Plant Cell Rep (2010) 29:887–894
DOI 10.1007/s00299-010-0874-0

ORIGINAL PAPER

Overexpression of *G10H* and *ORCA3* in the hairy roots of *Catharanthus roseus* improves catharanthine production

Cui-Ting Wang · Hua Liu · Xiao-Shu Gao · Hong-Xia Zhang

Received: 24 February 2010 / Revised: 20 April 2010 / Accepted: 13 May 2010 / Published online: 10 June 2010

Abstract A number of genes that function in the terpenoid indole alkaloids (TIAs) biosynthesis pathway have been identified in *Catharanthus roseus*. Except for the geraniol 10-hydroxylase (G10H) gene, which encodes a cytochrome P450 monooxygenase, several of these genes are up-regulated by ORCA3, a jasmonate-responsive APETALA2 (AP2)-domain transcript factor. In this study, the *G10H* gene was transformed independently, or co-transformed with *ORCA3* into *C. roseus*, using *Agrobacterium rhizogenes* MSU440. Hairy root clones expressing the *G10H* gene alone, or both the *G10H* and *ORCA3* genes, were obtained. Alkaloid accumulation level analyses showed that all transgenic clones accumulated more catharanthine, with the highest accumulation level in the transgenic clone OG12 (6.5-fold higher than that of the non-expression clone). Following treatment with ABA, accumulation of catharanthine reached 1.96 mg/g DW in the transgenic clone OG12. The expression levels of TIAs biosynthesis genes in transgenic and non-transgenic clones were also investigated.

Keywords *Agrobacterium rhizogenes* · *Catharanthus roseus* · Terpenoid indole alkaloids (TIAs) · Transformation

Communicated by K. Chong.

C.-T. Wang · H. Liu · X.-S. Gao · H.-X. Zhang (✉)
National Key Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China
e-mail: hxzhang@sippe.ac.cn

Introduction

As an important medicinal plant, *Catharanthus roseus* (Madagascar periwinkle) produces a large amount of terpenoid indole alkaloids (TIAs). Among them, vinblastine and vincristine are important antitumor bisindole alkaloids. However, these two anticancer compounds are produced at a very low level in *C. roseus* leaves, about 5.8 μg/g for vinblastine and 0.9 μg/g (fresh weight) for vincristine, leading to their high price in the market (Favretto et al. 2001). Biotechnological methods may provide an efficient alternative for producing natural products since a number of genes involved in the TIAs' biosynthetic pathway have been cloned (Pasquali et al. 2006). The multistep TIAs' biosynthetic pathway is not only quite complex but also strictly regulated by some transcriptional factors (Da Costa e Silva et al. 1993; Pauw et al. 2004; van der Fits and Memelink 2000).

Due to the genetic and biochemical stability and fast growing character in a medium consisting of sucrose and simple salts, hairy root culture has been used as research material for secondary metabolites in vitro. Recently, successful efforts have been made using hairy root cultures to improve secondary metabolism compounds in *Hyoscyamus niger* (Zhang et al. 2004) and p-hydroxybenzoic acid (pHBA) glucose ester production in hairy roots of *Beta vulgaris* (Rahman et al. 2009), express foreign proteins or vaccine in tobacco (Shadwick and Doran 2007; Woods et al. 2008), and produce "unnatural" products in *C. roseus* (Runguphan and O'Connor 2009). Several TIAs' biosynthesis genes have also been overexpressed in *C. roseus* hairy root cultures. Ectopic overexpression of anthranilate synthase holoenzyme ($AS\alpha\beta$) increased the amount of tryptophan, and resulted in the accumulation of several phenolic compounds (Chung et al. 2007). In the

TDC-overexpressing hairy root line, serpentine accumulation was increased as much as 129% (Hughes et al. 2004). Co-transformation of *ASαβ* and *TDC* genes in *C. roseus* hairy root cultures remarkably increased the production of both tryptamine and alkaloids (Hong et al. 2006). Very recently the *DAT* gene, which is responsible for the terminal step of vindoline biosynthesis in *C. roseus*, was overexpressed in *C. roseus* hairy roots. Interestingly, overexpression of *DAT* did not increase vindoline production but improved the accumulation of another monoterpenoid indole alkaloid, hörhammericine (Magnotta et al. 2007).

Previously, ORCA3, a jasmonate-responsive AP2-domain regulator, enhanced the expression of several metabolite biosynthetic genes, such as *CPR*, *TDC*, and *STR*, and increased the accumulation of TIAs consequently in *ORCA3*-tagged *C. roseus* cell line (Van der Fits and Memelink 2000; Fig. 1). Whereas the transcription of *G10H* was not regulated by ORCA3 (Van der Fits and Memelink 2000). *G10H* encodes a cytochrome P450 monooxygenase, which hydroxylates geraniol to form 10-hydroxy-geraniol, and thus controls the first committed step in the biosynthesis of secologanin and TIAs (Collu et al. 2001). Further research revealed that the *G10H* promoter contains unique binding sites of several transcriptional factors, suggesting that the *G10H* promoter may be regulated by a different transcriptional cascade (Suttipanta et al. 2007).

