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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

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ABSTRACT

Jasmonic acid (JA) activates the transcriptional regulator ORCA3, which has a role in regulating the terpenoid indole alkaloid (TIA) pathway within *Catharanthus roseus*. The TIA pathway leads to the production of the anticancer drugs vinblastine and vincristine. This work explores the transient effects of overexpressing ORCA3 under the control of a glucocorticoid-inducible promoter system in *C. roseus* hairy roots along with the simultaneous feeding of JA. The changes in TIA metabolites and in mRNA transcripts of pathway genes and regulators were tracked for 72 h. Upon induction of ORCA3 expression and elicitation with JA, ORCA3 transcripts increased 170-fold whereas ORCA3 expression caused an 89-fold increase and JA elicitation caused a 5-fold increase in ORCA3 transcripts. JA treatment caused the largest increase in TIA metabolites and transcripts of pathway genes. These transcripts displayed a transient response with the maximum expression reached between 12 and 24 h. In the samples overexpressing ORCA3, the largest increase in the transcripts of ZCT1 and ZCT2 (ZCT—zinc finger-binding protein), TIA transcriptional repressors, coincided with the largest increase in ORCA3 transcripts. This counter response of transcriptional repressors may explain why the large increase in ORCA3 transcripts do not correspond with larger increases in transcripts of TIA pathway genes.

1. Introduction

The plant hormone jasmonic acid (JA) has been shown to regulate the terpenoid indole alkaloid (TIA) pathway. This pathway produces compounds that are used by the plant for protection against pathogens and UV radiation (Meijer et al., 1993). Two of these bioactive compounds, vinblastine and vincristine from *Catharanthus roseus* (Fig. 1), have been used extensively as anticancer drugs in the treatment of lymphoma and leukemia (Gidding et al., 1999). Like most secondary metabolites, they are produced in small quantities within the plant and are expensive to harvest and purify.

Attempts to increase TIA production by media optimization (Moreno et al., 1993; Rijhwani and Shanks, 1998), precursor feeding studies (Facchini and DiCosmo, 1991; Kargi and Ganapathi, 1991; Knobloch and Berlin, 1980; Moreno et al., 1993; Morgan and Shanks, 2000; Peebles et al., 2006; Zenk et al., 1977), and genetic manipulations of pathway genes (Canel et al., 1998; Hughes et al., 2004a, b; Whitmer et al., 2002a, b) have brought limited success in both cell suspension and hairy root cultures of *C. roseus*. Likely the TIA pathway is highly regulated, so that changes in precursor concentration or protein concentration are tightly controlled by the plant. One example of this regulation is the feedback inhibition of anthranilate synthase alpha subunit (ASα) by increased levels of tryptophan (Verpoorte et al., 1999). When a tryptophan feedback-insensitive mutant of ASα was expressed in *C. roseus* hairy roots, tryptophan and tryptamine levels increased significantly but little effect was seen in TIA production (Hughes et al., 2004a). Feeding terpenoid precursors to this line while overexpressing ASα did little to increase TIA concentrations, further indicating the possibility of tight control of the TIA pathway (Peebles et al., 2006).

In recent years a number of likely regulators of the TIA pathway have been discovered. ORCA2 and ORCA3 are jasmonate-responsive AP2-domain transcription factors that promote transcription of TIA genes. In *C. roseus* cell suspension cultures, the constitutive overexpression of ORCA3 led to an increase in ASα, tryptophan decarboxylase (TDC), 1-deoxy-D-xylulose synthase

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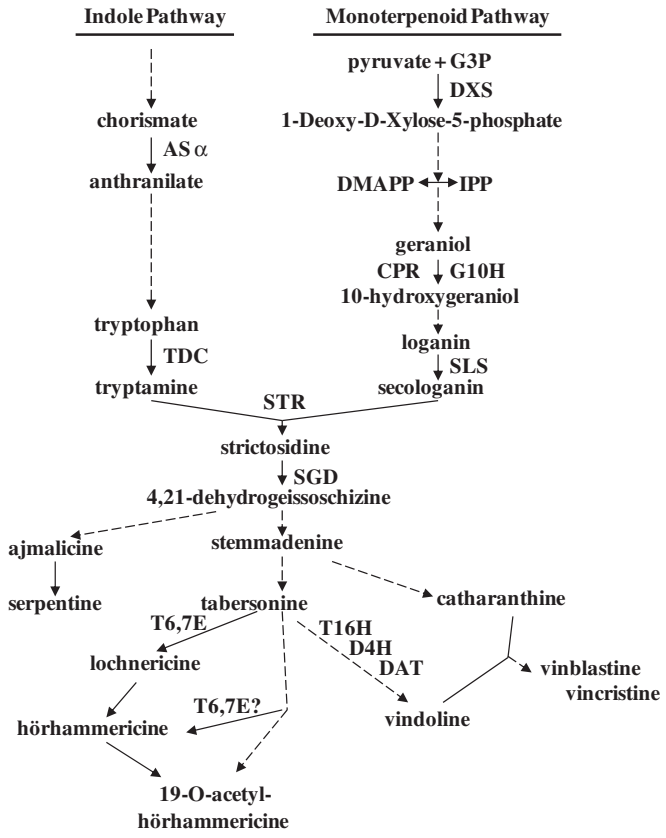

Fig. 1. Terpenoid indole alkaloid pathway. DXS, 1-deoxy-D-xylulose 5-phosphate synthase; G10H, geraniol 10-hydroxylase; CPR, cytochrome P450 reductase; SLS, secologanin synthase; Asα anthranilate synthase α subunit; TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGD, strictosidine β-D-glucosidase; T16H, tabersonine 16-hydroxylase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline acetyltransferase; T6,7E, tabersonine 6,7-epoxidase; T6,7E?, proposed activity (Rodriguez et al., 2003).

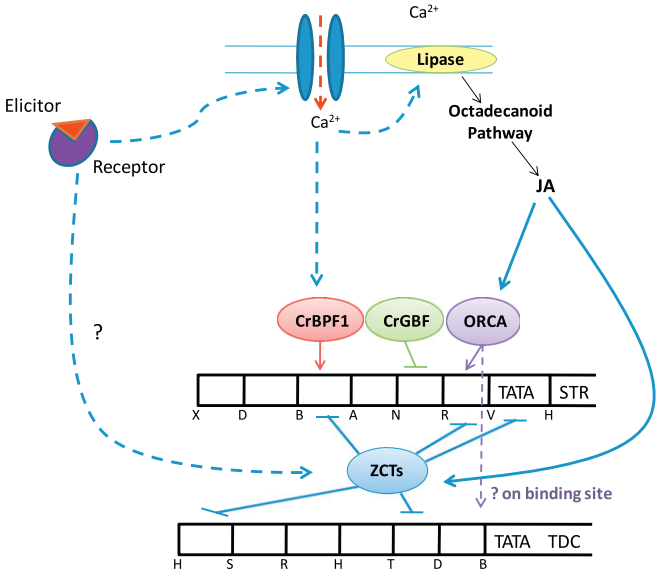

Fig. 2. Regulation of the STR and TDC promoters in *Catharanthus roseus*. The binding of the elicitor activates an influx of Ca^{2+} into the cell. The increase in Ca^{2+} concentration activates the octadecanoid pathway leading to the production of jasmonic acid (JA). Jasmonic acid activates ORCA transcription factors which in turn drives the expression of STR and TDC (Menke et al., 1999a, b; van der Fits and Memelink, 2000, 2001). Jasmonic acid also causes an increase in ZCT transcription factors which inhibit STR and TDC expression. The ZCTs are also increased by an unknown signaling cascade from the receptor (Pauw et al., 2004). The Ca^{2+} influx also causes the increase of the box P-binding factor (CrBPF1) (van der Fits et al., 2000). The G-box-binding factor (CrGBF) has been shown to inhibit STR expression (Siberil et al., 2001).

