical GUS activity assays were performed with seven putative transgenic and five negative control hairy root cultures from cefotaxime-resistant hairy root lines. All the putative transgenic hairy root cultures were stained blue and different samples showed diverse GUS activity with different blue spots. The control hairy root samples showed no significant GUS activity (Figure 5). These results indicate that the *Gus* gene in the T-DNA had already been integrated into the genome of the transgenic *C. roseus* hairy root cultures.

HPLC analysis of TIAs accumulation in hairy root cultures

TIAs profiles (catharanthine, vindoline and VLB) of the putative seven hairy root cultures (samples No.1, 2, 4, 6, 7, 8, 12) with relative high level expression of *Orca2* gene, and negative control hairy root cultures were determined by HPLC analysis in this study. The results showed that the putative transgenic hairy root extracts accumulated more catharanthine and vindoline in comparison with the control hair root cultures. However, VLB content could not be detected in the transgenic and control hairy root cultures by HPLC method. The average catharanthine content in the transgenic hairy root extracts was 4.7869 ± 0.59 mg/g DW; over 2.03-fold higher than that in the control cultures (2.357 ± 0.415 mg/g DW). The sample No.7 accumulated the maximum level of catharanthine content (5.986 ± 0.672 mg/g DW) among the seven samples, while the sample No.4 accumulated the lowest level of catharanthine content (3.815 ± 0.376 mg/g DW) (Figure 6, upper panel). The average vindoline content in the putative transgenic hairy root cultures were 0.144 ± 0.0157 mg/g DW, over 3.67-fold higher than that in the control cultures (0.0392 ± 0.0054 mg/g DW). It is noteworthy that the transgenic sample No.2, but not the sample No.7, accumulated the highest level of vindoline content (0.1888 ± 0.0024 mg/g DW) (Figure 6, lower panel).

DISCUSSION

Transcription factors are regulatory proteins that modulate the expression of specific groups of genes through sequence-specific DNA binding and protein-protein interactions. They can act as activators or repressors of gene expression, mediating either an increase or a decrease in the accumulation of messenger RNA (Broun, 2004). More than ten transcription factor genes have been cloned and characterized in *C. roseus* till now. Among them, *Orca1*, *Orca2* and *Orca3* were characterized to control closely the expression of some genes involved in TIAs biosynthesis. *ORCA1* had been approved to be expressed constitutively and had little effect on *Str* promoter activity. *ORCA2* activated the *Str* promoter and its expression was rapidly inducible with jasmonate (JA) and elicitor (Menke et al., 1999). *Orca3* was isolated via a T-DNA activation tagging approach applied to a *C. roseus* cell culture (van der Fits and Memelink, 2000; van der Fits et al., 2001) and activates *Str* gene expression by binding to the special sequence of *Str* gene promoter (van der Fits and Memelink, 2001). Overexpression of ORCA3 in *C. roseus* cultured cells increased the expression of the TIA biosynthesis genes *Tdc*, *Str*, *Sgd*, *Cpr*, *D4h*, *Asα* and *Dxs*. However, ORCA3 was not found to regulate *G10h* and *Dat* gene. The fact indicates that ORCA3 is a central regulator of TIA biosynthesis, acting on several steps of the TIA pathway and also regulating the biosynthesis of TIA precursors (Memelink and Gantet, 2007). The function of ORCA3 had been approved in *C. roseus* cultured cells, however, the function of ORCA2 during the TIAs biosynthesis has not been reported till now. Therefore, *Orca2* gene was chosen to transform *C. roseus* leaves to investigate the transgenic effect of overexpressing ORCA2 on the TIAs biosynthesis in *C. roseus* hairs roots. The results indicated that the accumulation of catharanthine and vindoline

