Appl Microbiol Biotechnol (2011) 91:1095–1105
DOI 10.1007/s00253-011-3288-1

Soybean transcription factor *Gm*MYBZ2 represses catharanthine biosynthesis in hairy roots of *Catharanthus roseus*

Mei-Liang Zhou · Hong-Li Hou · Xue-Mei Zhu · Ji-Rong Shao · Yan-Min Wu · Yi-Xiong Tang

Received: 11 December 2010 / Revised: 31 March 2011 / Accepted: 5 April 2011 / Published online: 17 May 2011

Abstract *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. Production of catharanthine in cell cultures or in hairy roots established by transformation with *Agrobacterium rhizogenes* is of interest because catharanthine can be chemically coupled to the abundant leaf alkaloid vindoline to form the valuable anticancer drug vinblastine. Here, we observed a high amount of catharanthine in hairy roots of *C. roseus*, established by infecting leaf explants with the *A. rhizogenes* >agropine-type A4 strain carrying plasmid pRi. T-DNA transfer from plasmid pRi into hairy roots was confirmed by PCR for the essential T-DNA genes *rolA* and *rolB* and the agropine synthesis gene *ags.* The results suggest that integration of T-DNA into the plant DNA plays a positive role on the catharanthine pathway in *C. roseus* hairy roots. Furthermore, co-transformation with the soybean transcription factor *GmMYBZ2* indicated that GmMYBZ2 reduces the catharanthine production by alteration of expression of a number of genes linked to the pathway. Transcription levels of the zinc-finger transcription factor 1 gene *ZCT1* were high, and the transcription levels of the anthranilate synthase gene *ASα*, the strictosidine synthase gene *STR*, and the key transcription factor gene octadecanoid-responsive *Catharanthus* APETALA2/ethylene response factor were low. In addition, GmMYBZ2 had a negative effect on the gene expression levels of A-type cyclin CYSA and B-type cyclin CYSB, which was correlated with a reduced growth rate of the hairy roots.

Keywords Catharanthine · Growth index · Hairy roots · Metabolic engineering · MYB transcription factor · Transcript profiling

M.-L. Zhou · J.-R. Shao (✉)
School of Life Science, Sichuan Agricultural University,
Yaan, Sichuan 625014, People's Republic of China
e-mail: shaojr007@163.com

M.-L. Zhou · Y.-M. Wu (✉) · Y.-X. Tang (✉)
Room 211, Biotechnology Research Institute, Chinese Academy of Agricultural Sciences (CAAS),
Zhongguancun south street No 12, Haidian district,
Beijing 100081, China

Y.-M. Wu
e-mail: wuyanmin211@yahoo.com.cn

Y.-X. Tang
e-mail: zhml39@hotmail.com

H.-L. Hou
Shenzhen Imported Center for Quality Crop Seed,
Shenzhen, Guangdong 518040, People's Republic of China

X.-M. Zhu
School of Resources and Environment, Sichuan Agricultural University,
Yaan, Sichuan 625014, People's Republic of China

Present Address:
M.-L. Zhou
Plant Cell Physiology, Institute of Biology, Sylvius Laboratory,
Sylviusweg 72, Leiden University,
P.O. Box 9505, 2300, RA Leiden, The Netherlands

Introduction

The production of secondary metabolites by plant cell and tissue cultures is an active field of study because of its potential as a source of valuable pharmaceutical compounds (Weathers et al. 2010). *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 monomeric compounds ajmalicine and serpentine are used in the treatment of cardiac and circulatory diseases, whereas dimeric TIAs, such as 3′, 4′-anhydrovinblastine, vincristine, and vinblastine, are powerful anticancer drugs (van der Heijden et al. 2004; Singh et al. 2001). These dimeric TIAs are synthesized from vindoline and catharanthine. A schematic representation of the catharanthine biosynthetic pathway in *C. roseus* adapted from our previous review is shown in Fig. 1 (Zhou et al. 2009). Vindoline is abundant in *C. roseus* plants, and catharanthine can be produced by *C. roseus* cell or hairy root cultures (Zhao et al. 2001). Purified catharanthine and vindoline can also be chemically coupled to form dimeric alkaloids as an alternative way to produce these compounds. Therefore, the production of catharanthine and its mechanism of biosynthesis in *C. roseus* cell or hairy root cultures have been extensively explored. In vitro cultures of hairy roots derived from *C. roseus* via *Agrobacterium rhizogenes* infection exhibited higher genetic stability as compared to cell suspension cultures. Hairy roots accumulate higher levels of TIAs and have been used widely in many laboratories. Additionally, they can grow in hormone-free and low-nutrient salt media (Georgiev et al. 2007; Zhou et al. 2009).

Coordinated transcriptional control of biosynthetic genes has emerged as a major mechanism dictating the accumulation of secondary metabolites in plant cells. Over the last decade, many efforts were directed at the elucidation of the regulatory role of transcription factors (TFs) in the TIA biosynthesis pathway in *C. roseus*. Octadecanoid-responsive *Catharanthus* AP2/ERF domain proteins (ORCAs), which are members of the AP2/ERF TF family, have been shown to regulate the MeJA-responsive activation of several TIA biosynthetic genes in *C. roseus* (Menke et al. 1999; van der Fits and Memelink 2000). In glucosinolate and phenylpropanoid biosynthesis, MYB TFs play important regulatory roles. The role of MYB TFs in TIA biosynthesis has not been studied until now. MYB TFs can be classified into three subfamilies based on the number of conserved imperfect repeats in the DNA-binding domain, i.e., R3 MYBs (MYB1R) with one repeat, R2R3 MYBs (MYB2R) with two repeats, and R1R2R3 MYBs (MYB3R) with three repeats (Rosinski and Atchley 1998; Jin and Martin 1999). Among these MYB TFs, the R2R3 MYBs form one of the largest TF gene families in plants, with 126 members identified in *Arabidopsis thaliana* (Stracke et al. 2001). Plant MYBs have been implicated in controlling processes as diverse as secondary metabolism, epidermal cell fate, formation of trichomes, development, signal transduction, disease resistance, and the control of the cell cycle (Jin

