The ORCA-ome as a key to understanding alkaloid biosynthesis in Catharanthus roseus

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Chapter 3

ORCA2 and ORCA3 differentially regulate specific metabolic sub-pathways in the terpenoid indole alkaloid biosynthetic network

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Abstract

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) synthesizes a structurally complex class of secondary metabolites known as terpenoid indole alkaloids (TIAs), several of which exhibit strong pharmacological activities. Despite decades of research efforts many steps in the pathway are still unknown and many enzymes , corresponding genes and even metabolic intermediates remain to be identified. ORCA2 and ORCA3 are two AP2/ERF-domain transcription factors regulating several known TIA biosynthesis genes. In addition sets of novel genes upregulated specifically by inducible expression of either one or both ORCAs were identified by cDNA-AFLP analysis. Although many of these genes are predicted to encode enzymes, assignment of these enzymes to specific metabolic steps in the TIA pathway is not obvious. Knowledge about the regulatory effects of each ORCA at the metabolite level might assist in assigning putative functions to orphan enzymes. Therefore we set out to explore the effects of overexpressing ORCA2 and ORCA3 under the control of the estradiol-inducible XVE system on TIA metabolism. To ensure precursor availability, we fed a set of samples with loganin and tryptamine. Overexpression of ORCA2 and ORCA3 alone did not affect alkaloid accumulation. Precursor feeding led to accumulation of high levels of strictosidine, the central precursor for all TIAs, independent of ORCA overexpression. Accumulation of downstream TIAs was differentially affected by ORCA2 and ORCA3 overexpression. TIA accumulation patterns allowed us to predict in which set of cDNA-AFLP tags genes encoding the corresponding enzymes should be present. In addition to assigning gene function the data allowed us to predict the existence of two regulatory steps controlled by ORCAs acting at the protein or metabolite level.

Introduction

Catharanthus roseus (L.) G. Don (Madagascar periwinkle), a tropical plant of the Apocynaceae family, produces a wide variety of terpenoid indole alkaloids (TIAs). Some of these compounds have pharmaceutical importance, such as ajmalicine used to treat circulatory disorders, and the bisindoles vinblastine and vincristine used in the treatment of various types of cancer. These bisindole alkaloids are produced in trace amounts in the leaf of the plant and it takes approximately 500 kg of dried leaves to extract 1 gram of vinblastine (Noble, 1990). The commercial importance of these compounds has led researchers to explore the biosynthesis of TIAs and its regulation with the ultimate goal to find ways to engineer increased production of these compounds.

The central intermediate in the biosynthesis of all TIAs is strictosidine (Fig. 1), formed by coupling of the iridoid glycoside secologanin and tryptamine. Tryptamine is derived from tryptophan. Downstream of strictosidine diverging biosynthetic routes give rise to different products such as ajmalicine, catharanthine and vindoline. Condensation of vindoline and catharanthine leads to the formation of dimeric alkaloids including vinblastine.

Cell suspension cultures were found to be incapable of synthesizing vindoline, vincristine and vinblastine, but they can produce several monomeric alkaloids including catharanthine, tabersonine, ajmalicine and serpentine (Vazquez-Flota et al., 2002). The limited production capacity has been attributed to tight developmental control of certain biosynthesis genes. In *C. roseus* plants expression of certain sets of genes was only observed in specific cell types, for example expression