Although several alkaloids have been successfully produced in *C. roseus* hairy roots, no study on improving catharanthine, one of the most important medical alkaloids, has been reported. In this study, a construct harboring the *G10H* gene driven by the constitutive cauliflower mosaic virus (*CaMV) 35S* promoter, or a combined construct harboring both the *G10H* driven by the *CaMV 35S* promoter and the *ORCA3* gene driven by the stress-inducible *rd29A* promoter from Arabidopsis (Yamaguchi-Shinozaki and Shinozaki 1994), was introduced into *C. roseus* hairy roots. The accumulation of four alkaloids, vindoline, catharanthine, vinblastine, vincristine, and the transcriptional levels of several TIA biosynthesis genes are investigated in the engineered hairy roots.

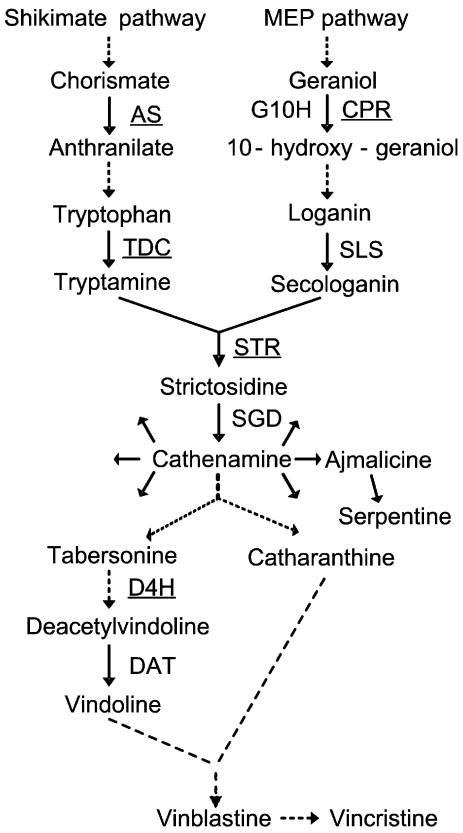

Fig. 1 Biosynthesis of TIAs in *C. roseus*. *Solid arrows* indicate single-step enzymatic conversions, whereas *broken arrows* indicate multiple-step reactions. *AS* anthranilate synthase, *TDC* tryptophan decarboxylase, *G10H* geraniol 10-hydroxylase, *CPR* cytochrome P450-reductase, *SLS* secologanin synthase, *STR* strictosidine synthase, *SGD* strictosidine *β*-D-glucosidase, *D4H* desacetoxyvindoline 4-hydroxylase, and *DAT* deacetylvindoline 4-*O*-acetyl transferase. Genes up-regulated by ORCA3 are *underlined*. (revised from van der Fits and Memelink 2000)

Materials and methods

Construction of plant expression vectors

Plasmids pGEMT-G10H and pGEM-T easy-ORCA3, carrying *C. roseus G10H* and *ORCA3* genes, respectively, were kindly provided by Professor J. Memelink. The *G10H* cDNA was isolated from pGEMT-G10H and inserted into the *Nco*I and *Pma*CI sites in pCAMBIA1301 (http://www.cambia.org) to generate the plant expression vector pG10H (Fig. 2a). To construct the pOG vector which carries both the *G10H* and the *ORCA3* genes, the stress/ABA-inducible *rd29A* promoter was cloned from Arabidopsis using the *rd29A* forward (5′-CCGGTACCCGACTCAAA ACAAA CTTACG-3′) and reverse (5′-C GCCGCGGAATCAAA CCCTTTA TTCCTG-3′) primers. The 824 bp PCR product was cloned into pUC19 for sequence confirmation. This intermediate plasmid was named pUC19-Prd29A. The *ORCA3* gene was excised from pGEM-T easy-ORCA3 and cloned into pUC19-Prd29A. Then, the P*rd29A-ORCA3* fragment was inserted just upstream of the OC*S* terminator in p1301P (via *Kpn*I and *Pst*I), a modified pCAMBIA1301 vector with a *CaMV 35S* promoter and an *OCS* terminator introduced into the multicloning sites. The whole expression cassette (P*rd29A-ORCA3-OCS*) was transferred (as a *Kpn*I-*Hind*III fragment) onto pCAMBIA1301 to form a new intermediate plasmid pORCA3. Finally, the plant expression vector pOG was generated by replacing the