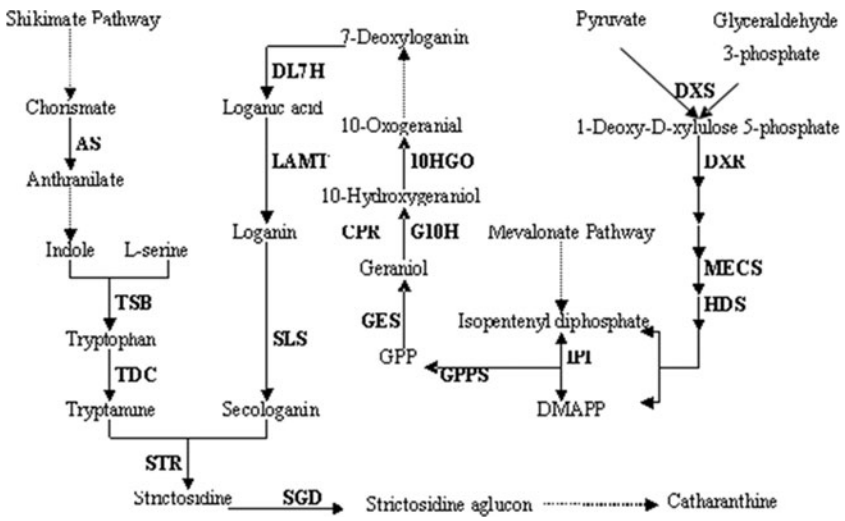

Fig. 1 Schematic representation of the biosynthesis pathway for catharanthine in *C. roseus*. *Solid lines* represent a single enzymatic conversion whereas *dashed lines* indicate multiple enzymatic conversions (Zhou et al. 2010). *Abbreviations*: *AS* anthranilate synthase, *CPR* cytochrome P450 reductase, *DL7H* 7-deoxy-loganin 7-hydroxylase, *DMAPP* dimethylallyl diphosphate, *DXR* 1-deoxy-D-xylulose 5-phosphate reductase, *DXS* 1-deoxy-D-xylulose 5-phosphate synthase, *GES* geraniol synthase, *G10H* geraniol 10-hydroxylase, *10HGO* 10-hydroxygeraniol oxidoreductase, *GPP* geranyl diphosphate, *GPPS* geranyl diphosphate synthase, *HDS* 1-hydroxy-2-methyl 2(*E*) butenyl 4-phosphate synthase, *IPI* isopentenyl diphosphate isomerase, *LAMT S*-adenosyl-L-methionine: loganic acid methyltransferase, *MECS* 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *SGD* strictosidine β-D-glucosidase, *SLS* secologanin synthase, *STR* strictosidine synthase, *TDC* tryptophan decarboxylase, *TSB* tryptophan synthase β

and Martin 1999; Kui et al. 2010). Consensus motifs in MYBs important for their function are just beginning to be elucidated. AtMYB4 has the motif NLELRISLPDDV in its C terminus, which is essential for its repressive activity on the cinnamate 4-hydroxylase promoter (Jin et al. 2000). This motif (pdLNLD/ELxiG/S) is also conserved in a number of R2R3 MYB proteins belonging to subgroup 4 which include AtMYB4, AtMYB6, AtMYB7 and AtMYB32, strawberry FaMYB1, *Antirrhinum* AmMYB308 and AmMYB330, and maize ZmMYB31 and ZmMYB42, which exert a similar repressive effect as AtMYB4 (Tamagnone et al. 1998; Aharoni et al. 2001; Fornalé et al. 2006). All these TFs possess, besides the repressor motif pdLNLD/ELxiG/S, a putative zinc-finger domain in their C terminus. The R2R3 GmMYBZ2 protein (DQ902861), which was previously isolated from soybean (*Glycine max*) in our lab, also has the pdLNLD/ELxiG/S motif and a putative zinc-finger domain in its C terminus, and it therefore probably acts as a transcriptional repressor (Du et al. 2009). Since, similar R2R3-type MYB genes have been shown to regulate secondary metabolite biosynthesis in *Arabidopsis*. We explored the effect of constitutive overexpression of *GmMYBZ2* on the expression of genes encoding TIA biosynthesis enzymes and regulatory TFs and on catharanthine biosynthesis in *C. roseus* hairy roots. The results showed that constitutive overexpression of GmMYBZ2 led to a significant decrease in the accumulation of catharanthine and in the expression level of several key genes acting in the catharanthine biosynthesis pathway. Additionally, GmMYBZ2 affected hairy root growth rate and the expression levels of the cell cycle genes *CYSA* and *CYSB* encoding A- and B- type cyclins, respectively. Finally, we found that genes from the T-DNA of the hairy root-inducing Ri plasmid affected the catharanthine biosynthesis pathway.

Materials and methods

Plant transformation and root cultivation

The plasmid pCAMBIA 2301-GmMYBZ2 (provided by Du Hai and Dr. Yang Wen-Jie, Biotechnology Research Institute, Chinese Academy of Agricultural Sciences) was electroporated into *A. rhizogenes* agropine strain A4 (obtained from the Agricultural Culture Collection of China) (Brillanceau et al. 1989), and transformants were selected on YEB media (5 g/L tryptone, 1 g/L yeast extract, 0.5 g/L $MgSO_4$ $7H_2O$, 10 g/L sucrose, 10 g/L agar, pH 7.4) containing 50 mg/L kanamycin and 50 mg/L rifampicin. The presence of pCAMBIA2301-GmMYBZ2 was confirmed by PCR amplification.

C. roseus seeds were obtained from the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (Beijing, China), and plants were grown at 25°C with a 16-h light/8-h dark photoperiod. Roots of wild-type plants served as controls (WT). Transformation of leaf explants was carried out with strains A4 and A4/pCAMBIA2301-GmMYBZ2 basically following the previously described method for tobacco leaf disk transformation (Horsch et al. 1989; Zhang et al. 2004). Roots developing at cutting edges 2–3 weeks after co-cultivation were excised from the explants and subcultured every 5 weeks at 25°C in the dark on solid, hormone-free, half-strength B5 medium (Gamborg et al. 1968), supplemented with 30 g/L sucrose. The selection media contained 400 mg/L cefotaxime and/or 100 mg/L kanamycin used for hairy roots (AR) and transgenic *GmMYBZ2* hairy root (Z), respectively. Rapidly growing bacteria-free kanamycin-resistant lines were used to establish liquid hairy root cultures. About 100 mg (±5 mg) of fresh roots (3 cm in length) were 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.

Investigation of root growth

Replicate flasks with the same inoculum size were cultured for 42 days, and roots from five flasks were collected in triplicate at days 3, 7, 14, 21, 28, 35, and 42. The growth index defined as (final dry biomass − initial dry biomass)/initial dry biomass was determined.