(DXS), cytochrome P450 reductase (CPR), strictosidine synthase (STR), strictosidine beta-glucosidase (SGD) and desacetoxyvindoline 4-hydroxylase (D4H) mRNA transcripts (Fig. 1). Geraniol 10-hydroxylase (G10H) and deacetylvindoline acetyltransferase (DAT) mRNA transcripts were not affected by ORCA3 overexpression (van der Fits and Memelink, 2000). The zinc finger-binding proteins ZCT1, ZCT2, and ZCT3 (member of the transcription factor IIIA-type zinc finger family) were found to bind to the promoters of STR and TDC (Fig. 2). This interaction repressed the activity of STR and TDC. The binding of the ZCTs to the STR promoter has been suggested to counteract the activation of STR by ORCA2 or ORCA3 (Pauw et al., 2004). Sequence analysis of the STR and TDC promoters show that they contain a G-box or G-box-like binding site. Two G-box-binding factors, Crgbf1 and Crgbf2, were subsequently identified in *C. roseus* and shown to repress the transcription of STR by binding to the G-box sequence (Siberil et al., 2001). Recently promoter analysis of G10H led to the identification of 3 unique regions that could act as potential enhancers to G10H transcript abundance. These regions differ from the elements found in the promoters of STR and TDC. These unique regions point to the possibility of another signaling cascade that regulates the activity of G10H in response to JA, thus complicating the regulatory landscape of TIA synthesis (Suttipanta et al., 2007).

The current work explores the effect of ORCA3 overexpression in the differentiated tissue of *C. roseus* hairy roots along with JA feeding. A glucocorticoid-inducible promoter system (Hughes et al., 2002) was used to control timing of the expression of ORCA3. The production of TIAs and the changes in mRNA expression of several TIA genes and regulators were tracked over time. This study showed that while ORCA3 overexpression is inducible, it did not significantly increase the accumulation of TIA metabolites within hairy roots. In response to ORCA3 overexpression, the mRNA transcripts of ASα, DXS, STR, secologanin synthase (SLS), and ZCTs increased; TDC, G10H, CPR, GBFs, and ORCA2 remained constant; and SGD decreased. The increase of ASα, DXS, and STR transcripts and the consistency of G10H transcripts are in agreement with the results observed in *C. roseus* cell cultures overexpressing ORCA3 (van der Fits and Memelink, 2000). The decrease in SGD transcripts and the constant level of TDC and CPR transcripts differ from the results observed in *C. roseus* cell cultures where all 3 transcripts were observed to increase (van der Fits and Memelink, 2000). The changes in transcript levels of SLS, ZCTs, GBFs, and ORCA2 were not reported for *C. roseus* cell culture. The differences in mRNA accumulation between hairy roots and cell cultures may indicate the existence of different control mechanisms of the TIA pathway between culture types. In contrast to ORCA3 overexpression, JA feeding alone increased both TIA metabolites and transcripts. These results support the complexity of the regulation of the TIA pathway and the need to discover the unknown regulatory elements.

2. Materials and methods

2.1. Clone generation

ORCA3 was amplified from genomic DNA through standard PCR technique, ligated into pCR2.1-TOPO (Invitrogen), and sequenced for verification. ORCA3 was moved from pCR2.1-TOPO into pBluescript as an EcoRV/SpeI fragment. It was then removed

as an XhoI/SpeI fragment and ligated into the glucocorticoid-inducible plasmid pTA7002 to form pTA7002/ORCA3. This plasmid was electroporated into *Agrobacterium rhizogenes* ATCC 15834 and selected on YEM media (1 g/L Yeast Extract, 0.2 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 4 mg/L $FeCl_3$, 0.66 g/L $K_2HPO_4 \cdot 3H_2O$, 10 g/L mannitol, 15 g/L agar) containing 50 mg/L kanamycin. The presence of pTA7002/ORCA3 was confirmed by restriction digest. A single colony was grown in a 5 mL culture at 28 °C and 200 rpm for 36 h. Scissors were dipped in the bacteria and used to infect aseptically grown plants as previously described (Bhadra et al., 1993). After 6 weeks, hairy roots were excised from the plants and selected on solid media as described below except that 30 mg/L hygromycin was added to the first solid subculture to select for clones carrying the transgenes of interest. Roots were then adapted to liquid culture and recultured every 5 weeks.

2.2. Culture conditions

Hairy roots were grown in a filter-sterilized solution of 30 g/L sucrose (Sigma), half-strength Gamborg's B5 salts (Sigma), and full-strength Gamborg's vitamins (Sigma) adjusted to a pH of 5.7. Cultures were initiated by placing five tips into a 250 mL Erlenmeyer flask with 50 mL of media. The cultures were grown at 26 °C at 100 rpm in the dark. Solid media plates were made from 30 g/L sucrose, half-strength Gamborg's B5 salts, full-strength Gamborg's vitamins (pH 5.7), and 6 g/L agar (Sigma).

2.3. Northern analysis

Hairy root flask cultures that were approximately 4 weeks old were used for transcriptional induction studies. Hairy roots were induced with 3 μM dexamethasone (Sigma) or with an equal volume of ethanol for the uninduced controls. Isolation of total RNA from hairy roots was performed using Trizol (Invitrogen) according to manufacturer's instructions. RNA was quantified by spectrophotometer according to absorbance at 260 nm and an ethidium bromide-stained test gel used to verify the quality of RNA. Twelve micrograms of total RNA were loaded per well. Northern analysis was performed according to a reference protocol (Brown and Mackey, 1997). All genes to be tested had been cloned into plasmids and the relevant section was removed by restriction digest and gel-purified with a Qiagen QIAEX II kit. Probes were made using Stratagene Prime-It II kit.

2.4. Induction and elicitation study

To study the inducible expression of ORCA3, EHIORCA3-4-1 hairy roots were treated with 3 μM dexamethasone (induced cultures) or with an equal volume ethanol (uninduced cultures) as a negative control. In addition to being induced or uninduced, the hairy roots were elicited with 50 mg/L JA or an equal amount of ethanol. Both treatments happened on day 35 after subculturing which corresponded to the exponential growth phase. Three flasks from each condition (uninduced, induced, uninduced fed with JA, and induced fed with JA) were harvested at 6, 12, 24, 48, and 72 h after treatment. In addition, three flasks were harvested at the time of treatment for a 0 h control. Three hundred milligram of fresh weight tissue was ground in liquid nitrogen and stored at −80 °C for further mRNA analysis. The remaining tissue was harvested for alkaloid analysis.

2.5. Alkaloid extraction and HPLC detection

The fresh weight of the harvested hairy root cultures were measured after cultures were blotted to remove excess media. The cultures were then frozen at −80 °C. The dry weight was recorded after lyophilization. Approximately 50 mg dry weight of the ground tissue was extracted in 25 mL of methanol by ultrasonication for 10 min (Model W-225, Heat Systems. Ultrasonics Inc., USA). The extracts were clarified by centrifugation at 1300*g* for 15 min at 4 °C. The supernatants were concentrated to 2 mL using a vortex evaporator, and passed through a 0.22 μm nylon filter (13 mm). Ten microliters of each samples was analyzed for the following terpenoid indole alkaloids: ajmalicine (Sigma), serpentine (Sigma), catharanthine (Qventas), hörhammericine (verified by Morgan and Shanks, 1999)), lochnericine (verified by Morgan and Shanks, 1999), and tabersonine (a gift from Dr. Hamada, Okayama University, Japan) on the HPLC as previously described (Peebles et al., 2005).

