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ORIGINAL ARTICLE

Engineering overexpression of *ORCA3* and strictosidine glucosidase in *Catharanthus roseus* hairy roots increases alkaloid production

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Abstract *Catharanthus roseus* produces many pharmaceutically important terpenoid indole alkaloids (TIAs) such as vinblastine, vincristine, ajmalicine, and serpentine. Past metabolic engineering efforts have pointed to the tight regulation of the TIA pathway and to multiple rate-limiting reactions. Transcriptional regulator ORCA3 (octadecanoid responsive *Catharanthus* AP2-domain protein), activated by jasmonic acid, plays a central role in regulating the TIA pathway. In this study, overexpressing *ORCA3* under the control of a glucocorticoid-inducible promoter in *C. roseus* hairy roots resulted in no change in the total amount of TIAs measured. RT-qPCR results showed that *ORCA3* overexpression triggered the upregulation of transcripts of most of the known TIA pathway genes. One notable exception was the decrease in strictosidine glucosidase (*SGD*) transcripts. These results corresponded to previously published results. In this study, *ORCA3* and *SGD* were both engineered in hairy roots under the control of a glucocorticoid-inducible promoter. Co-overexpression of *ORCA3* and *SGD* resulted in a significant ($p<0.05$) increase in serpentine by 44 %, ajmalicine by 32 %, catharanthine by 38 %, tabersonine by 40 %, lochnericine by 60 % and hörhammericine by 56 % . The total alkaloid pool was increased significantly by 47 %. Thus, combining overexpression of a positive regulator and a pathway gene which is not controlled by this regulator provided a way to enhance alkaloid production.

Keywords Terpenoid indole alkaloid · Madagascar periwinkle · Transcription factors · Plant secondary metabolism

Abbreviations

7-DLGT	7-deoxyloganetic acid-*O*-glucosyl transferase
7-DLH	7-deoxyloganic acid hydroxylase
ADH	alcohol dehydrogenase
ASα	anthranilate synthase alpha subunit
BPF	box P-binding factor
CPR	cytochrome P450 reductase
CRSDH4H	desacetoxyvindoline 4-hydroxylase
D4H	desacetoxyvindoline 4-hydroxylase
DAT	deacetylvindoline acetyltransferase
DMAPP	dimethylallyl pyrophosphate
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose 5-phosphate synthase
G10H	geraniol 10-hydroxylase
GBF	G-box binding factor
GPPS	geranyl diphosphate synthase
IO	iridoid oxidase
IPP	isopentenyl pyrophosphate
IS	iridoid synthase
JA	jasmonic acid
LAMT	loganic acid methyltransferase
MAT	minovincinine 19-hydroxy-*O*-acetyltransferase
MEP	2-C-Methyl-D-erythritol 4-phosphate
MYC2	MYC2 transcription factor
ORCAs	octadecanoid responsive Catharanthus AP2-domain proteins

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Prx1	peroxidase
SGD	strictosidine beta-glucosidase
SLS	secologanin synthase
STR	strictosidine synthase
T16H	tabersonine 16-hydroxylase
T19H	tabersonine 19-hydroxylase
TDC	tryptophan decarboxylase
TIA	terpenoid indole alkaloid
WRKY1	WRKY transcription factor 1
ZCT	transcription factor IIIA-type zinc finger family

Introduction

The terpenoid indole alkaloids (TIAs) are plant secondary metabolites with diverse chemical structures containing an indole moiety and a terpenoid moiety (O'Connor and Maresh 2006). TIAs not only help plants defend against biological and environmental stress (Wasternack 2014), but also exhibit interesting pharmaceutical activities such as anticancer, antimalarial, and antiarrhythmic functions (van der Heijden et al. 2004). *Catharanthus roseus* (*Madagascar periwinkle*) produces approximately 130 identified TIAs. Among this group of TIAs, ajmalicine (Fulzele and Heble 1994) and serpentine (Gendenshtein and Mikhailenko 1964) were applied as antihypertensive and antiarrhythmic drugs separately. Vinblastine and vincristine (Jordan et al. 1991; Ngan et al. 2001), exclusively synthesized in *C. roseus*, have been widely used clinically as anticancer agents to treat lymphoma and leukemia since the 1970s (Dostal and Libusova 2014; Rowinsky and Donehower 1991). In recent years, vinblastine and vincristine have been listed on the drug shortage list by the FDA (Food and Drug Administration) due to increasing demand and shortage of supply (Viale 2013). These useful alkaloids accumulate in plants at very low levels (Liscombe and O'Connor 2011). These facts drive a need for metabolic engineering efforts to develop more efficient production platforms.

The first alkaloid in TIA pathway, strictosidine, is synthesized by strictosidine synthase (STR) through the condensation of tryptamine from the indole pathway and secologanin from the terpenoid pathway (Fig. 1) (Pasquali et al. 1992). Strictosidine is further hydrolyzed by strictosidine glucosidase (SGD) to the unstable metabolites strictosidine aglycone (Luijendijk et al. 1998). Then this highly reactive ring-opened dialdehyde intermediate can be converted to a wide range of TIAs by different branched downstream pathways (El-Sayed et al. 2007). Some of these downstream TIA pathways are tissue, cell-type, or organelle specific (Giddings et al. 2011; Shukla et al. 2010; Verma et al. 2012). The genes leading to the synthesis of these alkaloids are largely unknown. The lack of pathway knowledge, the location of the pathway (Verma et al. 2012), the highly branched downstream pathways, and the complex transport of the metabolites (Verma et al. 2012) make engineering *C. roseus* to achieve expected increases in useful alkaloids extremely challenging.