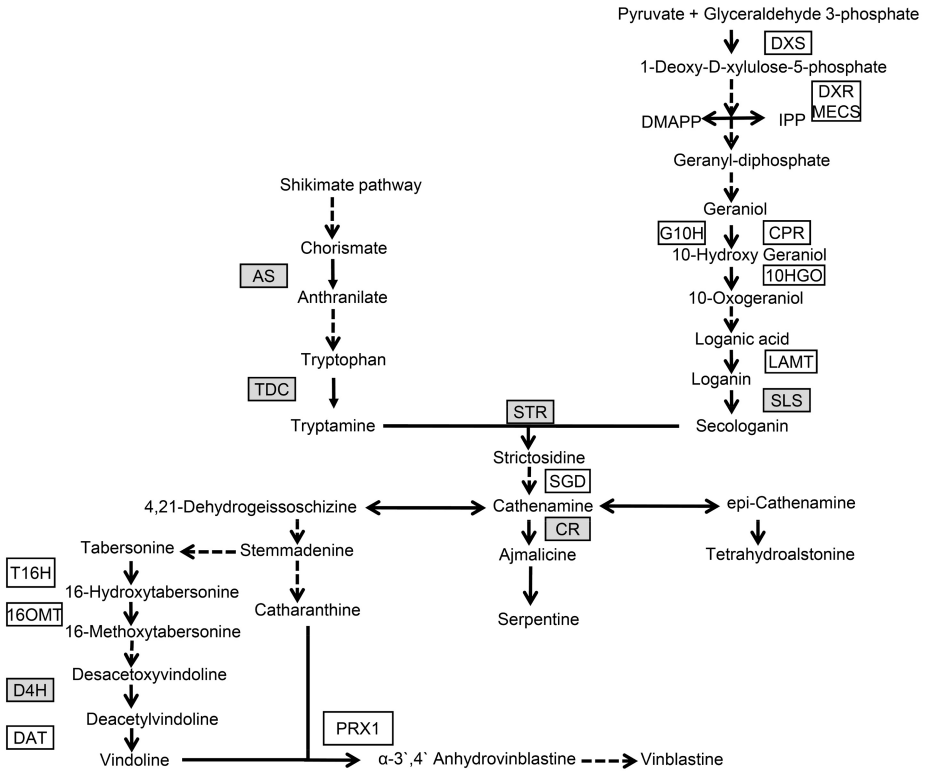

Figure 1. Biosynthetic pathway for terpenoid indole alkaloids in *Catharanthus roseus*.Solid arrows indicate single enzymatic conversions, whereas dashed arrows indicate multiple enzymatic conversions. Indicated are enzymes, for which the corresponding genes were cloned. Enzymes encoded by genes regulated by ORCA2 and 3 are shown against a pink background (according to chapter 2). **AS**: anthranilate synthase, **CPR**: cytochrome P450 reductase, **D4H**: desacetoxyvindoline 4-hydroxylase, **DAT**: acetyl-CoA:4-O-deacetylvindoline 4-O-acetyltransferase, **DXS**: 1-deoxy-D-xylulose-5-phosphate synthase, **DXR**: 1-deoxy-D-xylulose-5-phosphate reductoisomerase, **MECS**: 2-*C*-methyl-D-erytritol 4-phosphate cytidyltransferase, **LAMT**: loganic acid methyltransferase, **G10H**: geraniol 10-hydroxylase, **PRX1**: anhydrovinblastine synthase, **SGD**: strictosidine β-D-glucosidase, **SLS:** secologanin synthase, **STR**: strictosidine synthase **TDC**: tryptophan decarboxylase, **T16H**: tabersonine 16-hydroxylase, **16OMT**: 16-hydroxytabersonine-16-O-methyltransferase, **CR**: Cathenamine reductase

of genes acting late in vindoline biosynthesis was only observed in laticifers and idioblasts(St-Pierre et al., 1999).

The plant defense hormone (methyl) jasmonate has a positive effect on expression of TIA biosynthesis genes and alkaloid production in *C. roseus*. MeJA induces all known TIA pathway genes in cell cultures (Chapter 2), showing that cultured cells actually do have the capacity to express genes which in intact plants are only expressed in specific cell types. MeJA-responsive expression of several TIA biosynthesis genes is controlled by two AP2/ERF-domain transcription factors called ORCA2 and ORCA3 (Menke et al., 1999; van der Fits and Memelink, 2000). Constitutive overexpression of ORCA3 resulted in elevated expression of several primary and secondary TIA pathway genes (van der Fits and Memelink, 2000). Inducible overexpression of ORCA3 resulted in elevated expression of largely the same set of genes, although a few differences were observed (Chapter 2). Comparison of cell lines inducibly overexpressing ORCA2 or ORCA3 showed that

ORCA2 and ORCA3 regulate different but overlapping sets of genes (Chapter 2). Several key genes in the TIA pathway were not controlled by either ORCA2 or ORCA3. These include *G10H* involved in the biosynthesis of secologanin, *T16H* and *DAT* involved in vindoline biosynthesis, and *PRX1* encoding a peroxidase responsible for formation of dimeric alkaloids.