PCR analysis

Genomic DNA was isolated from hairy roots by using the modified acetyl trimethyl ammonium bromide method (Zhou et al. 2010). DNA was used in PCR analysis for detecting the presence of the *A. rhizogenes rolA*, *rolB*, *ags*, *vir C* and the soybean *GmMYBZ2* genes. Primers were RolAF (5′-ATGGAATTAGCCGGACTAAACG-3′) and RolBR (5′-ATGGATCCCAAA TTGCTATTCC-3′), AgsF (5′-CGGAAATTGTGGCTCGTTGTGGAC-3′) and AgsR (5′-AAT CGTTCAGAGAGCGTCCGAAGTT-3′), VirCF (5′-GGCTTCGCCAACCAATTTGGAGAT-3′) and VirCR (5′-TTTTGCTCCTTCAAGGGAGGTGCC-3′), GmMYBZ2F (5′-TTTTGAACAGAGGAATCGACCCTGCA-3′) and GmMYBZ2R (5′-ATTTCCAAGCGGGTGCAATCCCA-3′). 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 units 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 1% agarose gel electrophoresis.

Southern blot hybridization

Putatively transformed *GmMYBZ2* hairy root clones were analyzed by PCR with the primers GmMYBZ2SF (5′-TTTTGAACAGAGGAATCGACCCTGCA-3′) and GmMYBZ2SR (5′-ATTTCCAAGCGGGTGCAATCCCA-3′). Amplified fragments were separated by 0.8% agarose gel electrophoresis, transferred to a Hybond-N^+ nylon membrane (Amersham), and hybridized with the digoxigenin-11-dUTP labeled *GmMYBZ2* gene according to the manufacturer's instructions (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche, Germany).

Histochemical GUS assays

To assay β-glucuronidase activity, sectioned transformed roots were incubated overnight at 37°C in Eppendorf tubes containing 2 mL staining solution with 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 $K_4Fe(CN)_6$ (Jefferson 1987).

Catharanthine extraction and HPLC detection

Approximately 200 mg (±5 mg) of fresh hairy roots were freeze-dried overnight. The dry roots were weighed, crushed in a tissue grinder, 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 prior to HPLC analysis as previously described by using a mobile phase consisting of a mixture of 5 mM NaH_2PO4–H_3PO4 pH 6 and acetonitrile (Tikhomiroff and Jolicoeur 2002; Zhou et al. 2010). Standards for catharanthine (98%, Sigma) were prepared in methanol at a final concentration of 1 mg/mL.

cDNA synthesis and Q-PCR amplification

Total RNA was isolated from the hairy roots samples stored at −80°C using a Plant RNA_{out} 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. 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 of some genes (*ASα*, *TDC*, *DXS*, *G10H*, *CPR*, *SLS*, *STR*, *SGD*, *ORCA2*, *ORCA3*, *ZCT1*, *ZCT2*, *ZCT3*, *GBF1*, *GBF2*, *DXR*, *MECS*, *HDS*, *PGGT-I*, *RSP9*) were previously described (Peebles et al. 2009; Zhou et al. 2010). Table 1 contains a list of primers of the other investigated genes (*CYSA*, *CYSB*). 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 (Shalel-Levanon et al. 2005). The gene encoding the 40S ribosomal protein S9 (RSP9) was used as control (Menke et al. 1999).

Statistical analysis

All data were examined using one-way analysis of variance (ANOVA) (General Linear Models on GLM procedure). The Student–Neuman–Keuls test was used to identify the means which differed if the ANOVA test indicated significance. A P value <0.05 was considered to be significant.

Results

Development of transgenic hairy root lines

The soybean *GmMYBZ2* gene was introduced into *C. roseus* leaf explants using the *A. rhizogenes* strain A4 carrying the plasmid pCAMBIA2301-GmMYBZ2. The abbreviations Z and AR refer to the transgenic hairy root lines generated from transformations with strains *A. rhizogenes* A4/pCAMBIA2301-GmMYBZ2 and *A. rhizogenes* A4 (blank transformation), respectively. Thirty-six kanamycin-resistant Z hairy root lines and 10 AR hairy root lines were generated, of which 22 Z and 10 AR lines survived the successive subculture steps. Some Z hairy root lines turned brown and aged considerably faster than AR hairy root lines (including Z_4, Z_5, Z_6, Z_7, Z_{10}, Z_{11}, Z_{12}, Z_{13}, Z_{19}, Z_{23}, Z_{24}, Z_{25}, Z_{28}, and Z_{30}). These lines were discarded, and the remaining hairy root lines were subcultured for 5 weeks in hormone-free, half-strength B5 liquid medium. Significant differences in growth characteristics were detected among independently transformed root lines. The 22 Z lines grew slowly and vigorously with thicker and fewer branches whereas AR lines grew fast with slender, dense, and white branches (Fig. 2). Significant differences (P<0.01) were found between Z lines and AR lines with respect to growth rate (Fig. 3). 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.

Table 1 List of genes and primer pairs used for Q-PCR

Gene	Accession no.	Primer pairs	PCR products
CYSA	D86385	5′-CTTATTGGATTATGGCTGTG-3′ 5′-TCCTTTAAGTCTGCTGGTTT-3′	150 bp
CYSB	D86386	5′-AATTCGTCGTGCTCTTGGAG-3′ 5′-GCTACTGCTGCTGCTTGTGC-3′	141 bp

Molecular analysis of genetically engineered hairy roots

To demonstrate the presence of T-DNA region genes of both pRi and pCAMBIA2301-GmMYBZ2 plasmids in the genome of transformed hairy roots, a PCR analysis of root genomic DNA was performed. PCR analyses performed on all 10 AR hairy root lines led to the amplification of the expected *rolA* and *rolB* fragment. PCR analysis confirmed the presence of the *ags* gene in five out of 10 AR hairy root lines (data not shown). PCR analysis performed on all 22 Z hairy root lines led to the amplification of the expected *rolA* and *rolB* fragment (1.5 kb), identical to that of the positive control (pRi A4 plasmid) (Fig. 4a). No PCR products were obtained with DNA from wild-type roots (negative control), from brown and aged Z hairy root lines (Z_4, Z_{25}, and Z_{28}) (Fig. 4a), and from all the tested transformed roots when using the *virC* primers (data not shown). 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. Ten out of 25 examined hairy root lines contained the *ags* gene which is on a separate T_R-DNA derived from plasmid pRi A4 (Fig. 4b). Our result showed that the T_L-DNA derived from pRi A4 was sufficient to induce the typical hairy root phenotype. PCR performed with primers specific for the *GmMYBZ2* gene resulted in the amplification of a single fragment with the expected size of 810 bp in 23 out of 25 investigated hairy root lines (Fig. 4c). No PCR products were obtained with DNA from brown and aged hairy root lines Z_4 and Z_{28}. Based on these results, we selected six hairy root lines (Z_1, Z_8, Z_{16}, Z_{18}, Z_{32}, and Z_{33}) for further study. PCR–Southern blot analysis and reverse transcription PCR confirmed the presence and expression of the *GmMYBZ2* gene in these six selected hairy root lines (Fig. 4d and e, respectively). Additionally, strong GUS staining was detected for these six Z hairy root lines confirming that they contained the T-DNA derived from plasmid pCAMBIA2301-GmMYBZ2 (Fig. 5).