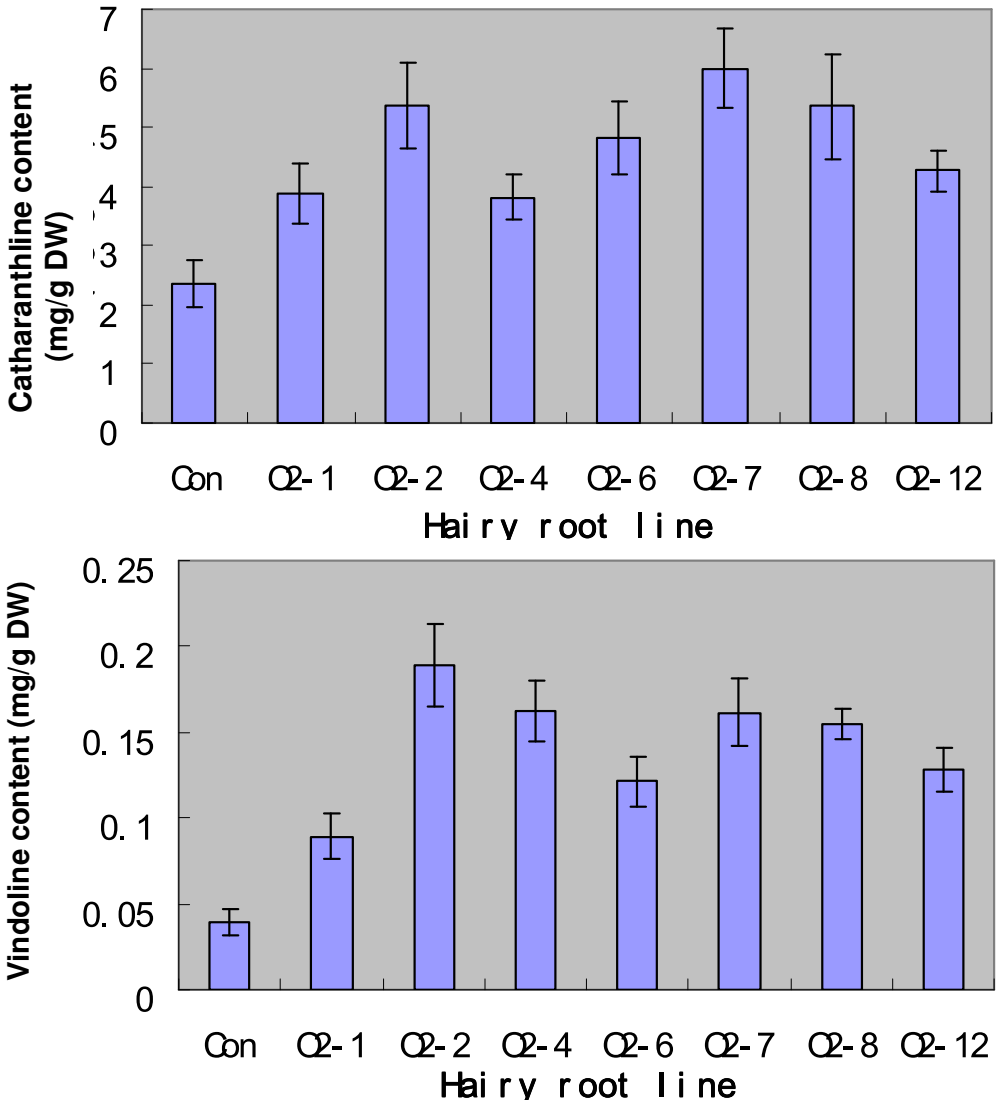

Figure 6. Catharanthline (upper panel) and vindoline contents (lower panel) in the transgenic hairy root cultures of *C. roseus* detected by HPLC. Con, untransformed (negative control) hairy root culture; O2-1,02-2,02-4,02-6,02-7,02-8,02-12, seven individual hairy root cultures transformed with *Orca2* gene; Data shown are means ± standard deviation of three replicate measurements.

are enhanced by overexpressing ORCA2 in *C. roseus* hairs roots.

It had been approved that catharanthine is distributed equally throughout the aboveground and underground tissues of *C. roseus*. However, vindoline, tabersonine as well as the dimeric alkaloids are restricted to leaves and stems because of the late steps of vindoline biosynthesis which require specialized cell types, idioblast and laticifer cells, which are located in stems and leaves (Westekemper et al., 1980; Deus-Neumann et al., 1987). Therefore, many cell and hairy root cultures produce catharanthine and tabersonine, but do not produce vindoline because there is a limitation in the conversion from tabersonine to vindoline (Shanks et al., 1998). This fact had been confirmed by some experiments (Bhadra and Shanks, 1997; Brillanceau et al., 1989; Toivonen et al., 1989).

In this study, however, the results demonstrated that the transgenic hairy root extracts accumulated 2.03 and 3.67-fold of catharanthine and vindoline more than the control hairy root cultures, respectively. Previously, Parr et al. (1988) reported a similar result: an experimental detection by immunoassay demonstrated that ajmalicine, serpentine, vindolinine and catharanthine were prominent components in *C. roseus* hairy root cultures during all stages of the growth cycle. Vinblastine could also be detected by a combination of HPLC and radioimmunoassay, though at a low level (0.05 µg/g DW). They

speculated that the differentiated characteristics of the hairy roots have offered the potential for the production of various monomeric indole alkaloids. It can be proposed that the main reason for these results is the activation of the enzymes in vindoline biosynthesis pathway in hairy root cultures by weak light during their subculture in liquid medium, since it was found that the hairy root samples had been lightened by some weak scattered light around in the culture room. It was noticed that some early experiments had already proven that light plays a critical role in TIAs biosynthesis in *C. roseus* plants during their growth and development. Further studies showed that phytochrome is involved in the activation of the last two enzymes in vindoline biosynthesis; D4H (Vazquez-Flota and De Luca, 1998) and DAT (Aerts and De Luca, 1992), in *Catharanthus* seedlings. In addition, Ramani and Jayabaskaran (2008) reported that catharanthine and vindoline increased 3 and 12-fold, respectively, on treatment with a 5-min UV-B irradiation in the suspension cultures of *C. roseus*. The other possible reason for the results is that the overexpression of ORCA2 in the hairy root might have triggered the flow of TIAs pools towards AVLB biosynthesis in which catharanthine and vindoline are needed as precursors. Therefore, our next research would focus on investigating the exact mechanism of light on the vindoline biosynthesis in *C. roseus* hairy root cultures and on the metabolic flow during TIAs biosynthesis in *C. roseus*.

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Abbreviations

TIAs, Terpenoid indole alkaloids; **PCR,** polymerase chain reaction; **MS,** Murashige and Skoog; **ORCA,** octadecanoid-responsive *Catharanthus* AP2/ERF-domain protein; **CTAB,** cetyltrimethyl ammonium bromide; **HPLC,** high performance liquid chromatography; **RT-QPCR,** real-time quantitative PCR; **GUS,** β-glucuronidase; **STR,** strictosidine synthase; **TDC,** tryptophan decarboxylase.

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