2.6. cDNA synthesis and Q-PCR amplification

Total RNA was isolated from the hairy root samples stored at −80 °C using Trizol (Invitrogen) according to the manufacturer's instructions. To ensure that all DNA was removed from the samples, the samples were treated with DNase I (Sigma) according to the manufacturer's instructions. RNA was quantified by spectrophotometer at an absorbance of 260 nm.

Reverse transcription was carried out using the Promega Reverse Transcription System in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA). One microgram of RNA was incubated at 70 °C for 10 min and placed on ice. The remaining reaction mixture (60 μL total volume) was added to each tube and incubated at room temperature for 10 min, followed by a 30 min incubation at 50 °C, a 5 min incubation at 95 °C, and a 5 min incubation at 6 °C. A no amplification control (without reverse transcriptase) was performed for each sample. The first strand cDNA was diluted to a final volume of 300 μL with nuclease-free water.

The Q-PCR amplification was carried out in a 96 well plate ABI Prism 7000 sequence detection system (Applied Biosystems). Each reaction contained a mixture of 3 μL diluted cDNA (final volume equivalent to 5 ng), 2 μL mixed primers (1.25 pmol/μL), 10 μL Power SYBR Green PCR Master Mix (Applied Biosystems), and 5 μL nuclease-free water. Table 1 contains a list of primers used. The reaction mixture was incubated for 2 min at 50 °C, 10 min at 95 °C, and for 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The relative gene expression was quantified by using the comparative C_T (threshold cycle) method as previously described (Shalel-Levanon et al., 2005). The 40S ribosomal protein S9 (rsp9) was used for the control gene (Menke et al., 1999b).

2.7. Jasmonic acid detection

To determine JA presence in the media at 72 h, 5 μL of the media samples was diluted in 995 μL of 85:15 mixture of 0.05% HOAc in H_2O:MeCN and filtered through a 0.22 μm nylon filter (13 mm). Three replicates of each sample were made. Ten microliters of the diluted media was analyzed by LCMS by injecting the sample onto a Phenomenex Luna 5A C18(2) HPLC column (250 mm × 4.6 mm) interfaced to a Shimadzu LCMS2010A system. The gradient system initially consisted of 85% solvent A (0.05% HOAc in H2O) and 15% solvent B (MeCN) at a flowrate of 0.2 mL/min (Segarra et al., 2006). This was held constant for the first 9 min, then ramped over a period of 6 min to 100% solvent B and held there for 3 min. The column was then re-equilibrated. The interface for the MS was the ESI mode with negative polarity. The CDL and heat block temperatures were 250 °C and 200 °C, respectively. The nebulizing gas flow rate was 1.5 L/min. JA was detected at an *m*/*z* ratio of 209.

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

Gene	Enzyme or function (accession no.)	Primer pairs	PCR product (bp)
ASα	Anthranilate synthase alpha subunit (AF441857)	5′-GCGAACATTTGCAGATCCAT-3′ 5′-GGCCGATTTGTTATTGTTCC-3′	156
TDC	Tryptophan decarboxylase (X67662)	5′-ATCCGATCAAACCCATACCA-3′ 5′-CGTCATCCTCGACCATTTTT-3′	143
DXS	1-Deoxyxylulose 5-phosphate synthase (AJ011840)	5′-TCGCTGCAGAACTTAGAGCA-3′ 5′-GCCAACATCCCAAATGATTC-3′	146
G10H	Geraniol 10-hydroxylase (AJ251269)	5′-TTATTCGGATTCTGCCAAGG-3′ 5′-TCCCCAAAGTGAATCGTCAT-3′	147
CPR	NADPH–ferrihemoprotein reductase (X69791)	5′-TGGCAGAAAAGGCTTCTGAT-3′ 5′-CTCAGCCTGTGTGCTATCCA-3′	152
SLS	Secologanin synthase (L10081)	5′-GTTCCTTCTCACCGGAGTTG-3′ 5′-CCCATTTGGTCAACATGTCA-3′	146
STR	Strictosidine synthase (X61932)	5′-ACCATTGTGTGGGAGGACAT-3′ 5′-ATTTGAATGGCACTCCTTGC-3′	152
SGD	Strictosidine beta-glucosidase (AF112888)	5′-GGAGGCTTCTTGAGTGATCG-3′ 5′-GCAAATTCACCAGTGGCATA-3′	149
ORCA2	AP2-domain DNA-binding protein (AJ238740)	5′-GAAGATGCGGCATTAGCTTT-3′ 5′-TTGAGGACGAAGATGACACG-3′	150
ORCA3	AP2-domain DNA-binding protein (AJ251249)	5′-CGGGATCCGAAATACAGAAA-3′ 5′-GCCCTTATACCGGTTCCAAT-3′	155
ZCT1	Zinc finger DNA-binding protein (AJ632082)	5′-AGCCGAAAACTCATGCTTGT-3′ 5′-CGCCTTTGCAACAGGTTTAT-3′	143
ZCT2	Zinc finger DNA-binding protein (AJ632083)	5′-CGTCAATTTCCATCGTTTCA-3′ 5′-CCGATAGCGAATTCAAGTCC-3′	155
ZCT3	Zinc finger DNA-binding protein (AJ632084)	5′-GACAAGCTTTGGGAGGACAC-3′ 5′-GGCAAGGCAGGTAAGTTCAA-3′	160
GBF1	G-box-binding protein (AF084971)	5′-AACAGGCTGAGACGGAAGAA-3′ 5′-GACCCGTGCATTTTTCAACT-3′	152
GBF2	G-box-binding protein (AF084972)	5′-GGAAGGTGCCATCTACTCCA-3′ 5′-CTAGATCGCCGAGCAGATTC-3′	142
rsp9	40S ribosomal protein S9 (AJ749993)	5′-GAGGGCCAAAACAAACTTGA-3′ 5′-CCCTTATGTGCCTTTGCCTA-3′	145

2.8. Statistical analysis

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

3. Results

3.1. Creation of inducible expression ORCA3 hairy root lines

A. rhizogenes ATCC 15834 caring the pTA7002/ORCA3 plasmid was used to infect aseptically grown *C. roseus* seedlings. The hairy roots that formed were excised and grown on solid media containing hygromycin to confirm the presence of the gene insert. Once the roots grew on solid media, they were adapted to liquid media and sub-cultured approximately every 5 weeks. The resulting hairy root lines were screened for activity. A line with low background and high inducible expression of ORCA3 called EHIORCA3-4-1 was chosen for further study. A northern blot (Fig. 3) was performed to confirm the inducible expression of ORCA3 mRNA.

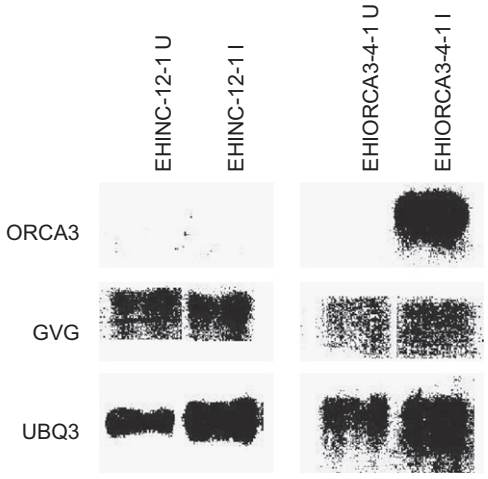

Fig. 3. Northern analysis of lines EHINC-12-1 and EHIORCA3-4-1. EHINC-12-1 is a negative control line containing the element of the glucocorticoid-inducible promoter but no gene (Hughes et al., 2004a). EHIORCA3-4-1 contains ORCA3 under the control of the glucocorticoid-inducible promoter. Lines were induced (I) at 3 μM for 72 h and RNA prepared with ethanol similarly added to uninduced (U) controls. Twelve microgram RNA was loaded and blotting performed. Probes were made for ORCA3, the glucocorticoid transcription factor (GVG), and ubiquitin 3 (UBQ3).