Previous effort to increase TIA production by media optimization (Mérillon et al. 1986), precursor feeding (Peebles et al. 2006), elicitor feeding (Zhao et al. 2001), and single rate-limiting gene (Peebles et al. 2006) or pathway regulator (Peebles et al. 2009) engineering had limited success in increasing overall alkaloids production in both cell suspension and hairy root cultures of *C. roseus* (Glenn et al. 2013). Often, the increase in some alkaloids was accompanied by decreases in other alkaloids. Overexpressing multiple pathway genes gave promising results. For example, the effect of co-expressing *DXS* (1-deoxy-D-xylulose 5-phosphate synthase) and *G10H* (geraniol 10-hydroxylase) or *DXS* and *AS* (anthranilate synthase) resulted in an increase in several downstream metabolites without significantly decreasing other alkaloids (Peebles et al. 2011). However, the increase in TIA production was still limited in magnitude.

Since transcription factors control the expression of multiple pathway genes, overexpressing positive pathway regulators is one way to coordinately upregulate multiple pathway genes which could lead to the increase in TIA metabolites. So far, increases and decreases in TIA metabolites were observed in hairy root lines overexpressing a single transcription factor. *ORCA3* overexpression in *C. roseus* hairy roots leads to a decrease in tabersonine, lochnericine, and hörhammericine (Peebles et al. 2009). The *ORCA2* engineered hairy roots produced more serpentine, 16-hydroxytabersonine and 19-hydroxytabersonine but less secologanin, strictosidine, tabersonine, and hörhammericine than the control (Li et al. 2013). Another jasmonate-induced transcription factor gene *WRKY1* was overexpressed in *C. roseus* hairy roots and resulted in an increase in serpentine but a decrease in catharanthine (Suttipanta et al. 2011). Further examination of the changes in mRNA transcripts of TIA genes and regulators showed that while many TIA genes were upregulated when a positive regulating transcription factor was overexpressed, some TIA genes expression remained unchanged or even decreased. Interestingly, the expression levels of some negative transcriptional factors were enhanced. In *ORGA3* overexpressing hairy root line, *G10H* and *TDC* did not show significant changes, while *SGD* was significantly downregulated and the negative regulators *ZCTs* (transcription factor IIIA-type zinc finger family) were upregulated (Peebles et al. 2009). Similarly, overexpression of *ORCA2* in *C. roseus* hairy roots upregulated *ZCTs*, while downregulated *SGD* and *DAT* transcription (Li et al. 2013).

These studies point out that the transcription factors regulate overlapping but distinct sets of TIA pathway genes due to their binding specificity. Overexpressing one regulator cannot stimulate the overexpression of all pathway genes. Also, the upregulation of positive regulators can trigger the upregulation of negative regulators. The genes that are not regulated by the positive regulator or are repressed by the upregulated negative regulator may limit the metabolic flux toward the desired

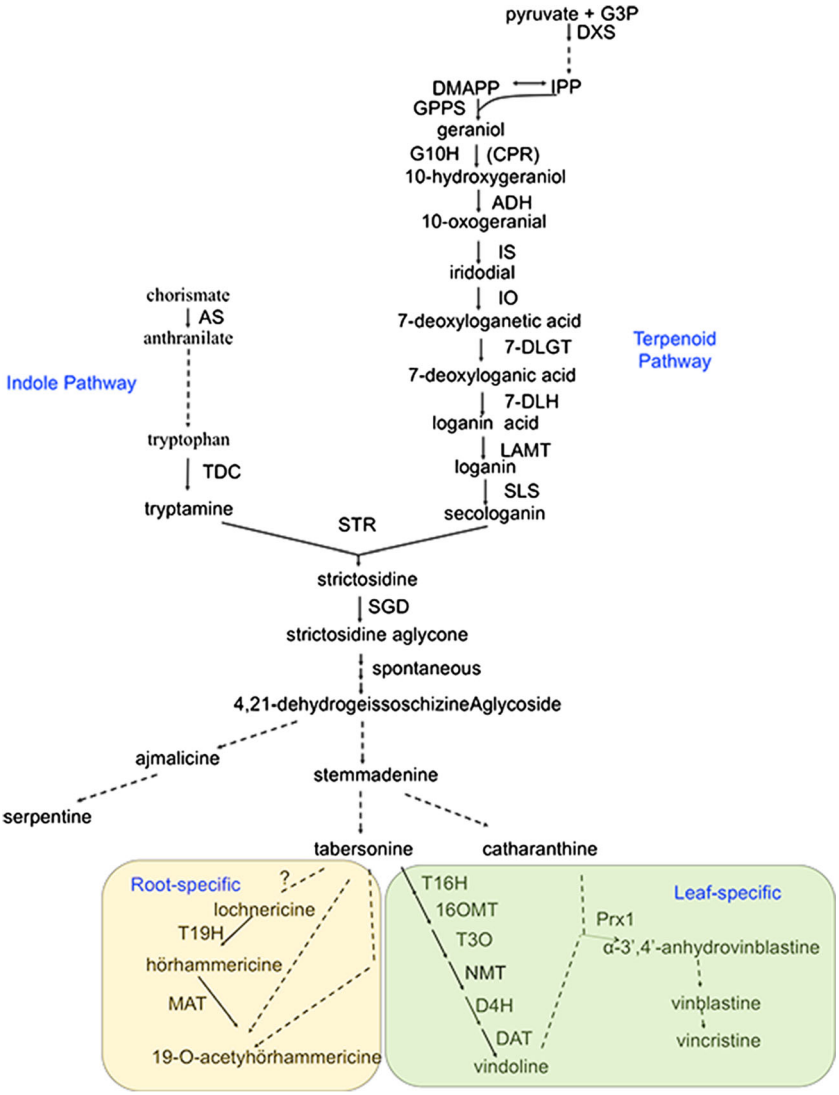

Fig. 1 Terpenoid indole alkaloid pathway. (Giddings et al. 2011; Zhu et al. 2014; Miettinen et al. 2014)

alkaloids. Therefore, this paper examines the effect on the TIA pathway in *C. roseus* of combining the overexpression of positive regulator ORCA3 and the critical pathway gene SGD which previously showed downregulation in the ORCA3 overexpressing hairy root line (Peebles et al. 2009). The results reported here demonstrate that paring overexpression of ORCA3 and SGD is sufficient to overcome endogenous negative regulation and results in increased TIA metabolite accumulation.