It is clear that due to the limited control of the ORCAs their overexpression in cell cultures will not lead to elevated levels of TIA. However, we reasoned that it is still useful to perform alkaloid analysis in ORCA-overexpressing cell lines. Genome-wide gene expression analysis has resulted in the isolation of a number of ORCA-controlled genes encoding enzymes (Chapter 2). However, prediction of the site of action of these putative TIA biosynthetic enzymes is difficult and at best speculative. Combination of gene expression and metabolite data may lead to clues about the site of action of some of the putative TIA biosynthetic enzymes. To ensure precursor availability we also fed the cells with tryptamine and loganin. We found that overexpression of ORCA2 or ORCA3 alone did not affect alkaloid accumulation but when combined with precursor feeding, increased levels of TIA were observed. Accumulation of downstream TIA was differentially affected by ORCA2 and ORCA3 overexpression. TIA accumulation patterns allowed us to predict in which set of cDNA-AFLP tags genes encoding the corresponding enzymes should be present. In addition to assigning gene function the data allowed us to predict the existence of two regulatory steps controlled by ORCAs acting at the protein or metabolite level.

Materials and Methods

Cell cultures and treatments

C. roseus cell suspension line MP183L was maintained by weekly 10-fold dilution in 50 mL of Linsmaier and Skoog (LS) medium containing 88 mM sucrose, 2.7 µM 1-NAA and 0.23 µM kinetin and was grown at 25°C in a 16/8 hour light/dark regime at 200 µE m^{-2} s^{-1} at 70% relative humidity on a rotary shaker at 120 rpm. Construction of transgenic cell lines is described in Chapter 2. For the induction and feeding of cell lines, 10 ml of cell culture 4 days after transfer to fresh medium were transferred to sterile flasks. The inducer β-estradiol (Sigma) dissolved in DMSO was added at a concentration of 10 µM. Control cell lines were treated with DMSO at a final concentration of 0.1% (v/v). Induced cells were harvested 24 h after estradiol addition. For feeding the precursors loganin (Extrasynthese, Genay, France) and tryptamine-HCl (Aldrich, Zwijndrecht, The Netherlands) were dissolved in water, filter-sterilized and added at 1 mM final concentration in the medium. Non-fed control samples received the same amount of sterilized water. Each treatment was performed in triplicate. Cells were harvested 24 h after treatment, frozen in liquid nitrogen and stored at -80 ºC.

NMR and multivariate data analysis

Freeze-dried cells (50 mg) from each flask were placed in a 2 ml micro-tube and extracted with 750 µl CH_3OH-d_4 and 750 µl KH_2PO_4-d_2 buffer in D_2O (pH 6.0). The mixture was vortexed (2,500 rpm, 1 min) using an electronic vortex mixer followed by sonication (20 min) in an ultrasonic bath and centrifugation (10,000×*g*, 15 min). The supernatant was transferred into a 5 mm NMR tube for NMR

measurements

^{1}H-NMR spectra was recorded at 25°C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany). CH_3OH-d_4 was used as an internal lock. Each spectrum consisted of 128 scans requiring 10 min acquisition time with the following parameters: 0.25 Hz/point, pulse width (PW) = 45º (6.6 μs), and relaxation delay (Dl) = 2.0 s. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz and the spectra were zero-filled to 32 K points. The window functions were optimized for the analysis. The resulting spectra were manually phased and baseline corrected, and calibrated to TMSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker).

The ^{1}H-NMR spectra was automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to TMSP and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ −0.4 to δ 10. Principal component analyses (PCA) partial least square-discriminant analysis (PLS-DA) were performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden).