3.2. Alkaloid concentrations in the roots

The metabolic effects of ORCA3 overexpression and/or JA elicitation on TIA production were evaluated by measuring the concentration of six different alkaloids shown in Fig. 4. The alkaloids analyzed include ajmalicine and serpentine from the Corynanthe family, catharanthine from the Iboga family, and tabersonine, hörhammericine, and lochnericine from the Aspidosperma family. Vinblastine and vincristine have not been observed in *C. roseus* hairy roots because of an absence of vindoline (Shanks et al., 1998). The alkaloids were evaluated at 4 different conditions at 0, 6, 12, 24, 48, and 72 h after the cultures were fed. The four conditions (UI, I, JA, and I JA) were: uninduced hairy roots which served as the control (UI); induced hairy roots where ORCA3 was overexpressed (I); JA-elicited hairy roots (JA);

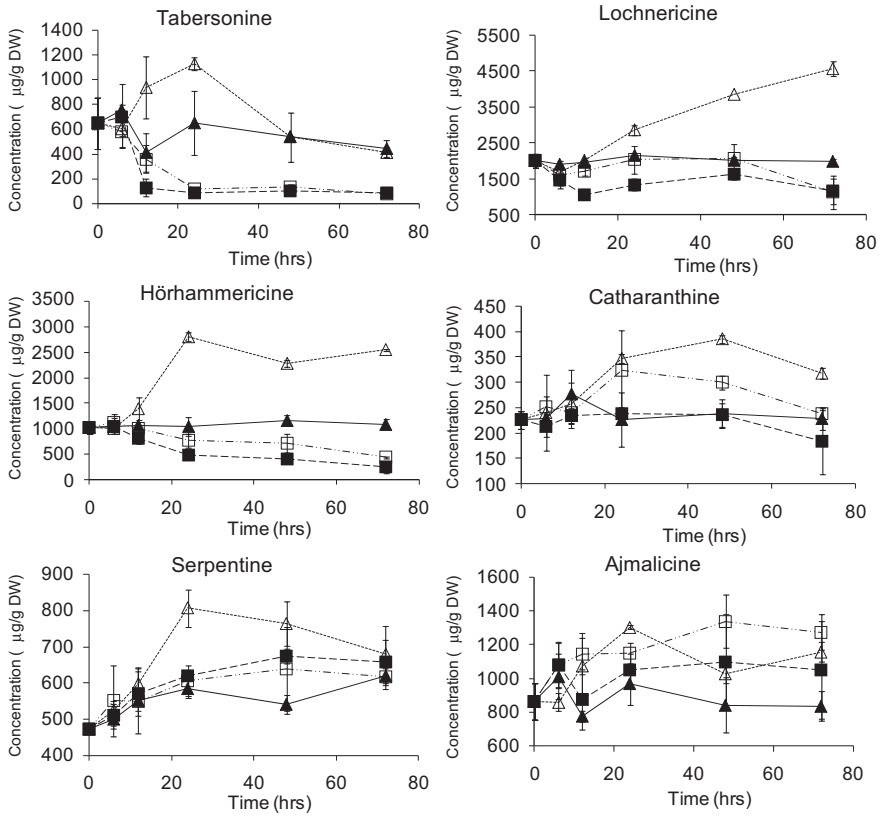

Fig. 4. TIA metabolites. Elicitation with jasmonic acid (△) caused the greatest increase in tabersonine, lochnericine, hörhammericine, catharanthine, and serpentine concentrations compared to the uninduced (▲) levels. In contrast induction of ORCA3 overexpression (■) or induction plus elicitation (□) caused a decrease in tabersonine, lochnericine, and hörhammericine. Error bars represent the standard deviation of triplicate shakeflask cultures.

and hairy roots that were both induced to overexpress ORCA3 and elicited with jasmonic acid (I JA).

In general, the JA cultures showed the greatest increase in the metabolites measured (Fig. 4). An initial increase in all six alkaloids was observed in the JA cultures. After 24 h it was observed that tabersonine concentrations decrease, lochnericine concentration continue to increase, and hörhammericine concentrations remain constant. An increase in the activity of tabersonine 6,7 epoxidase (T6,7E) maybe responsible for the conversion of tabersonine to lochnericine (Fig. 1). JA feeding has previously been shown to increase the activity of T67E in *C. roseus* hairy roots (Rodriguez et al., 2003). The I cultures overexpressing ORCA3 and the I JA cultures show a significant ($p<0.05$) decrease in tabersonine, hörhammericine, and lochnericine. Few other significant changes in the other alkaloids measured are observed for the I and I JA cultures.

For *C. roseus* cell suspension cultures overexpressing ORCA3, an increase in TIAs was not observed. This was due to the fact that G10H was not up-regulated under ORCA3 overexpression (van der Fits and Memelink, 2000). Only when loganin was fed to cells overexpressing ORCA3 was an increase in TIAs observed (van der Fits and Memelink, 2000). In hairy roots, neither TDC nor G10H are up-regulated when ORCA3 is overexpressed (Figs. 6 and 7). When loganin, tryptophan, or loganin and tryptophan were fed to *C. roseus* hairy roots overexpressing ORCA3, no significant increases in TIA metabolites were observed (Appendix Table A1). These results are consistent with the decrease in SGD mRNA transcripts that was observed in the cultures overexpression ORCA3, I (Fig. 8). The TIA metabolites measured are downstream of the SGD enzyme so a decrease in SGD transcripts could limit the flux through the pathway causing the feeding of upstream metabolites to be ineffective.

3.3. Transcript analysis

A variety of gene transcripts were analyzed by Q RT PCR from the TIA pathway and its regulators under the four conditions UI, I, JA, and I JA in the hairy root line EHIORCA3-4-1. The following changes in transcript levels were tracked over a 72 h period for the following genes: the transcriptional regulators ORCA2 and ORCA3; the zinc finger-binding proteins ZCT1, ZCT2, and ZCT3; the G-box-binding proteins GBF1 and GBF2; ASα and TDC from the indole pathway; DXS, G10H, CPR, and SLS from the monoterpenoid pathway; STR and SGD from the TIA pathway; and 40S ribosomal protein S9 (rsp9) to serve as the control gene.

In the following results, the relative expression levels of each gene are compared between the four experimental conditions and normalized to the cultures harvested at the start of the experiment (0 h). This means that for each gene the relative level at 0 h is always 1. It is important to note that the relative expression levels between genes cannot be compared since absolute concentrations of mRNA was not determined. The results represent the average of 4 samples that were prepared using the same mRNA. The mRNA content from 1 sample out of the triplicate for each condition and time point was examined. After examining the alkaloid results for each triplicate, the sample where most of the TIA compounds detected fell in the middle of the range for that time point and condition were chosen. This should give a representative sampling of mRNA content at each time point and condition. Even though errors in mRNA isolation were not determined, when comparing the levels of rsp9

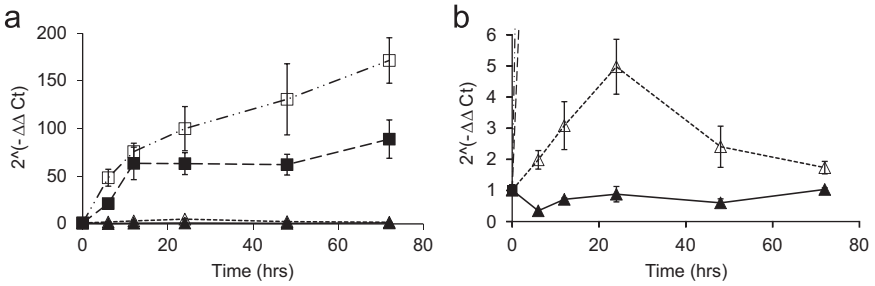

Fig. 5. ORCA3 mRNA transcripts over time. (a) Compared to the uninduced control (▲), induction of the hairy root cultures with dexamethasone (■) showed an 89-fold increase in ORCA3 mRNA transcripts by 72 h after treatment while induction with dexamethasone and elicitation with jasmonic acid (□) showed a 170-fold increase in ORCA3 mRNA transcripts during the same time period. (b) Elicitation with jasmonic acid (△) showed a transient response where ORCA3 transcripts reached a 5-fold increase by 24 h before returning to the uninduced levels by 72 h. The data was normalized to the 0 h time point using the comparative threshold cycle method. Error bars represent the standard deviation of triplicate runs for Q PCR.

transcripts, which should remain constant from sample to sample, errors in isolation should be lower than 6%.