Materials and methods

Clone generation

Plasmid *pTA7002*/*ORCA3* (Fig. 2) was obtained from Dr. Ka-Yiu San, and its construction was described previously (Peebles et al. 2009). *SGD* was amplified from cDNA prepared from RNA purified from *C. roseus* hairy roots using polyT primers and GoScript reverse transcriptase according to manufacturer's instructions (Promega). The primers used for *SGD* amplification were 5′-CCT TAA AGA GCG GTT CAG ATC-3′ and 5′-CAT TAT CTA AAA TAA GAA GAG AAA TAT G-3′, the *SGD* PCR product was ligated into StrataClone PCR cloning vector *pSC-A-amp*/*kan* (Agilent Technology), and verified by sequencing. The sequencing result of this gene matched the published sequence for *SGD* (GenBank: AF112888). *SGD* was then moved to the intermediate plasmid *pUCGALA* (Hughes et al. 2004b) at the XhoI/SpeI site to construct *pUCGALA*/*SGD*. *SGD* along with the promoter sequence *GAL4-UAS* was cut from *pUCGALA*/*SGD* with restriction enzyme SbfI, and constructed next to the right border in the T-DNA region of *pTA7002*/*ORCA3* (Fig. 2). The *cis* orientation and sequence of SGD in *pTA7002*/*ORCA3*/*SGD* was further verified by sequencing. Both *ORCA3* and *SGD* genes are under the control of a glucocorticoid-inducible promoter (Hughes et al. 2002). Plasmids *pTA7002*/*ORCA3* and *pTA7002*/*ORCA3*/*SGD* were transferred into *Agrobacterium rhizogenes* ATCC 15834 separately using

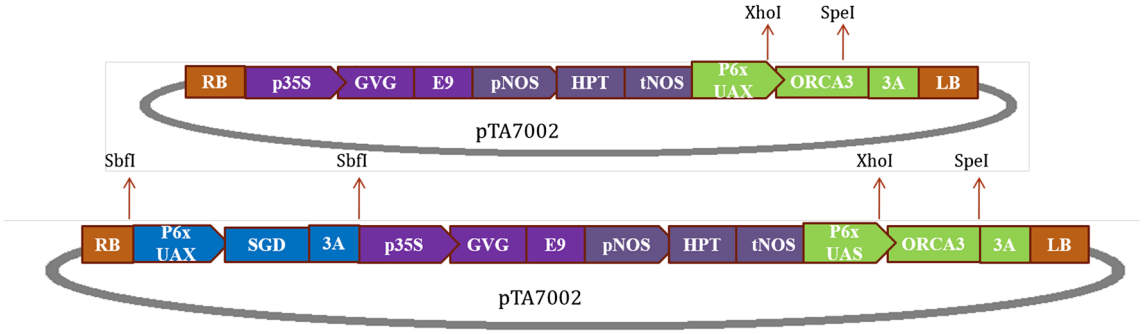

Fig. 2 Plasmid constructs used to transform *C. roseus* hairy roots. *RB*, right border of T-DNA; *LB*, left border of T-DNA; *p35S*, cauliflower mosaic virus 35S promoter; *GVG*, chimeric transcription factor containing GAL4 DNA-binding domain and VP16 transactivating domain and rat-GR HBD; *E9*, pea rbcS-E9 polyadenylation sequence; *pNOS*, nopaline synthase promoter; *HPT*, hygromycin phosphotransferase gene; *tNOS*, nopaline synthetase polyadenylation sequence; *p6xUAX*, GVG-regulated promoter; and *3A*, pea rbcS-3A polyadenylation sequence. (Hughes et al. 2002)

eletrotransformation method. The presence of the plasmids was confirmed by sequencing. *Agrobacterium* containing each plasmid was cultured in 6 ml YEM media at 28 °C and 225 rpm for 36 h. Forceps were dipped in the agrobacteria and used to infect the stem of the *C. roseus* seedlings as preciously described (Bhadra et al. 1993). After 6 weeks, hairy roots were harvested from the plants and grown on selection plates (hairy roots media described below with 350 mg/L cefotaximine and 30 mg/L hygromycin). Hairy roots growing on selection media were transferred to new hairy roots plates and then adapted to liquid culture as preciously described (Bhadra et al. 1993).

Hairy roots culture, induction study, and HPLC analysis

Hairy roots were cultured as previously described (Peebles et al. 2005). Hairy roots at late exponential growth stage (18 days after sub-culture) were treated with 3 μM inducer dexamethasone (induced cultures) to induce overexpression of the genes or with an equal volume of ethanol (uninduced cultures) as a negative control. After 72 h of induction, hairy roots were blotted dry and frozen at −80 °C. The frozen hairy roots were then lyophilized for 3 days using Freeze Dry Systems (LABCONCO). Approximately 50 mg dry weight of the ground tissue was extracted in 25 mL of methanol by ultrasonication (MISONIX, Sonicator S4000) for 10 min. The extracts were centrifuged at 1300×*g* for 15 min at 4 °C. The supernatants were concentrated to 2 mL using a vortex evaporator (LABCONCO, RapidVap 7670520) and filtered by 0.22 mm nylon film. Ten microliters of each sample was injected on the HPLC (SHIMADZU, Japan) for the analysis of the following metabolites: ajmalicine (AvaChem Scientific), serpentine (AvaChem Scientific), catharanthine (gift from Dr. Ka-Yiu San at Rice University), hörhammericine (gift from Dr. Jacqueline V. Shanks at Iowa State University), lochnericine (gift from Dr. Jacqueline V. Shanks at Iowa State University), and tabersonine (AvaChem Scientific) as previously described (Morgan and Shanks 1999; Peebles et al. 2006). Quantification of lochnericine and hörhammericine were based on tabersonine standard curve as preciously described (Morgan et al. 2000).