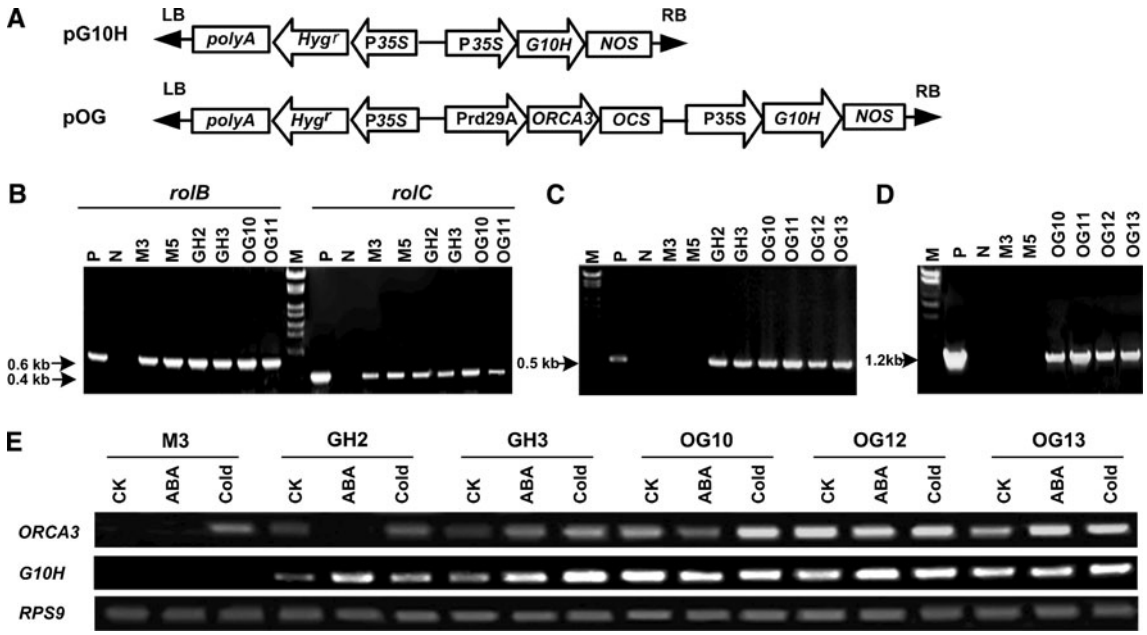

Fig. 2 Plant expression constructs and molecular analyses of transgenic hairy root lines. **a** Schematic map of pG10H and pOG constructs used to transform *C. roseus*. PCR analyses for the presence of *rolB* and *rolC* genes (**b**), *G10H* gene (**c**) and *ORCA3* gene (**d**) in different hairy root lines. *M* λ/*Eco*T14 I digested DNA marker; *P* positive control; *N* negative control; *M3*, *M5* control hairy root lines; *GH2*, *GH3* hairy root lines transformed with pG10H; *OG10*, *OG11*, *OG12* and *OG13* hairy root lines transformed with pOG. **e** Expression analyses of *G10H* and *ORCA3* in transgenic lines and control hairy root lines under different treatments. RT-PCR assays were performed and the *RPS9* gene was analyzed to serve as a quantitative internal control. *CK* control; *ABA* hairy roots treated with 100 μmol/l ABA for 24 h; Cold, hairy roots cultured at 4°C for 24 h

β-glucuronidase (*GUS*) gene with the *G10H* fragment excised from the plasmid pG10H. The plant expression vectors (pG10H and pOG) were transformed into *A. rhizogenes* MSU440 as described previously (Wise et al. 2006).

Plant transformation and hairy root cultivation

Plant materials (*C. roseus* L. G. Don) were grown in the greenhouse under a 16 h light/8 h dark photoperiod (~1,450 μmol m^{-2} s^{-1}), with 50 ± 10% relative humidity. The temperatures were set to 26°C at daytime and 21°C at night. The *Agrobacterium rhizogenes* strain MSU440 harboring pG10H or pOG was used for *C. roseus* transformation as described previously (Hughes et al. 2002). Hairy roots formed at the infected site in about 3 weeks after inoculation. The hairy roots were excised from the plant when they were 2–4 cm in length, and transferred onto hormone-free, half-strength B5 (1/2 B5) solid medium supplemented with 30 g/l sucrose, 200 mg/l timentin (GlaxoSmithKline, China), and 10 mg/l hygromycin (Gamborg et al. 1968). All hairy root cultures were grown on petri dishes in the dark at 26°C. The rapidly growing hygromycin-resistant lines were used to establish hairy root lines. Root tips 2–4 cm in length of well-established hairy root lines were transferred to fresh solid media without hygromycin every 6 weeks. Wild-type hairy root lines formed after infection with the *A. rhizogenes* strain MSU440 were established to be used as control.

PCR analysis

Genomic DNA was extracted from control and transformed hairy roots with 400 μl of homogenizing buffer (250 mM NaCl, 25 mM EDTA, 0.5% SDS, 200 mM Tris–HCl, pH 7.4). The supernatant of a 10,000*g* centrifugation was mixed with 300 μl of isopropanol. DNA was collected at the interface, washed, and re-suspended in double-distilled water. The quality and the concentration of the extracted DNA were confirmed using a 0.8% (w/v) agarose gel. To detect the presence of *Agrobacterium rol* (*B*, *C*), *G10H* and *ORCA3* genes in the hairy root cultures, PCR analyses were performed. The primers for *rol B* and *rol C* gene detection are as follows: rolB forward (5′-CGAGGGGATCCGATT TGCTTT-3′), rolB reverse (5′-GACGCCCTCCTCGCCTT CCT-3′), rolC forward (5′-TC GCCATGCCTCACCAA CTCAC-3′), and rolC reverse (5′-CCTTGATCG AGCCGGGT GAGAA-3′). Primers for *G10H* and *ORCA3* gene detection are 35S-F (5′-TTCGCAAGACCCTTC CTC-3′) and G10H-R (5′-CCGCTTCTCCGCTCTGGC TA-3′), and Prd29A-F and ORCA3-R (5′-CGTCGTA GAAGGCTCCGCAGG-3′), respectively. For PCR reaction,

an aliquot of 10 ng of DNA was combined with 2.5 μl of 10× buffer, 1.5 μl of 15 mM $MgCl_2$, 1 U of *Taq* polymerase (Sangon, Shanghai, China), 0.5 μl of 2.5 mM dNTP and 2 μl of 10 mM each primer. PCR programs were set as following: 3 min at 94°C, then 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min and holding at 4°C. Five microliters of PCR products were visualized on 0.8% (w/v) agarose gel.