Catharanthine content in hairy roots

The catharanthine contents of wild-type roots, AR hairy root lines, and Z hairy root lines were measured as shown in Fig. 6. Catharanthine contents in three AR lines (AR_1, AR_8, and AR_{10}) were variable, ranging from 0.38 to 0.56 mg/g fresh weight (FW). In wild-type roots, catharanthine was undetectable. This demonstrates that the T-DNA genes derived from the pRi A4 plasmid positively affected the catharanthine biosynthesis pathway. The catharanthine levels in the six Z lines were lower compared to those of the A lines, ranging from 0.20 to 0.25 mg/g FW. This indicates that GmMYBZ2 might down-regulate the biosynthesis of catharanthine in *C. roseus* hairy roots.

Analysis of gene expression

The expression levels of several genes encoding enzymes acting in the catharanthine biosynthesis pathway or in precursor pathways were determined in WT roots and hairy root lines AR1 and Z1. These genes were *ASα* and *TDC* from the indole pathway; *DXS*, *DXR*, *MECS*, *HDS*, *G10H*,

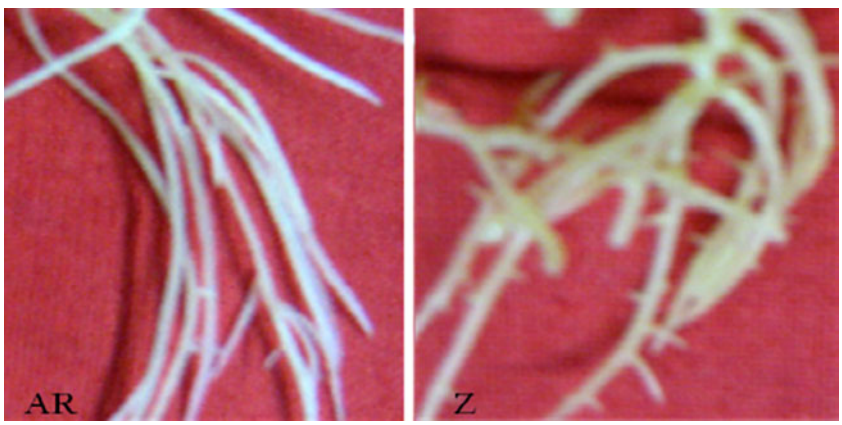

Fig. 2 The phenotype of *C. roseus* hairy roots. The abbreviations *Z* and *AR* refer to transgenic hairy root lines Z_1 and AR_1 generated from transformations with strains *A. rhizogenes* A4/pCAMBIA2301-GmMYBZ2 and *A. rhizogenes* A4, respectively

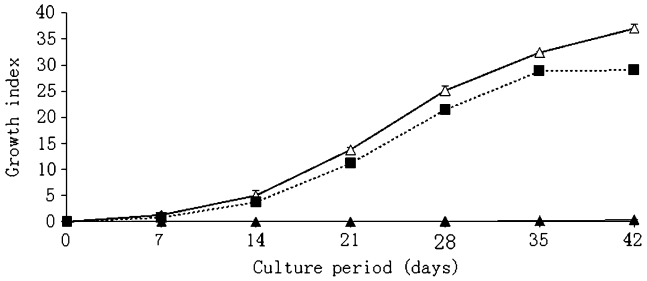

Fig. 3 Growth index of *C. roseus* hairy roots. *White triangles*, *black squares*, and *black triangles* refer to the AR_1 and Z_1 hairy root lines and WT roots, respectively. *Error bars* represent the standard deviation of triplicate analyses

CPR, and *SLS* from the monoterpenoid pathway; and *STR* and *SGD* from the TIA pathway. In addition, the expression levels of several genes encoding regulatory proteins and of two genes (*CYSA* and *CYSB*) encoding cell cycle regulators were determined. TF genes were *ORCA2* and *ORCA3*, the zinc-finger protein-encoding genes *ZCT1*, *ZCT2*, and *ZCT3*, and the G-box-binding protein-encoding genes *GBF1* and *GBF2*. The gene *PGGT-I* encodes type I protein geranyl-geranyltransferase, which plays a regulatory role by prenylating proteins. The *RPS9* gene encoding the 40S ribosomal protein S9 served as the control gene. For each gene, the expression level in WT roots was set at 1, and the expression levels in hairy root lines were normalized to the WT expression level. The results represent the average of three replicate measurements using the same RNA.

Expression of indole pathway genes

The expression levels of the indole pathway genes *ASα* and *TDC* for the three different types of roots are shown in Fig. 7a. The AR_1 line showed the highest *ASα* transcript level. The Z_1 line showed the highest *TDC* transcript level; however, it had a significantly lower *ASα* transcript level than the AR_1 line. The WT root showed the lowest transcript levels for both *ASα* and *TDC*. These results indicated that the T-DNA genes derived from plasmid pRi A4 could positively regulate the transcription levels of indole pathway genes *ASα* and *TDC*. TF GmMYBZ2 appeared to positively affect *TDC* gene expression, but it had a negative effect on *ASα* gene expression.

Expression of terpenoid pathway genes

The expression levels of the terpenoid pathway genes *DXS*, *DXR*, *MECS*, *HDS*, *G10H*, *CPR*, and *SLS* for the three different types of roots are shown in Fig. 7b. The Z_1 and AR_1 lines had similar levels of *DXS*, *DXR*, *MECS*, *HDS*, and *SLS* transcripts. The Z_1 line had a significantly lower level of *G10H* transcript compared to the AR_1 line. WT roots had the lowest transcript levels for all terpenoid pathway genes. These results indicated that the T-DNA genes derived from plasmid pRi A4 could positively affect the expression of terpenoid pathway genes. TF GmMYBZ2 had a positive effect on *CPR* gene expression but a small negative effect on *G10H* gene expression.

Expression of TIA pathway genes

The expression levels of TIA genes *STR* and *SGD* for the three different types of roots are shown in Fig. 7a. The *SGD* gene was expressed at a similar level in the three root types. The AR_1 line had the highest level and WT roots the lowest level of *STR* transcripts. These results indicated that the T-DNA genes derived from the plasmid pRi A4 could positively affect *STR* and *SGD* expression. TF GmMYBZ2 had a negative effect on the *STR* gene expression level.