cDNA synthesis and RT-qPCR amplification

Fresh hairy roots were blotted with a paper towel to remove excess liquid media. Flash-frozen hairy root powder was collected by grinding hairy roots with liquid nitrogen in a mortar. Total RNA was isolated from the frozen hairy root powder using TRIzol reagent according to manufacturer's instructions (Ambion RNA by Life Technologies). DNA was removed from the sample with TURBO DNA-free according to the manufacturer's instructions (Ambion RNA by Life Technologies). RNA concentration and quality was detected with NanoDrop 2000 (Thermo Scientific). cDNA was synthesized from 500 ng RNA using random primers and GoScript reverse transcriptase according to manufacturer's instructions (Promega). A *no-amplification control* (without reverse transcriptase) was performed for each sample. cDNA was diluted 10 times to 200 μL with nuclease-free water. Each qPCR reaction (20 μL) contained 1 μL diluted cDNA, 1.25 pmol/mL mixed primers, 10 μL SsoAdvanced SYBR green super mix (BIO-RAD) and nuclease-free water. The primers used for qPCR which were not previously described (Peebles et al. 2009) are listed in Table 1. The qPCR amplifications were carried out in BIO-RAD CFX Connect™ Real-Time PCR Detection System with the program: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 60 °C. The relative gene expression was quantified by using the comparative threshold cycle CT method as previously described (Shalel-Levanon et al. 2005). The *40S ribosomal protein S9* (*RSP9*) was used for the control gene (Menke et al. 1999).

Statistical analysis

Data were analyzed using the Student's *t* test.

Table 1 List of genes and primer pairs used for qPCR

Gene	Protein name (accession number)	Primer pairs	PCR product (bp)
LAMT	Loganic acid methyltransferase (EU057974)	5′-CCAATGAAAGGTGGTGATGA-3′ 5′-AATGCGGAAAGGTTTGATTG-3′	159
T19H	Tabersonine/lochnericine 19-hydroxylase (HQ901597.1)	5′- CTTCATTTGCAATCCCCATT-3′ 5′- AAACGAGAGAGGGTTTTGG-3′	150
Gbf3	G-box binding factor bZIP (AY027510)	5′-GCTTCCACTGTTGCTTCTCC-3′ 5′-CCTGGAGTCGTTGCCATAGT-3′	149
BPF	MYB-like DNA-binding protein (AJ251686)	5′-CCAATGATGCATTTGATTCG-3′ 5′-TGCAGGAAGAGTGACCAGTG-3′	143
LAMT	Loganic acid methyltransferase (EU057974)	5′-CCAATGAAAGGTGGTGATGA-3′ 5′-AATGCGGAAAGGTTTGATTG-3′	159
MAT	Minovincinine 19-hydroxy-*O*-acetyltransferase (AF253415.1)	5′-AGGATTGGGCTGCTTCTACA-3′ 5′- TATGGCTTCCGGAGAGAAGA -3′	167
MYC2	An early jasmonate-responsive bHLH transcription factor (AF283507)	5′-CTGGGTTCAACGGAATTGAT-3′ 5′-CGATGGATCAGTAAGCCACA-3′	147
WRKY1	WRKY1 transcription factor (HQ646368.1)	5′-GAAACTCTCGCCGTACTTGG-3′ 5′-CCGAAACATTCCTTCGTTTG-3′	159

Results

Creating hairy root lines

Two stable transgenic hairy root lines, ORCA3-26 and ORCA3/SGD-7, were obtained from *A. rhizogenes* transformation of *C. roseus* seedlings. The expression of *ORCA3* and *SGD* are controlled by the same glucocorticoid-inducible promoter (Hughes et al. 2002). The use of an inducible promoter system allows a single transgenic line to be used as the negative control and the experimental lines, which reduces the uncertainties that result from the random T-DNA insertion and hairy root line variations (Peebles et al. 2009). An *ORCA3* engineered hairy root line called ORCA3-26 demonstrated a 143.3±29.8 fold increase in *ORCA3* mRNA transcripts after 72 h induction comparing to the uninduced control. An *ORCA3*/*SGD* engineered hairy root called ORCA3/SGD-7 showed increased expression in both *ORCA3* and *SGD* after 72 h induction. In ORCA3/SGD-7 hairy roots, *ORCA3* mRNA was increased 36.5±1.9 times and *SGD* was increased 10.2±1.2 times after 72 h induction. These two hairy root lines were chosen for further study. It is important to note that RT-qPCR results are a qualitative measure of mRNA changes and the differences in fold increase could be due to differences in the background level of expression of ORCA3 in the two different hairy root lines. *Agrobacterium* mediated transformation randomly inserts the DNA of interest into the nuclear chromosome. This results in significant clonal variation between independent transformation events (Gelvin 2003). Evidence of this is well documented in literature and is seen by differences in morphology and basal TIA concentrations between hairy root lines (Hughes et al. 2004b; Peebles et al. 2009, 2011). Due to the inherent clonal variation, we utilized a glucocorticoid-inducible promoter to induce gene expression which allows us to compare the effects of increased expression to that of the control within the same background.