3.4. ORCA3 expression levels

The time profile of the ORCA3 expression level for the UI, I, JA, and I JA culture conditions are shown in Fig. 5. The expression level was calculated relative to the expression level at the start of the experiment (time 0 h) according to the Materials and methods (Section 2.6). These results suggest that upon the addition of the inducer dexamethasone (I) ORCA3 transcript levels exhibit a 60 plus fold increase. The addition of JA along with dexamethasone (I JA) gives an even larger increase in ORCA3 transcripts. These results for I JA provide support for the model proposed by Pauw and Memelink (2005) where JA activates ORCA3 and another transcription factor (identity unknown) through posttranslational modifications. The active transcription factor (identity unknown) binds to the promoter region of ORCA3 driving transcription and increasing ORCA3 transcripts. The active ORCA3 then activates the expression of TIA biosynthetic genes. In contrast, elicitation with JA causes a 5-fold increase in ORCA3 by 24 h before declining to the uninduced (UI) levels by 72 h. At least 30% of the initial amount of JA fed was detected in the media after 72 h in both JA- and I JA-treated cultures (data not shown).

3.5. Effects on the indole pathway

The expression levels of the indole genes anthranilate synthase (ASα) and TDC for the UI, I, JA, and I JA culture conditions are shown in Fig. 6. Elicitation by JA showed the largest increase in the mRNA levels for both ASα and TDC. The initial response to JA is rapid with over 50% of the maximum transcript levels occurring by 6 h. The response to JA is also transient with a maximum reached around 24 h followed by a return to the UI levels. The I cultures display an increase in ASα but not TDC transcripts. This response for TDC differs from the results observed for *C. roseus* cell suspension cultures where an increase in ORCA3 resulted in an increase in TDC transcripts (van der Fits et al., 2000).

3.6. Effects on the terpenoid pathway

The expression levels of the terpenoid genes DXS, G10H, and SLS for the UI, I, JA, and I JA culture conditions are shown in Fig. 7. Elicitation by JA for DXS and G10H transcripts displayed a similar trend to ASα and TDC transcripts. The JA cultures showed the largest increase in DXS and G10H transcripts with the maximum response occurring between 6 and 12 h followed by a decline to UI levels by 72 h. The I and I JA cultures showed a smaller increase in

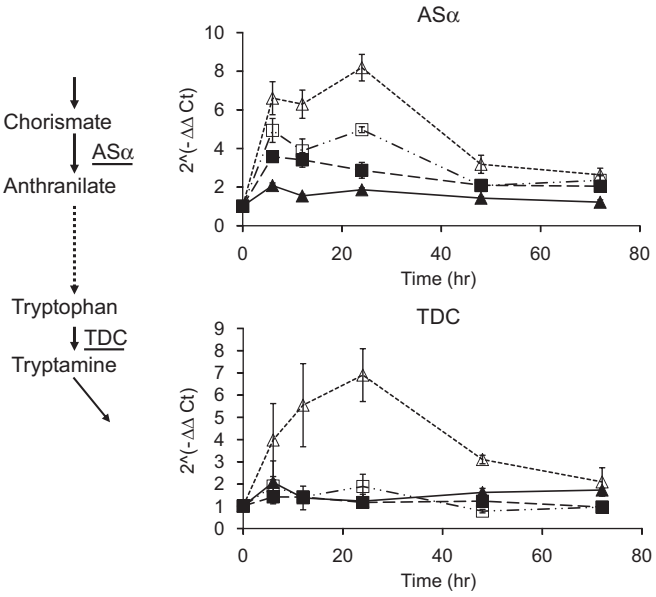

Fig. 6. Indole pathway transcripts. Elicitation by jasmonic acid (Δ) causes the greatest increase in ASα and TDC transcripts with a maximum occurring by 24 h after elicitation and transcript levels returning to the uninduced (▲) levels by 72 h. The induced cultures overexpressing ORCA3 (■) and the cultures that were induced and elicited (□) showed a slight increase in ASα transcripts but no increase in TDC transcripts. The data was normalized to the 0 h time point using the comparative threshold cycle method. Error bars represent the standard deviation of triplicate runs for Q PCR.

transcript levels than the JA cultures. The I JA cultures showed a slight increase in G10H transcripts while I cultures show no change in G10H transcripts. In regard to SLS transcripts, the JA and I JA cultures demonstrated a similar increase in transcripts by 24 h followed by a decrease to the UI levels. The transcripts for CPR do not vary by more than ~2 fold for all 4 conditions (Appendix Fig. A1). The results for CPR when ORCA3 was overexpressed differ from the results in *C. roseus* cell suspension cultures where an increase in ORCA3 expression caused an increase in CPR transcripts (van der Fits et al., 2000).

3.7. Effects on the TIA pathway

The expression levels of the TIA genes STR and SGD for the UI, I, JA, and I JA culture conditions are shown in Fig. 8. All three conditions, I, JA, and I JA, show an increase in STR transcripts compared to the UI levels. The JA cultures show a transient increase in SGD transcripts with the maximum occurring at 24 h

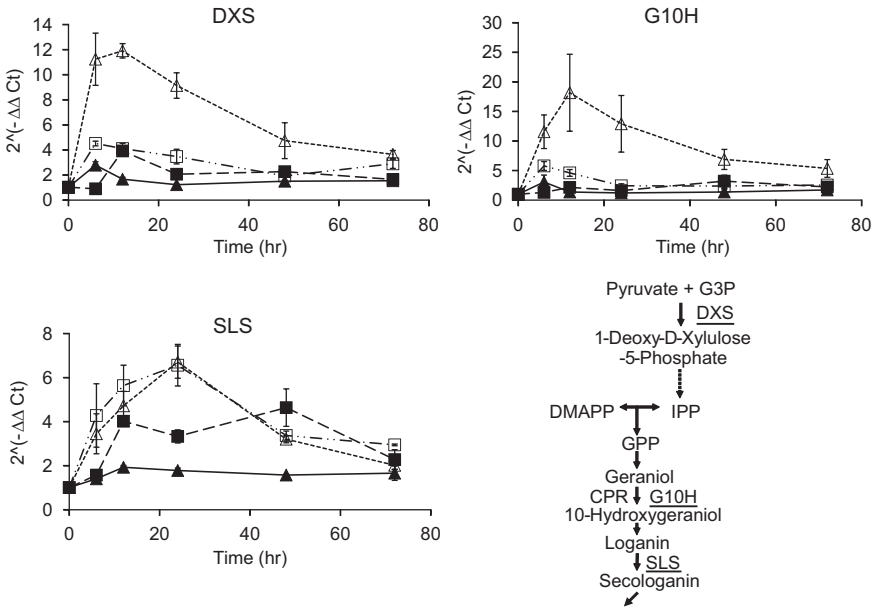

Fig. 7. Terpenoid pathway transcripts. Elicitation by jasmonic acid (△) causes the greatest increase in DXS and G10H transcripts with a maximum occurring by 6–12 h after elicitation and transcript levels returning to the uninduced (▲) levels by 72 h. The induced cultures overexpressing ORCA3 (■) and the cultures that were induced and elicited (□) showed a slight increase in DXS and G10H transcripts. Both elicitation and elicitation with induction showed similar increase in SLS transcripts with the max occurring 24 h after feeding. The induced cultures also showed an increase in SLS transcripts over the 72 h time period. The data was normalized to the 0 h time point using the comparative threshold cycle method. Error bars represent the standard deviation of triplicate runs for Q PCR.