Abscisic acid (ABA) or cold treatment

Hairy roots from each line were divided into three groups: one was for cold treatment, the second for ABA treatment, and the third was used as control. About 300 mg (fresh weight, FW) hairy roots of each line were transferred into a 250 ml culture bottle containing 30 ml 1/2 B5 liquid media. For cold treatment, cultures were kept at 4°C for 24 h in the dark, and the control group was cultured at 26°C for 24 h in the dark without shaking. For ABA treatment, hairy root cultures were grown under the same conditions as control, but the liquid media were supplement with 100 μmol/l ABA (Sigma).

RT-PCR analysis of gene expression

Total RNA was isolated from the hairy roots of each line grown under different conditions (control, ABA or cold treatment) using the RNAiso reagent (TaKaRa, Japan), and then digested with RNase-Free DNase (Promega). cDNA was prepared from 2 μg total RNA per reaction using ReverTra Ace (TOYOBO, Japan). Expression levels of *G10H* and *ORCA3* in transgenic hairy root lines were analyzed using primers G10H-F (5′-CCATCGCC ATTGCCGTTCAT-3′) and G10H-R, and ORCA3-F (5′-G GCGGCCGGAGGAGGTTGTTC-3′) and ORCA3-R, respectively. The expression level of *RPS9* (40S ribosomal protein S9) was investigated with primers RPS9-F (5′-AGCGCCGCCTTCAAACCCTT-3′) and RPS9-R (5′-GCCAGGACGACCACCACCGA-3′) to serve as a quantitative internal control.

Alkaloid extraction and HPLC analysis

Lyophilized hairy roots, 100 mg (dry weight, DW) of each hairy root line, were weighted and used for the alkaloid extraction as described previously (Tikhomiroff and Jolicoeur 2002). For HPLC analysis, individual stock solutions of standard samples, catharanthine, vindoline (Aladdin-Reagent, China), vinblastine, and vincristine (Sigma), were prepared at a concentration of 5 mg/ml in methanol, and stored at −20°C. The stock solutions were diluted in methanol at different concentrations (0.5–20 μg/ml for vindoline, vinblastine and vincristine, and 10–200 μg/ml for catharanthine). The HPLC analysis was performed using an Agilent 1100 Series HPLC System. A Zorbax Eclipse XDB-C_{18} (150 mm × 4.6 μm) column, coupled with a 1 cm × 4.3 mm ODS guard column, was used at a column temperature of 35°C. The injection volume was 10 μl. The mobile phase and eluent profile were performed as described earlier (Tikhomiroff and Jolicoeur 2002). The flow-rate was 0.9 ml/min and the DAD detection wavelength was 220 nm. The peaks' purity was determined using the Agilent ChemStation A09.01 software. Quantification analysis was repeated for three replicates of each line in parallel, and the means and standard deviations were calculated.

RT-PCR analyses

The transcripts of several marker genes involved in *C. roseus* TIAs' biosynthesis such as, *ASα*, *CPR*, *SLS*, *TDC*, *STR*, *D4H* and *DAT*, were analyzed using the following primers: ASα-F: 5′-GGCGGCGAAGCATGGGAACT-3′, ASα-R: 5′-CCTCGGTCCGCTGG GG TTTC-3′; CPR-F: 5′-TCGGCCCTGGTACTGGACTAGC-3′, CPR-R: 5′-TG GCATCACCGCAGACGTAAAC-3′; SLS-F: 5′-CTGCCA GCTTTTGCCATATGC-3′, SLS-R: 5′-CCAGCCTTCAA AGCCTTCATTC-3′; TDC-F: 5′-TCGGCATCTCACCTC AAGTTCT-3′, TDC-R: 5′-TCGGGACATATACAGGC GCTT-3′; STR-F: 5′-TTCGCCTACGCATCTCCCTTCT-3′, STR-R: 5′-CCGCCGGGAACATGTAGCTCTT-3′; D4H-F: 5′-TGGCGGCCTTCAAATTCTTCTT-3′, D4H-R: 5′-TCC GTACAATCTTGGCGAAACC-3′; DAT-F: 5′-CGGAGG TTTGGCGGTTGCTT-3′ and DAT-R: 5′-CCGTGGCAC ATCGACTGAGAAA-3′. The expression levels of *G10H* and *ORCA3* were also analyzed at the same time. The *RPS9* served as a quantitative internal control.

Statistical analysis

All data in this work were obtained from at least three independent experiments with three replicates each. For data analyzed with the Student's *t* test, the difference between treatments was considered as significant when $P < 0.05$, 0.01 or 0.001 in a two-tailed analysis.

Results and discussion

Generation of transgenic hairy root lines

The *G10H* gene alone or integrated with *ORCA3* gene was introduced into *C. roseus*. Hairy root lines resistant to hygromycin were selected. A total of 25 hygromycin-resistant hairy root lines transformed with pG10H (GH) and 38 hygromycin-resistant hairy root lines transformed with pOG (OG) were obtained, of which 12 GH and 9 OG

lines survived the subsequent selection process (three to five rounds of selection). Two GH lines (GH2 and GH3) and three OG lines (OG10, OG12 and OG13), which showed fast growth were chosen for further examinations. As control, two wild-type hairy root lines (M3 and M5) were also generated following inoculation with MSU440, which contains neither pG10H nor pOG.

Molecular analysis of genetically engineered hairy roots

PCR analysis of *Agrobacterium rol* genes revealed that both control and transgenic hairy roots contained the *rolB* and *rolC* genes (Fig. 2b). To eliminate the interference from *C. roseus* endogenous genes, both forward primers for the integration confirmation of *G10H* and *ORCA3* into the transformed hairy root genome land on the promoter sequences. PCR analyses confirmed the integration of *G10H* and/or *ORCA3* in all the tested transgenic lines (Fig. 2c, d).