Changes in terpenoid indole alkaloid concentrations

The TIA concentrations of ORCA3-26 and ORCA3/SGD-7 were analyzed after a 72 h induction study. This timeframe was chosen because previous studies show that TIAs reached high and stable production levels by this time after induction (Peebles et al. 2005, 2009). The terpenoid indole alkaloids analyzed include ajmalicine and serpentine from the coryanthe family; catharanthine from the iboga family; and tabersonine, hörhammericine, and lochnericine from the aspidosperma family. Upon induction, the changes in TIA metabolite concentrations for hairy root line ORCA3-26 (Fig. 3) follow the same trends for most metabolites as previously reported (Peebles et al. 2009). The main exception is lochnericine where the concentration was increased by 56 % ($p<0.05$). In the previous study, lochnericine was decreased by 54 % ($p<0.05$) (Peebles et al. 2009). In contrast to lochnericine, tabersonine showed a significant decrease of 97 % ($p<0.05$) when *ORCA3* was overexpressed (Fig. 3). Although lochnericine level was significantly increased in ORCA3 hairy roots, the aspidosperma family, the corynanthe family, and the total TIA pool of measured alkaloids did not show significant changes when *ORCA3* was overexpressed (Fig. 4).

In the ORCA3/SGD-7 hairy root line, all measured TIA metabolites showed significant increase compared to the uninduced control (ORCA3/SGD-7 UI) upon induction of *ORCA3* and *SGD* for 72 h. Serpentine concentration was increased by 44 % ($p<0.05$), ajmalicine by 32 % ($p<0.05$), catharanthine by 38 % ($p<0.05$), tabersonine by 40 %

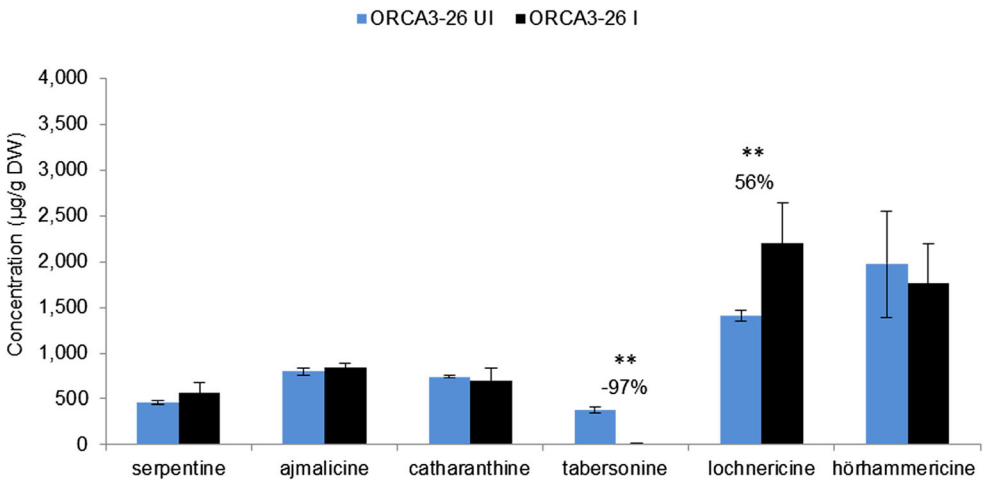

Fig. 3 The terpenoid indole alkaloid levels in ORCA3-26 *C. roseus* hairy roots. The hairy roots overexpressing ORCA3 (ORCA3-26 I) were induced with 3 μM dexamethasone during exponential growth phase and harvested 72 h later. The control (ORCA3-26 UI) was the same root line fed with an equivalent volume of ethanol. HPLC analysis was used to determine the concentration of serpentine, ajmalicine, catharanthine, tabersonine, lochnericine and hörhammericine. ** Significant results ($p<0.05$) between the UI and I cultures. *Error bars* represent the standard deviation of triplicate cultures

($p<0.05$), lochnericine by 60 % ($p<0.05$), and hörhammericine by 56 % (Fig. 5). Unlike overexpressing *ORCA3* alone, no significant decrease in any alkaloid was observed in the induced ORCA3/SGD-7 hairy root line. Co-overexpression of ORCA3 and SGD resulted in a 55 % ($p<0.05$) increase in the aspidosperma family, a 38 % ($p<0.05$) increase in the corynanthe family, and a 47 % ($p<0.05$) increase in the total TIA pool of measured alkaloids (Fig. 6).

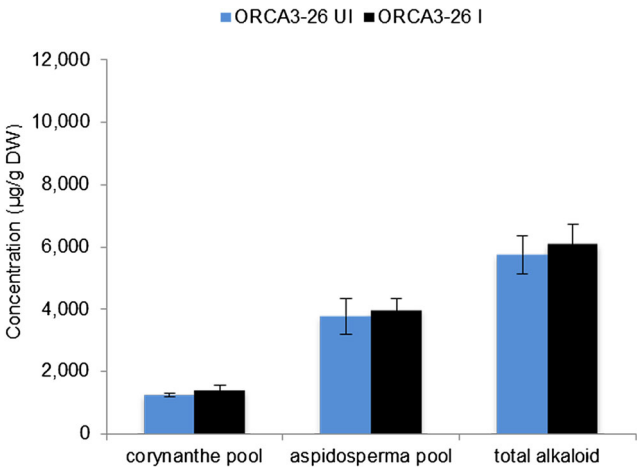

Fig. 4 The terpenoid indole alkaloid pools in ORCA3-26 *C. roseus* hairy roots. The hairy roots overexpressing ORCA3 (ORCA3-26 I) were induced with 3 μM dexamethasone during exponential growth phase and harvested 72 h later. The control (ORCA3-26 UI) was the same root line fed with an equivalent volume of ethanol. HPLC analysis was used to determine the TIA pools belong to corynanthe family (serpentine and ajmalicine), aspidosperma family (tabersonine, lochnericine, and hörhammericine) and the total alkaloids (serpentine, ajmalicine, catharanthine, tabersonine, lochnericine, and hörhammericine). *Error bars* represent the standard deviation of triplicate cultures