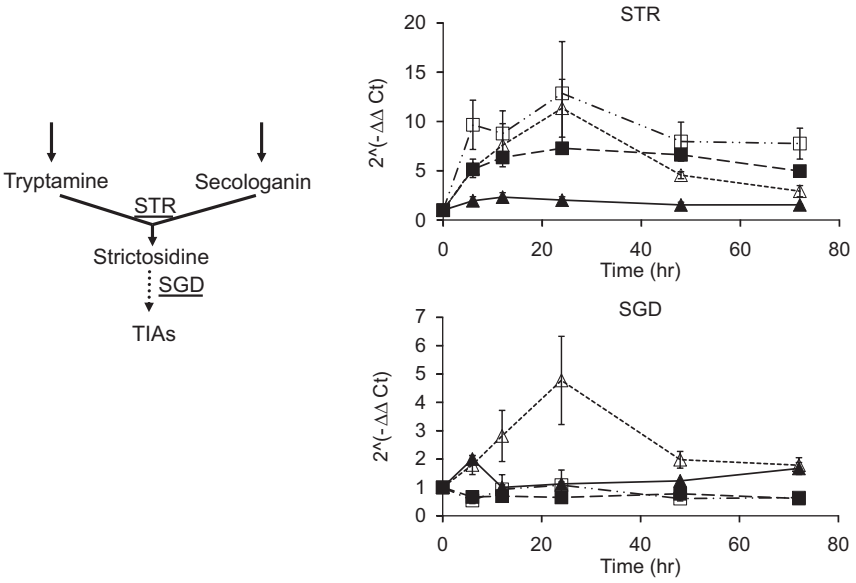

Fig. 8. Terpenoid indole alkaloid pathway transcripts. Elicitation by jasmonic acid (△), induction of ORCA3 overexpression (■), and elicitation and induction (□) caused an increase in STR transcripts compared to the uninduced (▲) levels. Elicitation also caused an increase in SGD transcripts reaching a max by 24 h before returning to the uninduced levels. The data was normalized to the 0 h time point using the comparative threshold cycle method. Error bars represent the standard deviation of triplicate runs for Q PCR.

followed by a decrease to the uninduced levels by 72 h. For the I and I JA cultures, the data suggests that SGD transcripts decrease when compared to the UI levels at 72 h.

3.8. TIA regulator genes

The expression levels of the TIA the transcriptional regulator ORCA2 and the zinc finger-binding proteins ZCT1, ZCT2, and ZCT3 for the UI, I, JA, and I JA culture conditions are shown in Figs. 9 and 10. Corresponding to the largest increase in ORCA3 transcripts (Fig. 5), the I JA cultures exhibit the largest increase in the ZCT transcripts. The next highest increase in ZCT transcripts occur for the I cultures corresponding to the next highest level of ORCA3 transcripts.

The cultures elicited with JA demonstrate a transient increase in ORCA2 transcripts similar to the effect JA had on ORCA3 transcripts (Fig. 5). The I and I JA cultures did not show an increase in ORCA2 transcripts. No significant changes in the G-box-binding proteins GBF1 and GBF2 were observed (Appendix Fig. A2).

4. Discussion

An inducible *C. roseus* hairy root line was created that exhibited an increase in ORCA3 transcripts with the feeding of the inducer

dexamethasone. In response to ORCA3 overexpression, this line exhibited a similar pattern of mRNA expression as *C. roseus* cell suspension with an increase in ASα, DXS, and STR (Figs. 6–8) and no change in the G10H transcripts (Fig. 7) (van der Fits and Memelink, 2000). These results are summarized in Table 2. Contrary to the data shown for the overexpression of ORCA3 in cell culture, no change in TDC and CPR transcripts and a decrease in SGD transcripts were observed (Figs. 6–8). These results point to the potential of different regulatory mechanisms of TIA biosynthesis between cell suspension cultures and differentiated tissues such as hairy roots. Currently the tissue-specific control mechanisms of the TIA pathway are not well understood.

TIA transcript levels are correlated to the metabolite concentrations. JA caused the largest increase in TIA transcripts and metabolites. This increase was a transient response that reached its maximum by 24 h for the mRNA transcripts and is echoed in the metabolite concentrations. Serpentine, catharanthine, and hörhammericine reach their maximum concentration by 24 h followed by an eventual return to the control level or a leveling off in concentration (Fig. 4). Tabersonine reaches its maximum concentration by 24 h and maybe converted into lochnericine after this period (Fig. 4). The I and I JA cultures do not show an increase in TDC and G10H transcripts, 2 important rate-limiting enzymes, and do not show significant increase in metabolites. In fact the observed decrease in SGD transcripts may explain why tabersonine, lochnericine, and hörhammericine concentrations decrease (Fig. 4).

In *C. roseus* hairy roots the flux is potentially pushed to lochnericine from tabersonine in response to JA treatment (Fig. 4). The activity of T6,7E was increased in JA-treated *C. roseus* hairy roots (Rodriguez et al., 2003). This enzyme was responsible for the conversion of tabersonine to lochnericine. These results differ from another study on *C. roseus* hairy roots where JA elicitation caused an initial spike in concentration of both tabersonine and lochnericine but concentrations fell to below the control by 72 h while hörhammericine level continued to increase over time (Rijhwani and Shanks, 1998). Clonal variation between hairy root lines may explain these conflicting results.

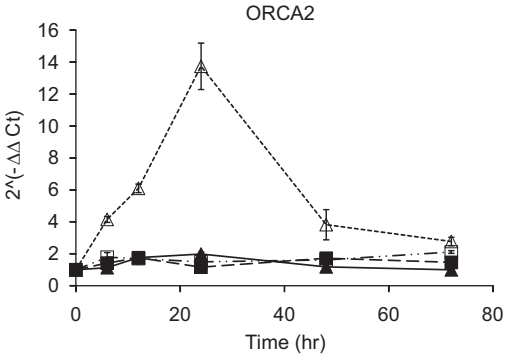

Fig. 9. ORCA2 transcripts. Elicitation by jasmonic acid (△) caused a 14-fold increase in transcripts when compared to the uninduced (▲) levels. There were no observed changes in ORCA2 transcripts in cultures induced of ORCA3 overexpression (■) or induced and elicited (□). The data was normalized to the 0 h time point using the comparative threshold cycle method. Error bars represent the standard deviation of triplicate runs for Q PCR.

Table 2
Comparison of the effect of ORCA3 overexpression on TIA genes and regulators in *Catharanthus roseus* cell suspension culture and hairy root culture.

Genes	Cell culture[a]	Hairy roots
ASα	+	+
TDC	+	No change
DXS	+	+
CPR	+	No change
G10H	No change	No change
SLS	Not reported	+
STR	+	+
SGD	+	–
ZCTs	Not reported	+
GBFs	Not reported	No change
ORCA2	Not reported	No change

[a] Data taken from van der Fits et al. (2000) (*Science* 289:295–297).