Alkaloid extraction and HPLC detection

Freeze-dried biomass (100 mg) was extracted in 5 ml ethanol by sonication for 1 minute in an ultrasonic bath, centrifuged at 15000 rpm for 30 min at room temperature. The supernatant was transferred to a new tube, dried under reduced pressure using a speedvac and the residue was suspended in 1 M phosphoric acid. The acidic alkaloid solution was centrifuged for 15 min at 15000 rpm and filtered through a 0.45 μm membrane (Nalgene) prior to HPLC analysis.

The alkaloid separation was performed on a (250 mm ×4.6 mm, 5 μm particles) Zorbax Eclipse XDB-C18 column (Agilent, USA) equipped with a SecurityGuard™ column (Phenomenex, USA). Eluent (1.2 ml/min) was delivered by means of a Waters 600S gradient controller pump (Waters, Milford, MA, USA). Eluents contained trifluoroacetic acid : water : acetonitrile in the proportions (0.01 : 79 : 21 v/v/v) for eluent A and (0.01 : 5 : 95 v/v/v) for eluent B. Elution was for 14 min with 100% solution A and then the percentage of solution B was increased to 10 % in 17 min and held for 8 min. The percentage of solution B was decreased to 0 % in 30 min and 100 % solution A was used for another 5 min. The alkaloids were eluted within 35 min. The online detection was performed with a Waters 990 photodiode-array (PDA) detector recording from 210 to 350 nm. Identification of alkaloids was done by comparison of retention times and UV spectra with those of our reference library created from authentic samples. This system allowed us to detect the alkaloids strictosidine, ajmalicine, serpentine, vinblastine, tabersonine, vincristine, and vindoline. For the detection of loganic acid, loganin, secologanin, tryptophan and tryptamine we used an isocratic eluent system containing 0.01 % phosphoric acid, 85 % water and 15 % acetonitrile. Otherwise the HPLC system was the same as for alkaloid separation and the compounds were eluted in 25 min.

LCMS analysis

For the LC-MS analysis the alkaloids were separated on a (250 x 4.6 mm i.d.) 5 µm particle size Vydac C18 column. The solvent at the flow rate of 0.5 ml/min were used: 95% H_2O/5% MeOH/0.1 % Formic Acid. The positive ion electrospray mass spectra were obtained by Agilent 1100 Series LC/MS system (Agilent Technologies, Palo Alto, CA, USA). Full scan spectra between m/z 150 and 850 were obtained in positive mode.

Results

Alkaloid accumulation

Construction and selection of *C. roseus* cell lines expressing ORCA2, ORCA3 or GFP under control of the estradiol-responsive XVE transcription factor (Zuo et al., 2000) is described in Chapter 2.

Initially we used a non-targeted approach to determine alkaloid accumulation in the transgenic cell lines. NMR technology with multivariate data analysis was applied to discriminate metabolites in our samples. This approach did not result in any separation of the samples for metabolites on PCA plots (data not shown). This might be because of the low sensitivity of NMR or because overexpression of ORCAs did not affect metabolite composition. Therefore we decided to use a targeted approach using high performance liquid chromatography (HPLC) technique which is more sensitive than NMR.

For the *G10H* gene it is known that is it is not controlled by ORCAs (Chapter 2). To circumvent this block in the terpenoid branch of the TIA pathway, we fed a set of samples with the two precursors tryptamine and loganin. *C. roseus* cells have a high constitutive capacity to convert loganin in secologanin (Whitmer et al., 2002). Strictosidine was present at very low levels in non-fed samples from all six transgenic cell lines (Fig. 2). Feeding the cells with tryptamine and loganin resulted in high accumulation of strictosidine to a level of 94 ± 8 µmol g^{-1} DW. This is in accordance with levels previously reported in precursor-fed cell lines (Whitmer et al., 1998; El-Sayed and Verpoorte, 2002). Accumulation of strictosidine was not affected by the overexpression of ORCA2 or ORCA3. It seems that in the absence of ORCA expression there is enough activity of the STR and SLS enzymes to convert the precursors efficiently in strictosidine.