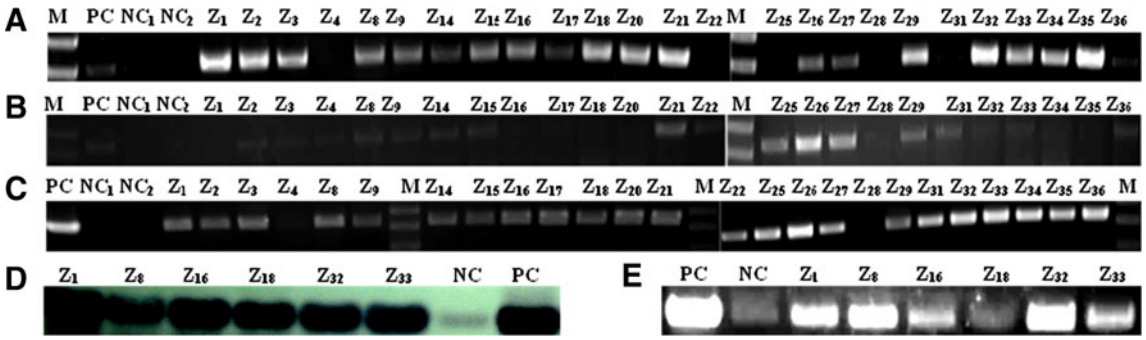

Fig. 4 Molecular analysis of transgenic hairy root lines. **a** Representative PCR analysis for the presence of *rolA* and *rolB* genes in transgenic hairy root lines. *M* DNA marker, *PC* pRiA4 (positive control), *NC* wild-type *C. roseus* roots (negative control), *Z* transgenic hairy root lines generated from transformations with strain *A. rhizogenes* A4/pCAMBIA2301-GmMYBZ2, Z_1 to Z_{36} different transgenic lines. **b** Representative PCR analysis for the presence of the *ags* gene in transgenic hairy root lines. **c** Representative PCR analysis for the presence of the *GmMYBZ2* gene in transgenic hairy root lines. *PC* pCAMBIA2301-GmMYBZ2 (positive control), *NC1* AR hairy root line, *NC2* wild-type *C. roseus* roots (negative control). **d** PCR–Southern blotting for the presence and (**e**) reverse transcriptase PCR analysis for the expression of the *GmMYBZ2* gene in transgenic hairy root lines. *PC* pCAMBIA2301-GmMYBZ2 (positive control), *NC* wild-type *C. roseus* roots (negative control)

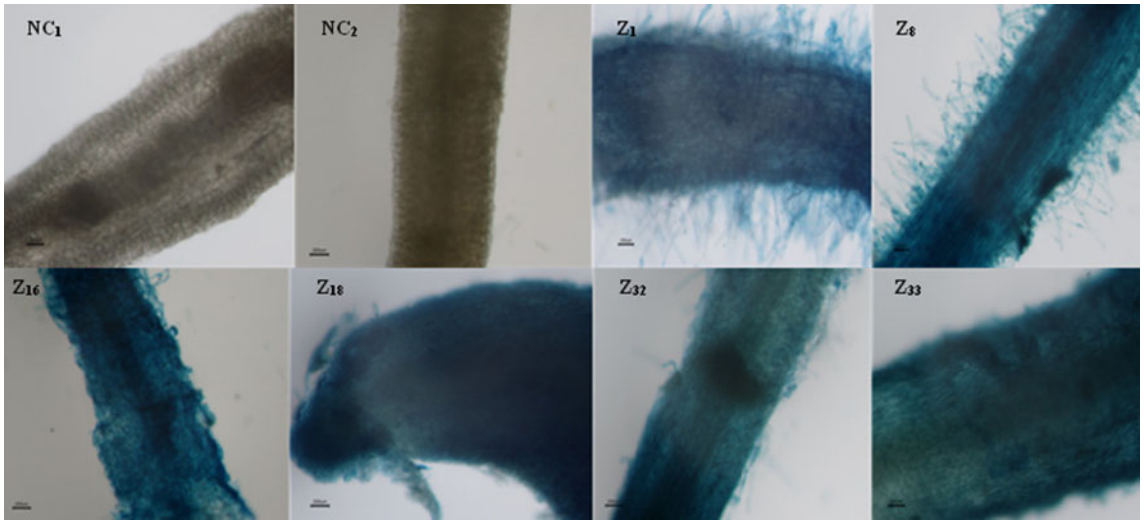

Fig. 5 GUS staining of transgenic hairy root lines. *NC1* AR hairy root line, *NC2* wild-type *C. roseus* root (negative control), *Z* different transgenic lines

Expression of TIA regulator genes

The expression levels of *ORCA2*, *ORCA3*, *ZCT1*, *ZCT2*, *ZCT3*, *GBF1*, *GBF2*, *and PGGT-I* for the three different types of roots are shown in Fig. 7c. *GBF1*, *GBF2*, *ZCT2*, *ZCT3*, and *PGGT-I* transcripts exhibited a similar level for all three root types. ZCT1 transcript levels were highest in WT roots and were twofold higher in the Z1 line than in the AR1 line. These results indicated that GmMYBZ2 positively affected *ZCT1* gene expression and that the T-DNA genes derived from the plasmid pRi A4 had a negative effect on *ZCT1* gene expression. ORCA3 mRNA levels were highest in the AR_1 line and lowest in WT roots. The Z_1 line exhibited a significant lower level of *ORCA3* mRNA transcripts than the AR_1 line. These results indicated that the T-DNA genes derived from the plasmid pRi A4 had a positive effect on *ORCA2* and *ORCA3* gene expression, whereas TF GmMYBZ2 had a negative effect on the *ORCA3* gene expression level.

Cell cycle regulators

The expression levels of the cell cycle regulator genes *CYSA* and *CYSB* for the three different types of roots are shown in Fig. 7a. The AR_1 line showed the highest levels and WT roots the lowest levels in *CYSA* and *CYSB* transcripts. The Z_1 line displayed lower levels of *CYSA* and *CYSB* transcripts than the AR_1 line. These results indicated that the T-DNA genes derived from the plasmid pRi A4 had a positive effect on *CYSA* and *CYSB* gene expression, whereas TF GmMYBZ2 had a negative effect on *CYSA* and *CYSB* gene expression.