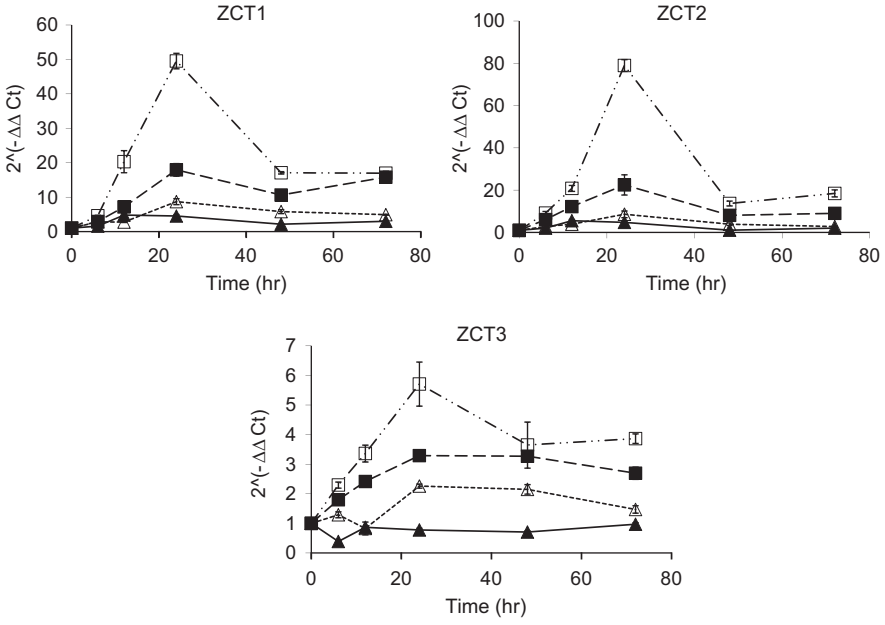

Fig. 10. Zinc-binding finger protein transcripts. Cultures that were elicited with jasmonic acid and induced to cause ORCA3 overexpression (□) showed the greatest increase in ZCT transcripts compared to the uninduced (▲) levels. Cultures that were induced (■) showed the next highest increase in ZCT transcripts followed by the elicitated cultures (△). The data was normalized to the 0 h time point using the comparative threshold cycle method. Error bars represent the standard deviation of triplicate runs for Q PCR.

The decrease in tabersonine, lochnericine, and hörhammericine for I and I JA cultures is possibly explained by the decrease in SGD transcripts suggesting that the flux toward tabersonine is being down regulated. In addition, these metabolite pools may be decreased by product degradation or conversion to other metabolites. Evidence for the conversion and/or degradation of these compounds was seen in an inhibitor study performed on the enzymes in this part of the pathway (Morgan and Shanks, 1999).

The large increase in ORCA3 transcripts in the ORCA3 overexpressing cultures may activate negative feedback regulation that can compensate for the increased supply of ORCA3 transcripts and compete for binding sites on the TIA promoters. One class of negative regulators is the ZCTs which have been shown to repress the expression of TDC and STR by binding to their promoter regions (Pauw et al., 2004). A large increase in the mRNA transcripts of ZCT1, ZCT2, and to a lesser extent ZCT3 is shown in Fig. 10. These increases paralleled the increase in ORCA3 transcripts with the largest increase in ORCA3 transcripts corresponding to the largest increase in ZCTs for the I JA cultures with the I cultures exhibiting a lesser amount of accumulation for both as shown if Fig. 11.

Considering the differences in transcript levels of the I JA cultures compared to the I cultures and the JA cultures can give us deeper insight into the TIA regulatory network (Figs. 6–11). For TDC, DXS, G10H, SGD, and ORCA2 transcripts, the I JA cultures show a similar response as the I cultures. In general, these transcripts are present in lower concentrations in the I and I JA cultures compared to the JA cultures. This suggests that a negative regulatory scheme is overriding the positive up-regulation that normally occurs with JA elicitation for these genes. The increase in ORCA3 transcripts in the I JA cultures causes a rise in the transcripts of the ZCTs and potentially other negative regulators that can counteract the positive regulators stimulated by JA. The concentration of ASα transcripts in the I JA cultures lie in between the transcript levels for the I and the JA cultures. This may indicate that ASα is partly regulated by the TIA regulatory network and partly by another regulatory network that responds positively to JA. ASα is involved in tryptophan biosynthesis, which is converted into numerous other metabolites besides tryptamine. The biosynthesis of any one of these metabolites could be controlled by another regulatory network that acts on ASα. For STR and SLS, all three conditions (I, JA, and I JA) give similar transcript levels. This suggests that ORCA3 is one of the dominate regulators of these 2 genes in the JA signaling cascade.

The transient response of the TIA transcripts and the TIA metabolites (Figs. 5–11) to JA indicates that *C. roseus* closely controls the TIA pathway and rapidly brings the system back to the basal levels even in the presence of exogenous JA. This transient response is the strongest in the JA cultures but is also evident in the I JA cultures as seen in Fig. 11. Recent reports in *Arabidopsis* describe the discovery of the JAZ protein family which was a missing link in the understanding of JA regulation (Chini et al., 2007; Thines et al., 2007). The JAZ proteins are normally bound to transcription factors inhibiting their activity. The JA–isoleucine complex interacts with COI1 allowing COI1 to bind to the JAZ protein. This binding event leads to the degradation of JAZ and allows transcription of both defense genes and JAZ proteins. The new production of JAZ leads back to the inhibition of the transcription factors (Farmer, 2007). A similar mechanism may control the response of the TIA pathway to JA. The JAZ family of proteins has not been discovered in *C. roseus*.

Since JA showed the largest increase in the TIA transcripts tested and ORCA3 overexpression does not increase G10H and TDC transcripts in hairy roots, the existence of other positive regulators of the TIA pathway is likely. JA feeding increases ORCA2 transcripts with similar dynamics as ORCA3 (Figs. 5 and 9). This suggests that ORCA2 also plays a key role in the increased accumulation of TIA transcripts. Recently the G10H promoter