Changes in mRNA concentrations of TIA genes and regulators

In the current research, transcriptional alteration of TIA pathway genes and transcription factors were assessed in the induced and uninduced hairy root lines by using RT-qPCR at 24 h after induction (Table 2). This time point was selected based on a previous study that demonstrated that most changes in the transcripts of TIA genes and regulators peak by 24 h after induction and can be captured at this time point in *C. roseus* hairy roots overexpression ORCA3 (Peebles et al. 2009). This previous study also demonstrated that transcripts from most TIA genes and regulators returned close to uninduced levels by 72 h. Many of the known terpenoid and indole pathway genes and most downstream TIA pathway genes such as *STR*, *T19H* and *MAT* were upregulated by *ORCA3* overexpression (Table 2). Interestingly, *SGD* mRNA transcript was downregulated in ORCA3-26 I hairy roots cultures. These results were consistent with previous studies in *ORCA3* tagged *C. roseus* cell cultures, seedlings and hairy roots (Memelink and Gantet 2007; Pan et al. 2012; Peebles et al. 2009). Co-overexpression of *ORCA3* together with *SGD* in the ORCA3/SGD-7 hairy root line resulted in the increase of transcripts from pathway genes including *G10H*, *TDC*, *DXS*, *SLS*, *LAMT*, *CPR*, *AS*, *STR*, and *T19H* after 24 h induction. No decreases in TIA transcripts were observed in this line after the 24 h induction period.

In addition, overexpression of *ORCA3* alone or paring overexpression of *ORCA3* and *SGD* also triggered the negative regulation response of TIA pathway. Negative regulators including *ZCTs*, *GBF2* (G-box binding factor 2) and *GBF3* (G-box binding factor 3) were upregulated in ORCA3-26

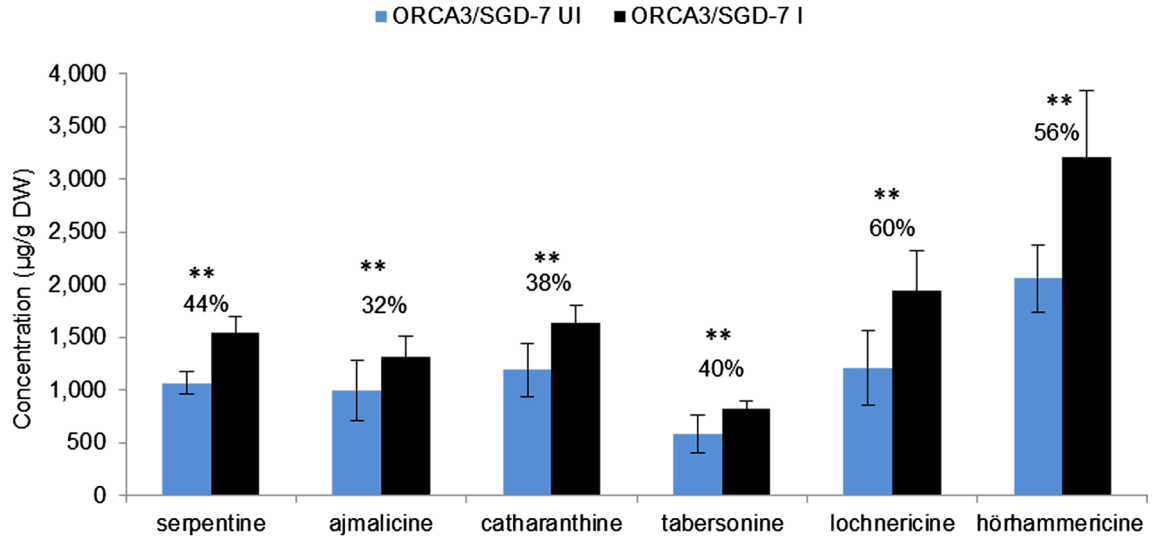

Fig. 5 The terpenoid indole alkaloid levels in ORCA3/SGD-7 *C. roseus* hairy roots. The hairy roots overexpressing ORCA3 and SGD (ORCA3/SGD I) were induced with 3 μM dexamethasone during exponential growth phase and harvested 72 h later. The control (ORCA3/SGD UI) was the same root line fed with an equivalent volume of ethanol. HPLC analysis was used to determine the concentration of serpentine, ajmalicine, catharanthine, tabersonine, lochnericine and hörhammericine. ** Significant results ($p<0.05$) between the UI and I cultures. *Error bars* represent the standard deviation of triplicate cultures

induced hairy roots (Table 2). ZCTs competitively bind to the ORCA3 targeted promoter region to weaken positive effects of ORCA3 on TIAs production (De Geyter et al. 2012). Similarly, most TIA pathway transcription factors including *ZCTs*, *ORCA2* and *MYC2* were upregulated in ORCA3/SGD-7 hairy roots after 24 h induction. The upregulation of the

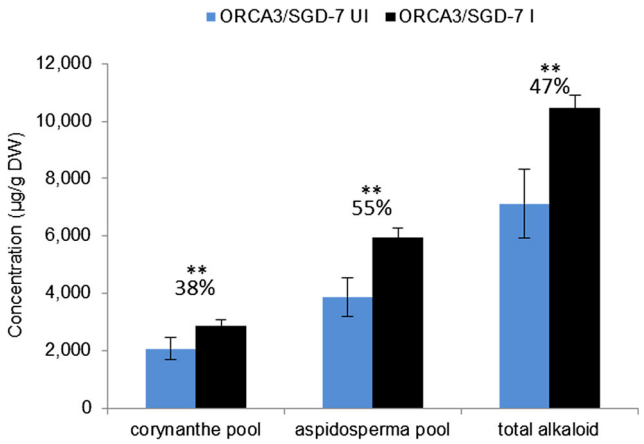

Fig. 6 The terpenoid indole alkaloid pools in ORCA3/SGD-7 *C. roseus* hairy roots. The hairy roots co-overexpressing ORCA3 and SGD (ORCA3/SGD-7 I) were induced with 3 μM dexamethasone during exponential growth phase and harvested 72 h later. The control (ORCA3/SGD-7 UI) was the same root line fed with an equivalent volume of ethanol. HPLC analysis was used to determine the TIA pools belong to corynanthe family (serpentine and ajmalicine), aspidosperma family (tabersonine, lochnericine, and hörhammericine) and the total alkaloids (serpentine, ajmalicine, catharanthine, tabersonine, lochnericine, and hörhammericine). ** Significant results ($p<0.05$) between the UI and I cultures. *Error bars* represent the standard deviation of triplicate cultures