Discussion

In dicots, hairy roots can be induced after infection with *A. rhizogenes*, a soil-borne pathogenic bacterium responsible for the development of the hairy roots disease (Georgiev et

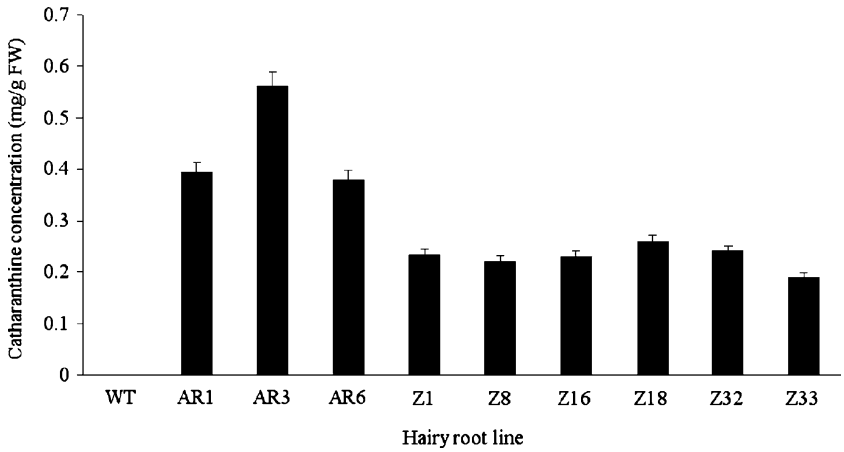

Fig. 6 Catharanthine content in three types of *C. roseus* roots. *WT* wild-type *C. roseus* roots. The abbreviations *Z* and *AR* refer to the transgenic hairy root lines generated from transformations with strains *A. rhizogenes* A4/pCAMBIA2301-GmMYBZ2 and *A. rhizogenes* A4, respectively. *Bars* represent the deviation of 5%

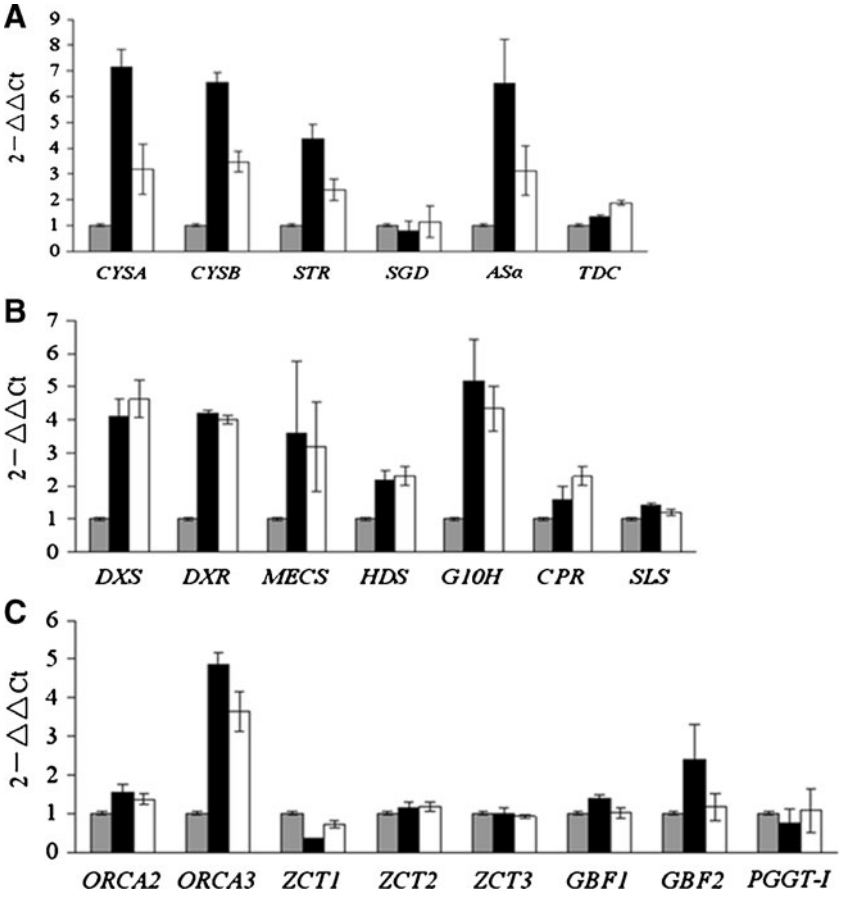

Fig. 7 Expression levels of genes involved in the catharanthine biosynthesis pathway. *Dark gray*, *black*, and *white* bars refer to the WT roots, and AR_1 and Z_1 hairy root lines, respectively. *2^(−ΔΔCt)* values represent the relative expression levels. The data were normalized to wild type (set at 1) using the comparative threshold cycle method. *Error bars* represent the standard deviation of triplicate runs for QRT-PCR

al. 2007). *A. rhizogenes*-induced hairy root cultures exhibit stable and fast growth rates, genetic and biochemical stability, and increased production of secondary compounds compared to cell suspension cultures. Moreover, transformed hairy roots can also be successfully cultured in large-scale bioreactors (Georgiev et al. 2007, 2009). *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 (co-transformation). However, it has been reported that the integration of the Ri-plasmid T-DNA and the T-DNA from the binary vector occur independently (Shahin et al. 1986). More recently, Kumar et al. (2005) have shown that during transformation, co-transfer of T-DNA from both Ri plasmid and binary plasmid is not obligatory in *Coffea canephora*. The presence of the *rolA*, *rolB*, and *GmMYBZ2* genes in Z lines demonstrates that the Ri T-DNA and the binary plasmid T-DNA were successfully transferred to *C. roseus* hairy roots.

We found variability in the distribution of GUS expression among the six selected Z hairy root lines. The strongest GUS expression was always seen in the root tip and in the central cylinder. This staining pattern has often been observed in transformed plants when the CaMV35S promoter was used (Terada and Shimamoto 1990; Diouf et al. 1995). However, in some root lines, all the tissues displayed GUS activity. This wide variability in the spatial distribution of GUS expression has often been reported in hairy roots (Alpizar et al. 2006). It might result from differences in the number of T-DNA copies, in T-DNA rearrangements (Kohli et al. 2003).