The conversion to downstream alkaloids such as ajmalicine was very low even at high strictosidine levels upon precursor feeding. Interestingly, the ajmalicine accumulation was increased in ORCA2 and ORCA3 cell lines as compared to GFP cell lines after precursor feeding in the absence of estradiol induction (Fig. 3). The concentration of ajmalicine was further increased when precursor-fed ORCA2 and ORCA3 cell lines were treated with estradiol. The highest ajmalicine concentration of about 1 µmol g^{-1} DW was observed in induced ORCA3 cell lines. It can be concluded that either ORCA2 or ORCA3 overexpression led to increased accumulation of ajmalicine in precursor-fed cells.

Catharanthine was not significantly increased in cell lines fed with precursors except in ORCA3 cell lines (Fig. 4). The catharanthine accumulation further increased 4-fold to 1.42 ± 0.072 µmol g^{-1} DW when precursor-fed ORCA3 cell lines were treated with estradiol. From this observation it can be

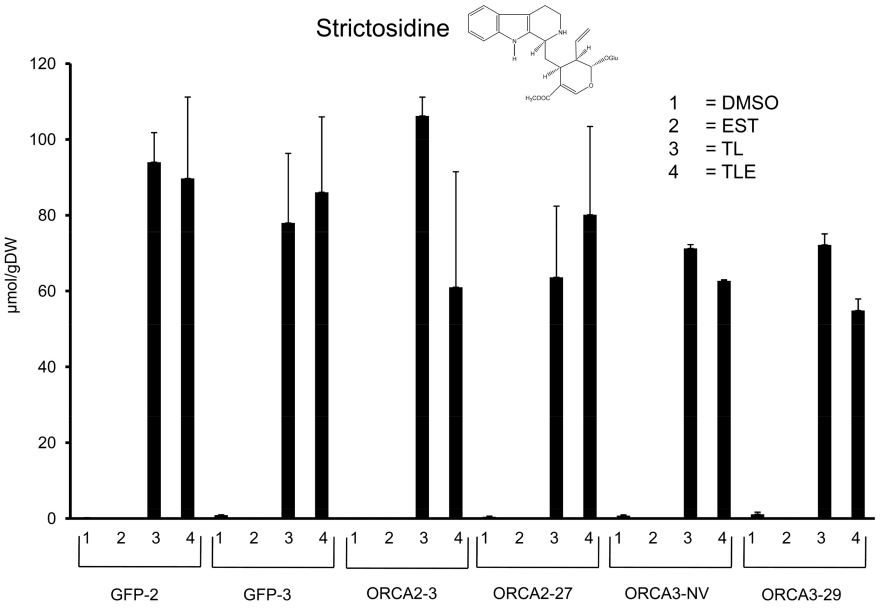

Figure 2. Strictosidine levels in transgenic *C. roseus* cell lines. Each cell line was treated with 10 µM estradiol (E), DMSO, 1 mM tryptamine and 1 mM loganin and DMSO (TL), and 1 mM tryptamine and 1 mM loganin and 10 µM estradiol (TLE). Bars represent the mean and the standard error of triplicate measurements.