Table 2 RT-qPCR of TIA pathway genes and transcription factors mRNA transcripts fold change in the 24 h induced root line compared to the uninduced root line

	Genes	ORCA3-24	ORCA3/SGD-7
Indole pathway	*AS*	2.5±1.2	2.0±0.1
	TDC	1.3±0.2	2.2±0.3
Terpenoid pathway	*G10H*	3.7±0.2	2.1±0.2
	CPR	2.7±0.5	1.3±0.2
	DXS	3.6±0.3	1.6±0.1
	SLS	4.5±0.3	2.2±0.1
	LAMT	4±0.3	1.8±0.1
Alkaloid pathway	*STR*	5.6±0.3	2.7±0.1
	SGD	0.7±0.1	10.2±1.2[a]
	T19H	4.8±0.3	6.9±0.6
	MAT	1.3±0.1	1.1±0.1
Positive regulators	*ORCA3*	143.3±29.8[a]	36.5±1.9[a]
	ORCA2	1.8±0.3	2.1±0.2
	BPF	1.0±0.1	1.0±0.1
	MYC2	1.8±0.1	1.2±0.1
	WRKY1	0.8±0.4	1.8±0.4
Negative regulators	*ZCT1*	6.4±0.6	2.2±0.1
	ZCT2	6.5±0.6	1.6±0.1
	ZCT3	4.2±0.4	1.4±0.1
	GBF1	1.1±0.1	1.1±0.1
	GBF2	1.7±0.2	1.0±0.3
	GBF3	2.8±0.2	1.0±0.2

Data represents the mean of triplicate ± standard deviation

[a] Genes that were engineered to be overexpressed in the hairy root line

negative regulator ZCTs may counterbalance the effect of the positive regulators and limit the increase of the alkaloids production in both transgenic *C. roseus* hairy roots. These interactions between positive and negative transcription factors point to the complex regulation of TIA production.

Discussion

The effect of ORCA3 overexpression

A *C. roseus* hairy root line with the inducible overexpression of *ORCA3* was successfully re-established. The previously published hairy root line EHIORCA3-4-1 had been lost due to contamination (Peebles et al. 2009). The new hairy root line name ORCA3-26 demonstrated a similar response to the inducible overexpression of *ORCA3* at both the metabolite and transcriptional levels. The most notable difference between EHIORCA3-4-1 and ORCA3-26 is in the concentration of lochnericine and hörhammericine. In EHIORCA3-4-1, the concentrations of lochnericine and hörhammericine significantly decreased ($p<0.05$) upon overexpression of *ORCA3* 72 h after induction (Peebles et al. 2009). This resulted in a significant decrease ($p<0.05$) in measured aspidosperma family metabolites in EHIORCA3-4-1. In contrast for ORCA3-26, the concentration of lochnericine significantly increased ($p<0.05$) and the concentration of hörhammericine did not change after 72 h of overexpressing *ORCA3* (Fig. 3). Here, the decrease in tabersonine was offset by the increase in lochnericine resulting in no difference in measured aspidosperma family metabolites.

Similarly, both increases and decreases in TIA metabolite levels were observed in other *C. roseus* hairy root lines that were engineered to overexpress other transcription factors. Overexpressing *ORCA2* led to the increase in serpentine, 16-hydroxytabersonine and 19-hydroxytabersonine, but decrease in secologanin, strictosidine, tabersonine, and hörhammericine (Li et al. 2013). In *WRKY1* tagged *C. roseus* hairy roots, serpentine level was enhanced while catharanthine level was reduced (Suttipanta et al. 2011). These results indicate that overexpressing a positive TIA pathway regulator does not necessarily lead to the significant overall increase in alkaloid production. This is most likely due to the tight and complicated regulation of TIA pathway or to other rate-limiting steps that are not upregulated by the overexpressed regulator.

In *C. roseus* cell cultures, ORCA3 is directly involved in transcriptional activation of *TDC*, *STR*, *CPR*, *ASa*, *D4H*, and *DXS* but not *SGD*, *G10H*, and *DAT* (van der Fits and Memelink 2000). Overexpressing *ORCA3* in *C. roseus* plants activated the expression of *AS*, *TDC*, *STR* and *D4H* but not *DXS* (Pan et al. 2012). The RT-qPCR analysis of hairy roots overexpressing *ORCA3* showed *AS*, *DXS*, *SLS*, and *STR* expression were increased, *G10H*, *TDC*, and *CPR* mRNA levels remains unchanged, and *SGD* showed decrease (Peebles et al. 2009). The differences in pathway gene expression between culture or tissue types may result from the existence of different control mechanisms of the TIA pathway between culture types or due to clonal variations. Currently, the tissue-specific regulation of the TIA pathway is not well understood.