The hairy roots from the AR lines grew slowly, were thin and straight, and had slender, dense, and white branches with thin tips, whereas hairy roots from the Z lines grew slowly with thick and fewer branches. Aging was observed along with the generation of brown pigments in some of the Z lines, most likely due to the accumulation of phenolic compounds. The growth rate of the AR lines was faster than that of the Z lines through analysis of the growth index. Plant growth is accomplished in two ways: by cell growth and by cell division, processes controlled by the G1 and M phases of the cell cycle, respectively (Nasmyth 1996). Cell cycle phases are initiated by cyclins which can be grouped as the A- and B-type mitotic (M) cyclins and the D-type G1 cyclins (Dewitte and Murray 1997). D-type cyclins might function as mediators of internal and environmental stimuli to drive cell division (Riou-Khamlichi et al. 1999). Both B- and A-type cyclins in plants are expressed during mitosis and are known as mitotic cyclins, and they contribute greatly to G2/M transition (Obaya and

Sedivy 2002). Many authors have also reported that the expression of plant mitotic cyclin genes is a good marker for mitotic activity since it is exclusively confined to mitotically active cells of meristematic tissues (Ferreira et al. 1994; Kouchi et al. 1995; Setiandy et al. 1997). One approach for a better understanding of how hairy roots regulate cell division is the molecular analysis of genes associated with the cell cycle. Our results demonstrated that the T-DNA genes derived from the plasmid pRi A4 positively affected *CYSA* and *CYSB* gene expression. TF GmMYBZ2 on the other hand had a negative effect on *CYSA* and *CYSB* gene expression levels. Therefore, there is a correlation between the growth rates of the AR lines and the Z lines, and the relative *CYSA* and *CYSB* gene expression levels.

Generally, a high content of secondary metabolites in plant tissues is associated with poor growth (Jouhikainen et al. 1999). Hairy root morphology also plays an important role in secondary metabolite production (Zhang et al. 2004). We observed that catharanthine contents in the three AR lines (AR_1, AR_8, and AR_{10}) were variable, ranging from 0.38 to 0.56 mg/g FW. Catharanthine levels in WT roots were undetectable, in agreement with a previous report (Ciau-Uitz et al. 1994). Catharanthine levels in the six selected Z lines were lower compared with those of the A lines, ranging from 0.20 to 0.25 mg/g FW. We conclude that the T-DNA genes derived from the plasmid pRi A4 had a positive effect on the catharanthine biosynthesis pathway, and TF GmMYBZ2 had a negative effect. The transcript levels of catharanthine pathway genes correlate with the catharanthine concentrations. The AR hairy root lines had higher transcript levels of *ASα*, *MECS*, *SLS*, *DXR*, *DXS*, *G10H*, *HDS*, *CPR*, *STR*, *ORCA2*, and *ORCA3*, lower levels of *ZCT1*, and no difference in the transcript levels of other genes studied (*TDC*, *PGGT-I*, *SGD*, *ZCT2*, *ZCT3*, *GBF1*, and *GBF2*) compared to WT roots. Canel et al. (1998) reported that overexpression of *TDC* did not improve the contents of catharanthine although it enhanced tryptamine levels and that a constitutively high TDC activity seemed to be detrimental to the growth of *C. roseus* cell cultures. *STR* overexpressing cultures showed 10 times higher STR activity than the wild-type line, resulting in higher TIA accumulation including catharanthine (Canel et al. 1998). *ASα* overexpression in *C. roseus* hairy roots under the control of a glucocorticoid inducible promoter system resulted in a large increase in the indole compounds tryptophan and tryptamine. Loganin feeding to these hairy roots led to an increase in catharanthine content of 26% (Peebles et al. 2005, 2006). *G10H* overexpression in cell cultures resulted in higher G10H activity and promoted the formation of 10-hydroxygeraniol (Collu et al. 2001). Overexpression of *G10H* in transgenic hairy root lines significantly enhanced the accumulation of ajmalicine, vincristine, and vinblastine (Gong et al. 2005). These reports show that key enzyme genes can limit the metabolic flux in the catharanthine pathway. The AR hairy root lines contained high levels of catharanthine, probably due to the fact that the expression levels of key genes in the catharanthine pathway were elevated. At least two reasons can be put forward to explain why GmMYBZ2 has a negative effect on catharanthine biosynthesis in Z lines. First, the Z lines had high levels of *ZCT1* transcripts. ZCT1 can bind to the promoter of *STR* and repress its activity (Pauw et al. 2004). Second, the Z lines had low transcript levels of *ASα* and *ORCA3*, which are key genes in catharanthine biosynthesis. GmMYBZ2 has the conserved amino acid motif pdLNLD/ELxiG/S (Du et al. 2009), which is a well-studied transcriptional repression motif. Our results reveal the potential of T-DNA genes derived from the plasmid pRi A4 to modify the flux in the catharanthine pathway. They also reveal the complexity of the regulation of the catharanthine pathway and indicate the necessity to find unknown regulators, of which the expression is affected by T-DNA genes derived from the pRiA4 plasmid.

In conclusion, the current work demonstrates the positive effect of T-DNA genes derived from the plasmid pRi A4 on genes encoding enzymes and regulators acting in the catharanthine pathway. Our work also demonstrates that GmMYBZ2 had a negative effect on catharanthine production which was correlated with higher transcript levels of *ZCT1* and lower expression levels of *ASα*, *STR*, and *ORCA3*. It is possible that this effect of GmMYBZ2 is indirect via reduction of the expression levels of T-DNA genes. These results suggest that establishment of a delicate balance between levels of cell cycle regulators, positive regulators, and negative regulators might be necessary to increase the flux through the TIA pathway to catharanthine in *C. roseus* hairy roots.

Acknowledgments We thank Pang Jun-Feng for technical assistance with RNA extraction, Ma Jiang-Tao for measuring the growth index of hairy roots, Du Hai for work discussions, and Dr. Yang Wen-Jie for providing the plasmid pCAMBIA2301-GmMYBZ2. We are also grateful to Prof. Dr. Johan Memelink, Dr. A. A. N. van Brussel, and Trillian Ross (Plant Cell Physiology, Institute of Biology, Leiden University, The Netherlands) for giving some useful comments on this manuscript. This work was supported by a grant from the National Transgenic Program (2009ZX08005-004B, 2009ZX08009055B, and 2008ZX08005-004).

References

Aharoni A, De Vos CH, Wein M, Sun Z, Greco R, Kroon A, Mol JN, O'Connell AP (2001) The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. Plant J 28:319–332

Alpizar E, Dechamp E, Espeout S, Royer M, Lecouls AC, Nicole M, Bertrand B, Lashermes P, Etienne H (2006) Efficient production of *Agrobacterium rhizogenes*-transformed roots and composite plants for studying gene expression in coffee roots. Plant Cell Rep 25:959–967

Brillanceau MH, David Ch, Tempé J (1989) Genetic transformation of *Catharanthus roseus* G. Don by Agrobacterium rhizogenes. Plant Cell Rep 8:63–66

Canel C, Lopes-Cardoso MI, Whitmer S, van der Fits L, Pasquali G, van der Heijden R, Hoge JHC, Verpoorte R (1998) Effects of overexpression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. Planta 205:414–419

Ciau-Uitz R, Miranda-Ham ML, Coello-Coello J, Chi B, Pacheco LM, Loyola-Vargas VM (1994) Indole alkaloid production by transformed and non-transformed root cultures of *Catharanthus roseus*. In Vitro Cell Dev Biol 30:84–88