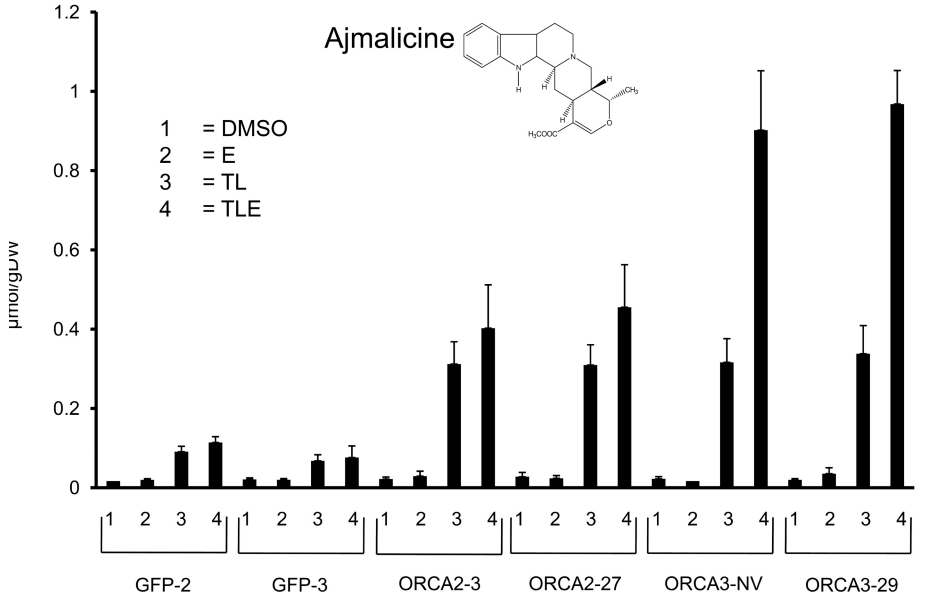

Figure 3. Ajmalicine levels in transgenic *C. roseus* cell lines. See legend of Fig.2 for explanation.

concluded that one or more genes in the pathway from cathenamine to catharanthine are specifically regulated by ORCA3. Interestingly, in ORCA3 samples there was a new chromatographic peak with a retention time of around 6 minutes with the same cell line-specific accumulation pattern as catharanthine (Figs. 5 and 6). The new peak has a unique UV spectrum with absorbance peaks at wavelengths of 242.2 nm and 308.5 nm (Fig. 5c). Similar to catharanthine this unknown compound was also further increased 2 to 3-fold in ORCA3 cell lines when treated with estradiol (Fig. 6). A literature search for the possible identity of this compound resulted in the discovery of vindolinine, a dihydroindole alkaloid found in *C. roseus* with reported UV absorbance peaks at 245 nm and 300 nm (Johnson et al., 1963). Determination of the molecular weight of this new compound using liquid chromatography and mass spectrometry (LC-MS) revealed a molecular weight of 336 which is identical to vindolinine. Further support for our identification of the unknown compound as vindolinine was the fact that Hallard et al. (2000) observed the same retention time for vindolinine with exactly the same HPLC system.

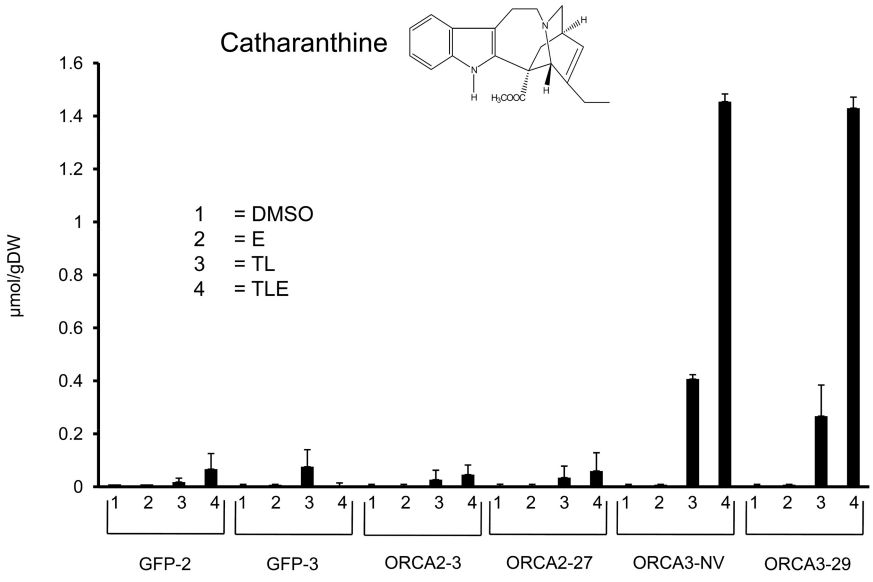

Figure 4. Catharanthine levels in transgenic *C. roseus* cell lines. See legend of Fig. 2 for explanation.