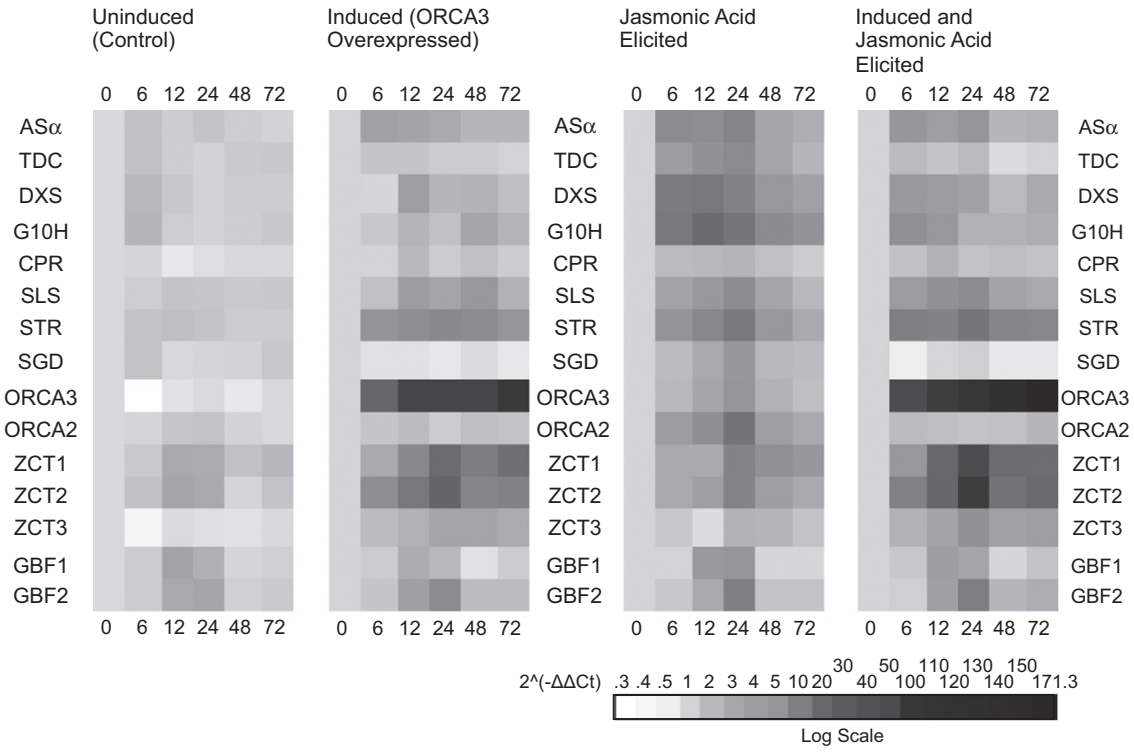

Fig. 11. Qualitative analysis of the transcripts of TIA genes and regulators. The box plot highlights the transient nature of the response of the transcripts of TIA genes and regulators to jasmonic acid (JA) elicitation over a period of 72 h. The parallel rise in ORCA3 transcripts with ZCT1 and ZCT2 transcripts is also evident in the cultures overexpressing ORCA3 with or without JA elicitation (I or I JA).

region was found to contain three unique enhancer regions that differed from the known regulatory elements of the promoter regions of STR and TDC (Suttipanta et al., 2007). Discovery of these regulatory elements and others will deepen our understanding of the regulation scheme of the TIA pathway.

The G-box-binding factors, GBF1 and GBF2, do not respond to an increase in JA concentration or in ORCA3 transcripts (Fig. A2). So far the signals that trigger the regulatory activity of the GBFs have not been discovered for *C. roseus*. In other plants, the G-box has been shown to respond to a wide variety of stimuli from plant hormones to UV light to anaerobiosis (Menkens et al., 1995). While in *C. roseus* it had been shown that the G-box-binding factor was an inhibitor of STR transcription. In the presence of another regulator such as ORCA3, there is a possibility that the GBFs could act as an activator (Siberil et al., 2001).

The current work demonstrates the positive effect that exogenously supplied JA has on both TIA transcripts and regulators. Since ORCA3 overexpression does not fully account for the increase in TIA transcripts seen with JA feeding, the study points to the existence of other positive regulators of the TIA pathway. It also suggests that a delicate balance of overexpressing positive regulators while repressing negative regulators may be necessary to increase the flux through the TIA pathway to vinblastine and vincristine in *C. roseus* hairy roots.

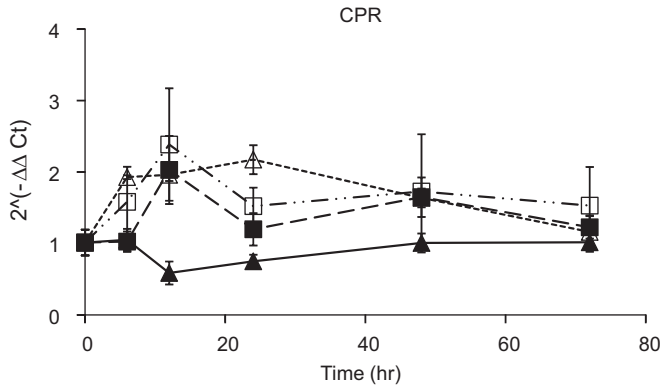

Fig. A1. CPR transcripts. (△) refers to the uninduced cultures. (■) refers to the induced cultures that overexpressed ORCA3. (△) refers to the jasmonic acid-elicited cultures. (□) refers to the induced and elicited cultures that overexpressed ORCA3 with jasmonic acid feeding. The data was normalized to the 0 h time point using the comparative threshold cycle method. Error bars represent the standard deviation of triplicate runs for Q PCR.

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Appendix

A.1. Materials and methods

A.1.1. Feeding study

EHIORCA3-4-1 hairy roots were fed with 3 μM dexamethasone (induced cultures) or with an equal volume ethanol (uninduced cultures) as a negative control. In addition to being induced or uninduced, the hairy roots were fed with 104 μM loganin, with

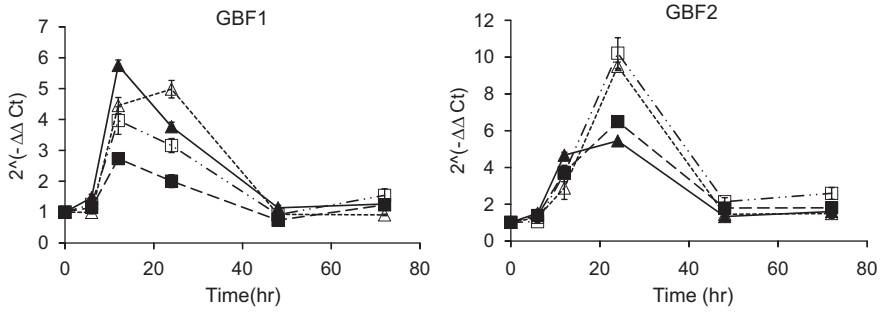

Fig. A2. G-box-binding factor transcripts. (▲) refers to the uninduced cultures. (■) refers to the induced cultures that overexpressed ORCA3. (△) refers to the jasmonic acid-elicited cultures. (□) refers to the induced and elicited cultures that overexpressed ORCA3 with jasmonic acid feeding. The data was normalized to the 0 h time point using the comparative threshold cycle method. Error bars represent the standard deviation of triplicate runs for Q PCR.

Table A1
The effect of feeding terpenoid and indole precursors on the production of TIAs when ORCA3 is overexpressed.

Sample	Serpentine (μg/g DW)	Catharanthine (μg/g DW)	Ajmalicine (μg/g DW)	Hörhammericine (μg/g DW)	Lochnericine (μg/g DW)	Tabersonine (μg/g DW)	Total TIAs (μg/g DW)
UI UF	446±61	141±13	925±114	961±212	1369±323	1153±167	4996±233
UI L	372±52	153±26	932±277	1012±144	1212±448	847±356	4528±487
UI T	407±42	174±28	865±20	1123±339	1292±106	1016±277	4877±200
UI LT	387±69	202±53	1034±254	1247±215	1130±494	652±373	4653±294
I UF	396±4	158±27	1216±102	616±68	1059±118	214±10	3659±110
I L	353±19	157±22	1069±118	608±72	1128±84	227±54	3542±35
I T	379±80	156±30	981±62	502±190	1071±249	207±44	3295±357
I LT	383±43	155±14	1170±163	584±107	1052±244	201±36	3545±247

Catharanthus roseus hairy roots containing an inducible ORCA3 gene were either induced with 3 μM dexamethasone (I) or fed an equal volume of ethanol (UI). The indole precursor, loganin (L), was fed at a concentration of 104 μM. The terpenoid precursor, tryptophan (T), was fed at a concentration of 104 μM. The unfed (UF) control was fed an equal volume of ethanol. Data represents the average of triplicate cultures ± standard deviation.

104 μM tryptophan, or with 104 μM loganin and 104 μM tryptophan (Figs. A1 and A2). The cultures were fed on day 35 after subculturing which corresponded to the exponential growth phase. The cultures were harvested 72 h after feeding for alkaloid analysis (Table A1).

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