To investigate the expression of the introduced *G10H* and *ORCA3* genes, total RNA was isolated from the generated hairy root lines following different treatments. We observed that *G10H*, which is driven by the constitutive *CaMV 35S* promoter, was overexpressed in all transgenic root lines (Fig. 2e); the expression of *G10H* was affected by ABA or cold treatment. As shown in Fig. 2e, the expression level of *G10H* increased in transgenic lines GH2 and GH3 following ABA or cold treatment. The transcriptional factor gene *ORCA3*, which is promoted by the stress-inducible promoter *Prd29A*, was also overexpressed in all the examined transgenic hairy root lines, especially following ABA or cold treatment (Fig. 2e). However, the transcripts of *ORCA3* were only detectable following cold treatment in the control hairy root line (M3), implying that the *rd29A* promoter from Arabidopsis was sensitive to environmental stress in the *C. roseus* hairy roots. ABA treatment increased *ORCA3* expression in OG13 whereas it decreased the expression slightly in OG10. In all hairy root lines, including M3, *ORCA3* was expressed at a comparatively higher level following cold treatment, suggesting that the transcripts of endogenous *ORCA3* gene could be induced by low temperature.

As a jasmonate-responsive transcriptional factor, transcription of *ORCA3* can be activated by jasmonic acid, a plant hormone, which acts as an intermediate signal response to stress, and overexpression of *ORCA3* led to increased accumulation of terpenoid indole alkaloids (Van der Fits and Memelink 2000). The Arabidopsis *rd29A* gene can be induced by ABA and abiotic stress such as low temperature, dehydration, and high salinity (Yamaguchi-Shinozaki and Shinozaki 1994). Thus the *Rd29A* promoter has been successfully used to promote inducible overexpression of different transcript factors in tobacco (Yamaguchi-Shinozaki and Shinozaki 1993), potato (Behnam et al. 2007; Pino et al. 2007) and peanut (Bhatnagar-Mathur et al. 2007). When compared with other constitutive promoters, it has fewer negative phenotype effects on transgenic plants (Pino et al. 2007). In the preliminary experiments, we transformed the *GUS* reporter gene driven by the *rd29A* promoter into *C. roseus* hairy roots, and confirmed the inducible expression of the *GUS* gene following ABA or cold treatment by histochemical dye staining (data not shown). The above results showed that the *rd29A* promoter can drive the overexpression of *ORCA3* following ABA or cold treatment. The different expression patterns of *ORCA3* in the OG lines, cultured under different conditions, (CK, ABA or cold) may be due to the effects of insertion sites where the heterologous gene was integrated or other unknown reasons.

Analysis of alkaloid concentration

The profiles of four kinds of alkaloids (catharanthine, vindoline, vinblastine, and vincristine) in both control and transgenic hairy root lines were determined by HPLC. Identification of alkaloids from the hairy roots' extract was established by comparison of the retention time and the UV spectra with those of authentic standards (Fig. 3). The purity of the peaks was determined using the Agilent ChemStation A09.01 software to make sure that a peak contained only one compound. Our results demonstrated that although vindoline, vinblastine, and vincristine were not detectable in either control or transgenic root lines, catharanthine was detected in all hairy root lines. Compared with the control hairy root lines (M3 and M5), the accumulation of catharanthine increased in all transgenic lines (Fig. 4a). Under normal conditions, the amount of catharanthine was 0.019 and 0.029% on a dry weight basis in the wild-type lines M3 and M5, but reached 0.063–0.107% in GH lines and 0.075–0.123% in OG lines. The range of the catharanthine amount was not significantly different between GH lines and OG lines, though the expression level of *ORCA3* in the OG lines was higher than in the GH lines under the control conditions (Fig. 2e), implies that the *G10H* gene plays a more important role in catharanthine synthesis. The transgenic line OG12 accumulated catharanthine at about 0.123% on a dry weight basis under normal conditions, which is about 6.5-fold of that of M3, and 4.2-fold of that of M5. ABA or cold treatment slightly increased the catharanthine accumulation in M3 and M5 root lines, but had a different influence on different transgenic hairy root lines. As for the GH lines, neither ABA nor cold treatment has an effect on the catharanthine amount. In the OG lines, ABA treatment significantly increased the catharanthine amount in OG12 and OG13, but had no effect on OG10, whereas under cold