Tabersonine accumulated in all cell lines when fed with precursors (Fig. 7). The concentration of tabersonine was high in precursor-fed ORCA2 and OCRA3 cell lines as compared to the control GFP cell lines in the absence of estradiol induction. Induction of ORCA cell lines with estradiol did not further stimulate tabersonine accumulation. Highest levels of 0.38 ± 0.02 µmol g^{-1} DW were observed in precursor-fed ORCA2 cell lines treated with estradiol.

Consistent with the proposed block in the terpenoid branch of the TIA pathway we could not detect loganin in the transgenic cell lines with or without induction by estradiol (Fig. 8). Feeding of loganin at 1 mM concentration in the medium resulted in the detection of cellular loganin levels. The cell

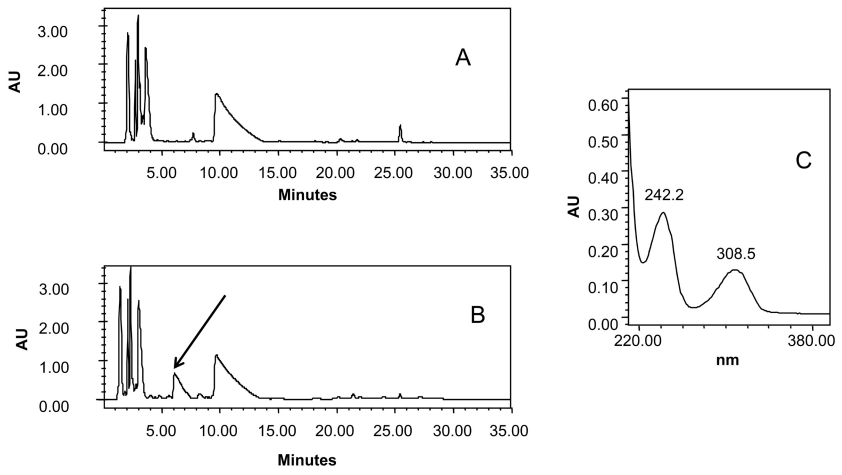

Figure 5. HPLC chromatogram and UV spectrum of an unknown compound accumulating in estradiol-treated precursor-fed ORCA3 cell lines.A, HPLC chromatogram of alkaloids extracted from ORCA3-NV cell line treated with DMSO and fed with precursors measure at 280 nm; B, HPLC chromatogram of alkaloids from ORCA3-NV cell line induced with 10 µM estradiol and fed with precursors. The peak corresponding to the unknown compound is indicated with an arrow; C, UV spectrum of the unknown compound. AU is absorption unit.

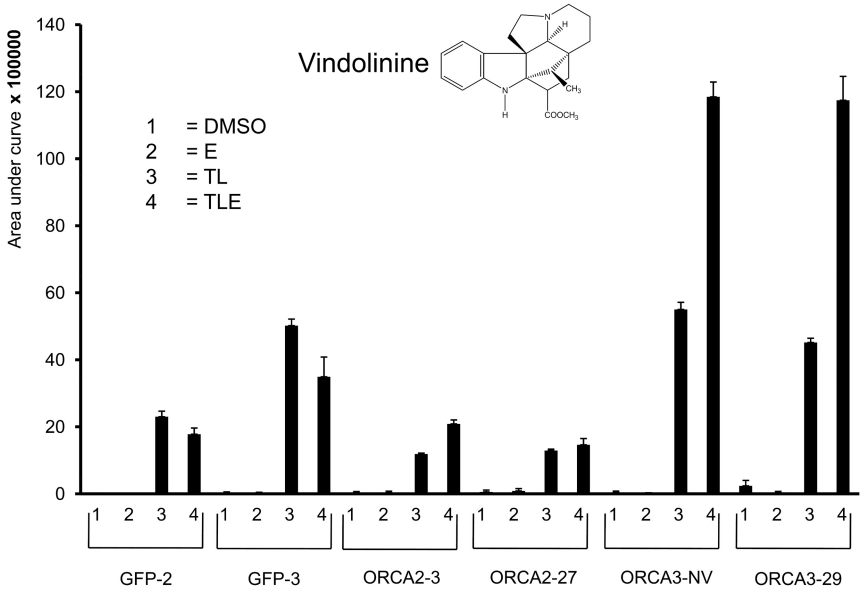

Figure 6. Vindolinine levels in transgenic *C. roseus* cell lines. See legend of Fig. 2 for explanation.