Collu G, Unver N, Peltenburg-Looman AMG, 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

Dewitte W, Murray JA (1997) The plant cell cycle. Annu Rev Plant Biol 54:235–264

Diouf D, Gherbi H, Prin Y, Franche C, Duhoux E, Bogusz D (1995) Hairy root nodulation of *Casuarina glauca*: a system for the study of symbiotic gene expression in an actinorrhizal tree. Mol Plant Microbe Interact 8:532–537

Du H, Liu L, Tang XF, Zhao YL, Gao J, Wu YM, Huang YB, Tang YX (2009) Characterization and mutation of soybean MYB transcription factor GmMYBZ2 and its prokaryotic expression. Chin J Agr Biotechnol 17:301–306

Ferreira PCG, Hemerly AS, JdA E, Montagu MV, Engler G, Inzé D (1994) Developmental expression of the *Arabidopsis* cyclin gene *cyc1At*. Plant Cell 6:1763–1774

Fornalé S, Sonbol FM, Maes T, Capellades M, Puigdomenech P, Rigau J, Caparros-Ruiz D (2006) Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid O-methyl-transferase genes by two new maize R2R3-MYB transcription factors. Plant Mol Biol 62:809–823

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

Georgiev MI, Pavlov AI, Bley T (2007) Hairy root type plant in vitro systems as sources of bioactive substances. Appl Microbiol Biotechnol 74:1175–1185

Georgiev MI, Weber J, Maciuk A (2009) Bioprocessing of plant cell cultures for mass production of targeted compounds. Appl Microbiol Biotechnol 83:809–823

Gong YF, Liao ZH, Pi Y, Guo BH, Miao ZQ, Sun XF, Tang KX (2005) Engineering terpenoid indole alkaloids biosynthetic pathway in *Catharanthus roseus* hairy root cultures by over-expressing the geraniol 10-hydroxylase gene. J Shanghai Jiaotong Univ (Sci) E-10, S1:8–13

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1989) Plant molecular biology manual. Kluwer, Dordrecht

Jefferson R (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Jin H, Martin C (1999) Multifunctionality and diversity within the plant MYB gene family. Plant Mol Biol 41:577–585

Jin H, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C (2000) Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. EMBO J 19:6150–6161

Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TH, Oksman-Caldentey KM (1999) Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208:545–551

Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P (2003) Transgene integration, organization and interaction in plants. Plant Mol Biol 52:247–258

Kouchi H, Sekine M, Hata S (1995) Distinct classes of mitotic cyclins are differentially expressed in the soybean shoot apex during the cell cycle. Plant Cell 7:1143–1155

Kui LW, Bolitho K, Grafton K, Kortstee A, Karunairetnam S, McGhie TK, Espley RV, Hellens RP, Allan AC (2010) An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. BMC Plant Biol 10:50

Kumar V, Satyanarayana KV, Itty SS, Indu EP, Giridhar P, Chandrashekar A, Ravishankar GA (2005) Stable transformation and direct regeneration in *Coffea canephora* P ex. Fr. by *Agrobacterium rhizogenes* mediated transformation without hairy-root phenotype. Plant Cell Rep 25:214–222

Menke FLH, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18:4455–4463

Nasmyth K (1996) Viewpoint: putting the cell cycle in order. Science 274:1643–1645

Obaya AJ, Sedivy JM (2002) Regulation of cyclin-CDK activity in mammalian cells. Cell Mol Life Sci 59:126–142

Pauw B, Hilliout FAO, Martin VS, Chatel G, de Wolf CJF, 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, Hong SB, Gibson SI, Shanks JV, San KY (2005) The transient effects of over-expressing anthranilate synthase alpha and beta subunits in *Catharanthus roseus* hairy root. Biotechnol Prog 21:1572–1576

Peebles CA, Hong SB, Gibson SI, Shanks JV, San KY (2006) Effects of terpenoid precursor feeding on *Catharanthus roseus* hairy roots over-expressing the α or the α and β subunits of anthranilate synthase. Biotechnol Bioeng 93:534–540

Peebles CAM, Erik HH, Jacqueline VS, 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

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science 283:1541–1544

Rosinski JA, Atchley WR (1998) Molecular evolution of the MYB family of transcription factors: evidence for polyphyletic origin. J Mol Evol 46:74–83

Setiandy YY, Sekine M, Yamamoto T, Kouchi H, Shinmyo A (1997) Expression pattern of tobacco cyclin genes. Plant Cell Rep 16:368–372

Shahin EA, Sukhapinda K, Simpson RB, Spivey R (1986) Transformation of cultivated tomato by a binary vector in *Agrobacterium rhizogenes*: transgenic plants with normal phenotypes harbour binary vector T DNA, but no Ri-plasmid T-DNA. Theor Appl Genet 72:770–777

Shalel-Levanon S, San KY, Bennett GN (2005) Effect of oxygen, and ArcA and FNR regulators on the expression of genes related to the electron transfer chain and the TCA cycle in *Escherichia coli*. Metab Eng 7:364–374

Singh SN, Vats P, Suri S, Shyam R, Kumria MML, Rangganathan S, Sridharan K (2001) Effect of an antidiabetic extract of

Catharanthus roseus on enzymatic activities in steptozotocin induced diabetic rats. J Ethnopharmacol 76:269–277

Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol 4:447–456

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C (1998) The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum* regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. Plant Cell 10:135–154

Terada R, Shimamoto K (1990) Expression of CaMV35S-GUS gene in transgenic rice plants. Mol Gen Genet 220:389–392

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

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11:607–628

Weathers PJ, Towler MJ, Xu JF (2010) Bench to batch: advances in plant cell culture for producing useful products. Appl Microbiol Biotechnol 85:1339–1351

Zhang L, Ding RX, Chai YR, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu TF, Pi Y, Wang ZN, Zhang HM, Kai GY, Liao ZH, Sun XF, Tang KX (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci USA 101:6786–6791

Zhao J, Zhu W, Hu Q (2001) Enhanced catharanthine production in *Catharanthus roseus* cell cultures by combined elicitor treatment in shake flasks and bioreactors. Enzyme Microb Technol 28:673–681

Zhou ML, Shao JR, Tang YX (2009) Production and metabolic engineering of terpenoid indole alkaloids in cell cultures of the medicinal plant *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle). Biotechnol Appl Biochem 52:313–323

Zhou ML, Zhu XM, Shao JR, Wu YM, Tang YX (2010) Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with MeJA/nitric oxide elicitation in *Catharanthus roseus* hairy root culture. Appl Microbiol Biotech 88:737–750