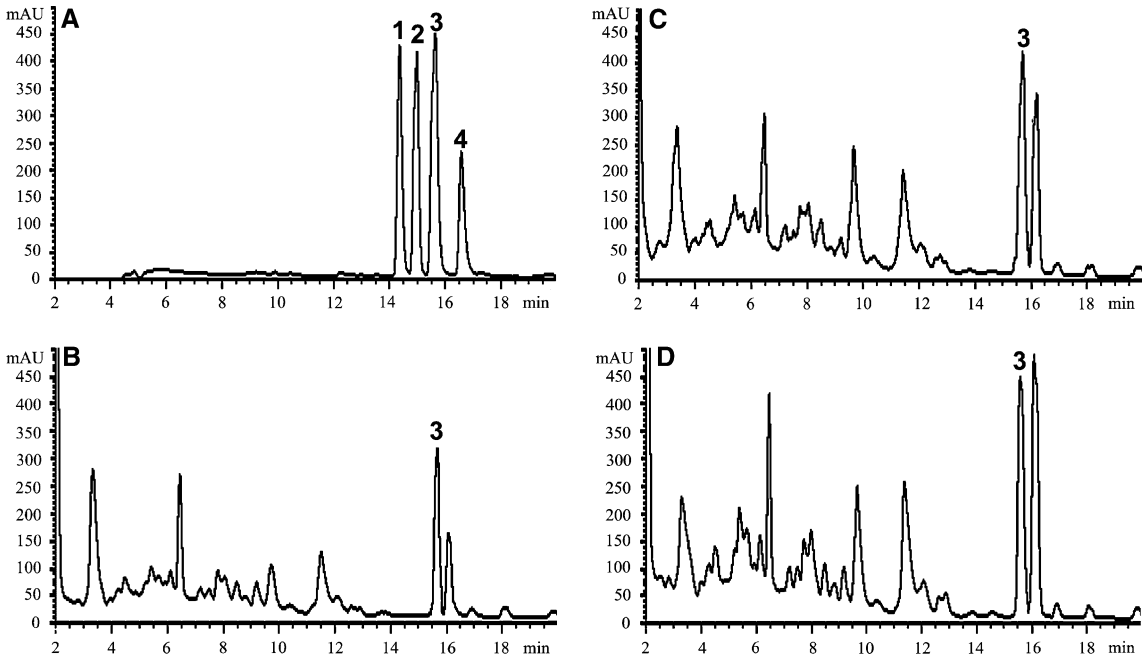

Fig. 3 HPLC analyses of TIAs accumulation in representative hairy root line OG12. **a** The pure standards. *1* vincristine, *2* vindoline, *3* catharanthine, *4* vinblastine. **b** Control. **c** 100 μmol/l ABA treatment for 24 h. **d** 4°C treatment for 24 h. The *abscissas* represent the retention time (min) and the *y*-axes represent the absorbance (mAU) at 220 nm. The injection volume is 10 μl

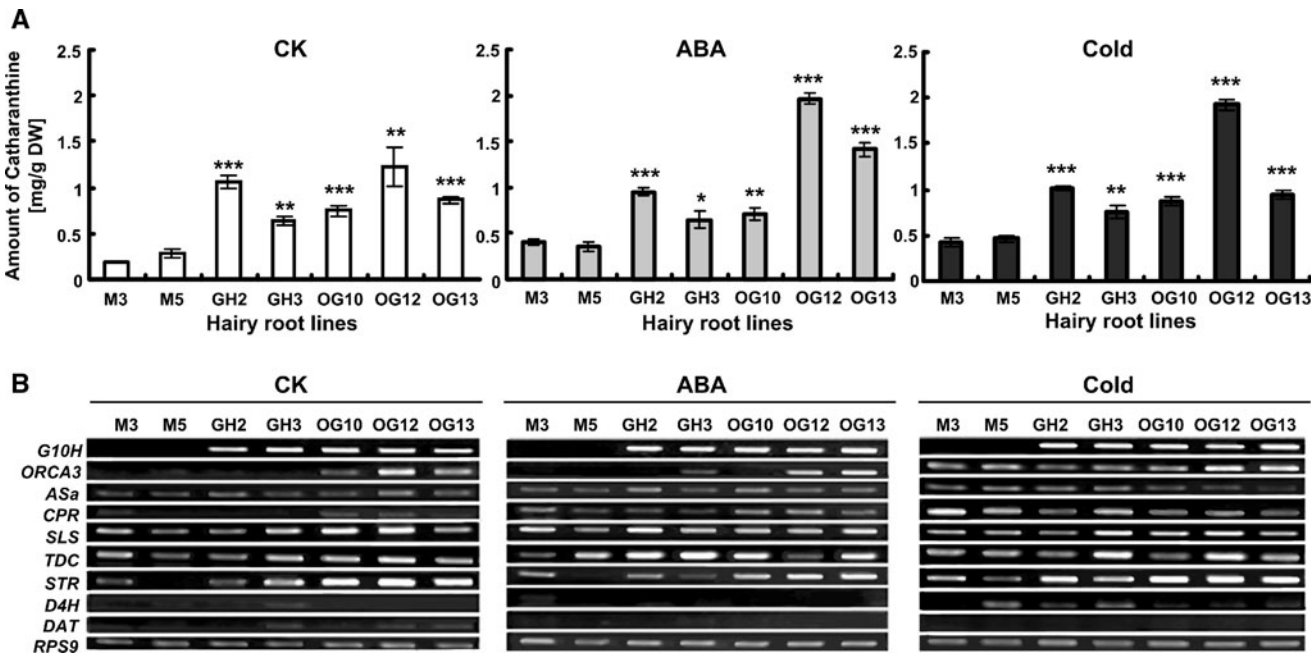

Fig. 4 Catharanthine contents (**a**) and RT-PCR analyses of the TIAs' biosynthesis genes (**b**) in hairy root lines after different treatments. *Asterisks* indicate statistically significant differences of transgenic lines compared with the control line M3 as determined with Student's *t* test (*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$)

treatment the increased catharanthine amount was observed only in OG12 but not in OG10 and OG13. The highest amount of catharanthine was observed in OG12 (0.196% on a dry weight basis) following treatment with ABA (Fig. 4a).