Although experimental results showed the upregulation of multiple pathway genes during *ORCA3* overexpression, *SGD* transcripts were downregulated (Table 2). The limited knowledge about the regulatory elements located in the promoters of most TIA pathway genes makes it hard to pinpoint the target TIA pathway genes of ORCA3. Only the interaction of ORCA3 with the promoters of *STR* (van der Fits and Memelink 2001), *TDC* (van der Fits and Memelink 2001), *DAT* (Makhzoum et al. 2011; Wang et al. 2010), and *G10H* (Suttipanta et al. 2007) have been investigated. ORCAs can bind to the *STR* and *TDC* promoters to enhance gene expression (van der Fits and Memelink 2001). Analysis of the *G10H* promoter showed that it contains unique binding sites for several transcriptional factors but did not include a binding site for ORCA3. This indicates that *G10H* may be regulated by a different transcriptional cascade (Suttipanta et al. 2007). Similarly, the regulatory elements and transcription factor binding sites of DAT does not contain AP2/ERF-domain responsive sequence (Makhzoum et al. 2011). These studies point out that ORCA3 does not directly influence the overexpression of all TIA pathway genes.

SGD mRNA level was downregulated in *ORCA3* overexpressing hairy roots in this study and not significantly changed in cell cultures (van der Fits and Memelink 2000) and seedlings (Pan et al. 2012) overexpressing *ORCA3*. This suggests *SGD* is not under the control of ORCA3. Feeding loganin, tryptophan, or loganin and tryptophan to *C. roseus* hairy roots while overexpressing *ORCA3* did not help to increase TIA metabolites (data not shown). This is consistent with previously published results (Peebles et al. 2009) and implies that the limitation of the TIA pathway is neither coming from the terpenoid pathway nor the indole pathway. The downstream TIA pathway is highly branched, and tabersonine is an important intermediate metabolite. The significant decrease in tabersonine, the lack of change in total alkaloid concentrations, and the upregulation of other TIA pathway gene transcripts suggest that the downregulation of SGD transcripts is a potential rate-limiting step in the production of downstream alkaloid biosynthesis in ORCA3 overexpressing hairy roots.

The effect of ORCA3 and SGD co-overexpression

SGD from *C. roseus* catalyzes the deglycosylation of strictosidine, an intermediate from which thousands of terpenoid indole alkaloids are derived (O'Connor and Maresh

2006). The above results suggested that SGD may be the rate-limiting step in *ORCA3* overexpressing hairy roots. To address this rate-limiting step, *ORCA3* and *SGD* were co-overexpressed under the control of a glucocorticoid-inducible promoter in *C. roseus* hairy roots. The co-overexpression of *ORCA3* and *SGD* in ORCA3/SGD-7 hairy root line resulted in a significant ($p<0.05$) increase in all measured TIA metabolite concentrations. Additionally, the corynanthe family, aspidosperma family, and total concentration of alkaloids measured showed a significantly ($p>0.05$) increased accumulation. The results indicate that the overexpression of both *ORCA3* and *SGD* alleviates the negative impact of *SGD* downregulation on some of the TIA metabolites in *ORCA3* overexpressing hairy roots.

Previously it was shown that overexpressing two key pathway genes in *C. roseus* hairy roots resulted in slight but significant increases in some TIAs (Peebles et al. 2011). For example, co-overexpressing two key genes *DXS* (1-deoxy-D-xylulose synthase) and *G10H* (geraniol 10-hydroxylase) from the terpenoid pathway overcame the regulation around the DMAPP and IPP branch point through a push-and-pull mechanism to lead the flux toward secologanin and downstream alkaloid synthesis which could not be realized by the individual modification of either *DXS* or *G10H* (Peebles et al. 2011). In addition, the overexpression of *DXS* and a rate-limiting indole pathway gene *AS* simultaneously resulted in an increase in several downstream metabolites by the quick turnover of both terpenoid and indole precursors. It alleviates the negative effect on some of the TIA metabolites from overexpressing *DXS* (Peebles et al. 2011) or *AS* alone (Hughes et al. 2004a). Similarly, in this study, overexpressing *SGD* in combination with *ORCA3* increased TIAs significantly. Thus, combining modifications of pathway genes and regulators showed an efficient way to balance flux through the TIA pathway and increase TIA metabolite production.

Conclusion

The lack knowledge of the TIA pathway in *C. roseus* makes it impractical to pinpoint all the rate-limiting steps of TIA biosynthesis, thus impeding rational design of engineering *C. roseus* to enhance interested alkaloid accumulation or to introduce the pathway into other organisms. The discovery of TIA pathway regulators allows for coordinate control of multiple pathway genes through the overexpression of one regulator. However, each transcription factor regulates shared but distinct set of pathway genes. Engineering single positive transcription factor such as *ORCA3*, *ORCA2*, and *WRKY1* in *C. roseus* did not simply lead to the overall increase in alkaloids. Interestingly, *SGD* was downregulated in *ORCA3* engineered and *ORCA2* engineered *C. roseus* hairy roots. In this study, co-overexpression of *ORCA3* and *SGD* strategy has been used in engineering *C. roseus* hairy roots and successfully results in increases in the total alkaloid concentration, the corynanthe family concentration and aspidosperma family concentration. Combining overexpression of *SGD* and *ORCA3* proved advantageous over single ORCA3 overexpression by driving metabolic flux toward downstream alkaloids.

Altering the expression of TIA pathway genes or regulators can induce the transcription of a combination of transcriptional activators and repressors. The upregulation of both positive and negative regulators was observed in both ORCA3-26 and ORCA3/SGD-7 hairy root lines. Their effects compete with each other serving to fine-tune the amplitude and timing of the pathway gene expression. This complicated and tight regulation is necessary for plants to control gene expression in order to conserve cellular resources and to prevent negative effects associated with high level accumulation of secondary metabolites. The further silencing of negative regulators and optimization of the expression of positive regulators may be necessary to maximize the production. Further, elucidation of pathway genes and regulatory mechanisms is necessary to rationally engineer TIA pathway and increase production dramatically.

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