An earlier study has demonstrated that although the production of tryptamine and tryptophan was increased in *C. roseus* cell suspension cultures overexpressing *ORCA3*, production of TIAs was increased only when loganin was supplemented in the medium (Van der Fits and Memelink

2000). This was due to the fact that *G10H* was not up-regulated under *ORCA3* overexpression (Fig. 1). Our observation that catharanthine production was increased in all transgenic hairy root lines implies that *G10H* gene plays an important role in TIA biosynthesis. Very recently, *ORCA3* was overexpressed in *C. roseus* hairy roots, but no significant increases in TIA metabolites were observed, even after the hairy roots were fed with loganin, tryptophan or loganin and tryptophan (Peebles et al. 2009). A possible explanation of this inconsistency is that only one hairy root line was analyzed in the article. Compared with the transgenic lines OG10 and OG13, *G10H*-overexpressing lines (GH2 and GH3) produced equivalent or even more catharanthine (except OG12). Furthermore, the amounts of catharanthine in different transgenic lines were not consistent with the expression levels of *G10H* and/or *ORCA3* (Fig. 2e), suggesting the complexity of TIA biosynthesis.

Expression analyses of TIA biosynthetic genes

In the *ORCA3*-tagged *C. roseus* cell suspension line, the expressions of *ASα*, *TDC*, *CPR*, *STR*, and *D4H* increased, whereas SLS was not analyzed (Van der Fits and Memelink 2000). To understand the relationship between catharanthine accumulation and TIA biosynthesis-related gene expression in the hairy root lines, total RNA was extracted from both transgenic and control hairy root lines cultured under different conditions, and RT-PCR analyses were performed (Fig. 4b).The same amplification cycles were carried out for the same gene under different treatments, and the amount of catharanthine in hairy root lines corresponding to the treatments was determined (Fig. 4a). Compared with the lines M3, M5, GH2, and GH3, *STR* was strongly expressed in OG10, OG12, and OG13 under normal conditions (CK) or following cold treatment. The *SLS* expression was mildly higher in OG10 and OG12 under normal conditions. The expression of the other analyzed genes (*ASα*, *CPR*, and *TDC*) showed no significant differences between transgenic lines and control lines, though slight expression variations existed. The expression of *D4H* and *DAT*, two downstream genes of TIA in the biosynthesis pathway, were hardly detectable in most hairy root lines. In this study, only *STR*- and *SLS*-enhanced expression was observed in *ORCA3*-overexpressing hairy root lines. This is in consistence with a recent study in which the authors explained that *ORCA3* is one of the dominant regulators of *STR* and *SLS* genes in the JA signaling cascade, and pointed the possibility of different regulatory mechanisms of TIA biosynthesis between cell suspension cultures and differentiated tissues such as hairy roots (Peebles et al. 2009).

The production of catharanthine paralleled the *STR* expression level of OG transgenic lines under normal conditions, but showed no correlation following ABA or cold treatment, suggesting that the *STR* gene plays an important role but not as a bottleneck in the TIA biosynthesis pathway. This was also confirmed by the GH2 line in which a highly increased catharanthine amount corresponds to a low expression of the *STR* gene. Strictosidine *β*-D-glucosidase (SGD) catalyzes the transformation from strictosidine to cathenamine, and the latter is the immediate precursor substance of the TIAs. As the *SGD* gene showed a large variable expression in the non-transgenic cell suspension lines (Van der Fits and Memelink 2000), it was not selected for analysis in this study. However, it was presumed recently that a decrease in *SGD* transcripts could limit the flux through the TIA biosynthesis pathway, causing the feeding of upstream metabolites to be ineffective in the *ORCA3*-overexpressed hairy root line (Peebles et al. 2009). Thus, further study on the *SGD* expression would be helpful for understanding the difference of catharanthine amount in the transgenic hairy root lines of this study.

In this study, the amount of catharanthine is increased through the overexpression of *G10H* or both *G10H* and *ORCA3* genes in *C. roseus* hairy roots. An increase of 6.5-fold was obtained when compared with the control lines. However, the increased catharanthine accumulation in transgenic hairy roots is still less than that in *C. roseus* plants (data not shown). The lack of vinblastine and vincristine in *C. roseus* hairy roots has been ascribed to an absence of vindoline (Bhadra et al. 1998). This may be due to the undetectable expression of the *D4H* and *DAT* genes in the transgenic hairy roots. In *C. roseus* plants, *D4H* and *DAT* mRNAs are preferentially expressed in idioblast and laticifer cells of leaves, stems, and flower buds, but not detectable in roots (St-Pierre et al. 1999). Thus, a more detailed study of the genes involved in the TIA biosynthesis pathway as well as a deep understanding of the differentiation mechanism between *C. roseus* plants and hairy roots would be helpful for improving bisindole alkaloid production in hairy root cultures by biotechnological methods.

Acknowledgment The authors are grateful to Professors J. Memelink (Leiden University, Netherlands) for providing the vectors containing *G10H* or *ORCA3* genes, and Dr. Wang Yan-zhang (Shanghai Institutes for Biological Sciences, China) for providing *A. rhizogenes* MSU440, and Chen Jun (Shanghai Institutes for Biological Sciences, China) for HPLC analysis. This work was supported by the following grants: the Knowledge Innovation Program of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Grant No. 2007KIP304); the National Basic Research Program of China (Grant No. 2006CB100106; 2010CB126600); the National Natural Science Foundation of China (Grant No. 30571196; 0933ZF11C1; 0933Z411C1); The Ministry of Science and Technology of China (Grant No. 2007AA10Z187); Shanghai Science and Technology Commission (Grant No. 08DZ2270800).

References

Behnam B, Kikuchi A, Celebi-Toprak F, Kasuga M, Yamaguchi-Shinozaki K, Watanabe KN (2007) Arabidopsis *rd29A:DREB1A* enhances freezing tolerance in transgenic potato. Plant Cell Rep 26:1275–1282

Bhadra R, Morgan JA, Shanks JV (1998) Transient studies of light-adapted cultures of hairy roots of *Catharanthus roseus*: growth and indole alkaloid accumulation. Biotechnol Bioeng 60:670–678

Bhatnagar-Mathur P, Devi MJ, Reddy DS, Lavanya M, Vadez V, Serraj R, Yamaguchi-Shinozaki K, Sharma KK (2007) Stress-inducible expression of *At DREB1A* in transgenic peanut (*Arachis hypogaea* L.) increases transpiration efficiency under water-limiting conditions. Plant Cell Rep 26:2071–2082

Collu G, Unver N, Peltenburg-Looman AM, van der Heijden R, Verpoorte R, Memelink J (2001) Geraniol 10-hydroxylase, a cytochrome P450 enzyme involved in terpenoid indole alkaloid biosynthesis. FEBS Lett 508:215–220

Da Costa e Silva O, Klein L, Schmelzer E, Trezzini GF, Hahlbrock K (1993) BPF-1, a pathogen-induced DNA-binding protein involved in the plant defense response. Plant J 4:125–135

Favretto D, Piovan A, Filippini R, Caniato R (2001) Monitoring the production yields of vincristine and vinblastine in *Catharanthus roseus* from somatic embryogenesis. Semiquantitative determination by flow-injection electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 15:364–369

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Hong SB, Peebles CA, Shanks JV, San KY, Gibson SI (2006) Terpenoid indole alkaloid production by *Catharanthus roseus* hairy roots induced by *Agrobacterium tumefaciens* harboring *rol ABC* genes. Biotechnol Bioeng 93:386–390

Hughes EH, Hong SB, Shanks JV, San KY, Gibson SI (2002) Characterization of an inducible promoter system in *Catharanthus roseus* hairy roots. Biotechnol Prog 18:1183–1186

Hughes EH, Hong SB, Gibson SI, Shanks JV, San KY (2004) Metabolic engineering of the indole pathway in *Catharanthus roseus* hairy roots and increased accumulation of tryptamine and serpentine. Metab Eng 6:268–276

Magnotta M, Murata J, Chen J, De Luca V (2007) Expression of deacetylvindoline-4-*O*-acetyltransferase in *Catharanthus roseus* hairy roots. Phytochemistry 68:1922–1931

Pasquali G, Porto DD, Fett-Neto AG (2006) Metabolic engineering of cell cultures versus whole plant complexity in production of bioactive monoterpene indole alkaloids: recent progress related to old dilemma. J Biosci Bioeng 101:287–296

Pauw B, Hilliou FA, Martin VS, Chatel G, de Wolf CJ, Champion A, Pre M, van Duijn B, Kijne JW, van der Fits L, Memelink J (2004) Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in *Catharanthus roseus*. J Biol Chem 279:52940–52948

Peebles CA, Hughes EH, Shanks JV, San KY (2009) Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of *ORCA3* along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. Metab Eng 11:76–86

Pino MT, Skinner JS, Park EJ, Jeknic Z, Hayes PM, Thomashow MF, Chen TH (2007) Use of a stress inducible promoter to drive ectopic *AtCBF* expression improves potato freezing tolerance while minimizing negative effects on tuber yield. Plant Biotechnol J 5:591–604

Rahman L, Kouno H, Hashiguchi Y, Yamamoto H, Narbad A, Parr A, Walton N, Ikenaga T, Kitamura Y (2009) HCHL expression in hairy roots of beta vulgaris yields a high accumulation of *p*-hydroxybenzoic acid (pHBA) glucose ester, and linkage of pHBA into cell walls. Bioresource Technol 100:4836–4842

Runguphan W, O'Connor SE (2009) Metabolic reprogramming of periwinkle plant culture. Nat Chem Biol 5:151–153

Shadwick FS, Doran PM (2007) Propagation of plant viruses in hairy root cultures: a potential method for in vitro production of epitope vaccines and foreign proteins. Biotechnol Bioeng 96:570–583

St-Pierre B, Vazquez-Flota FA, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell 11:887–900

Suttipanta N, Pattanaik S, Gunjan S, Xie CH, Littleton J, Yuan L (2007) Promoter analysis of the *Catharanthus roseus* geraniol 10-hydroxylase gene involved in terpenoid indole alkaloid biosynthesis. Biochim Biophys Acta 1769:139–148

Tikhomiroff C, Jolicoeur M (2002) Screening of *Catharanthus roseus* secondary metabolites by high-performance liquid chromatography. J Chromatogr A 955:87–93

Van der Fits L, Memelink J (2000) *ORCA3*, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289:295–297

Wise AA, Liu Z, Binns AN (2006) Three methods for the introduction of foreign DNA into *Agrobacterium*. Methods Mol Biol 343:43–53

Woods RR, Geyer BC, Mor TS (2008) Hairy-root organ cultures for the production of human acetylcholinesterase. BMC Biotechnol 8:95

Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol Gen Genet 236:331–340

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6:251–264

Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun X, Tang K (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci USA 101:6786–6791