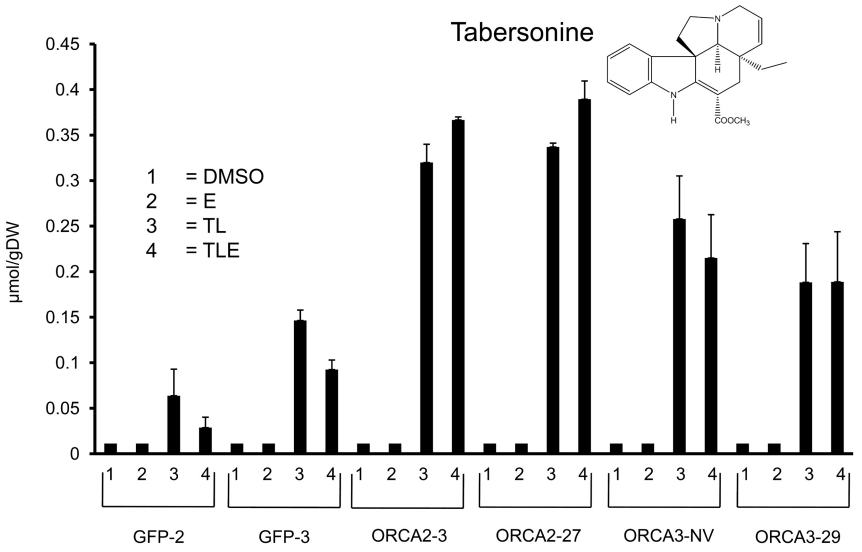

Figure 7. Tabersonine levels in transgenic *C. roseus* cell lines.See legend of Fig.2 or explanation.

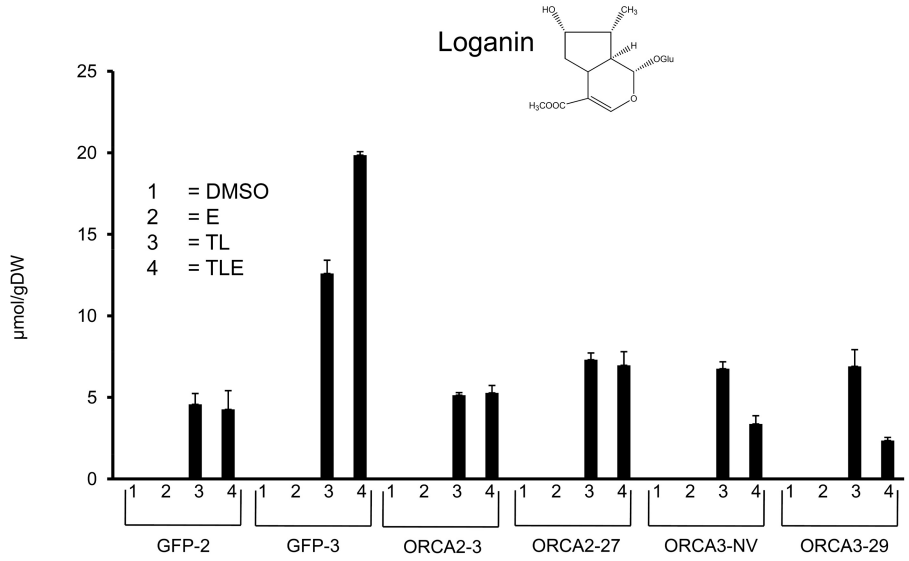

Figure 8. Loganin levels in transgenic *C. roseus* cell lines. See legend of Fig. 2 for explanation.

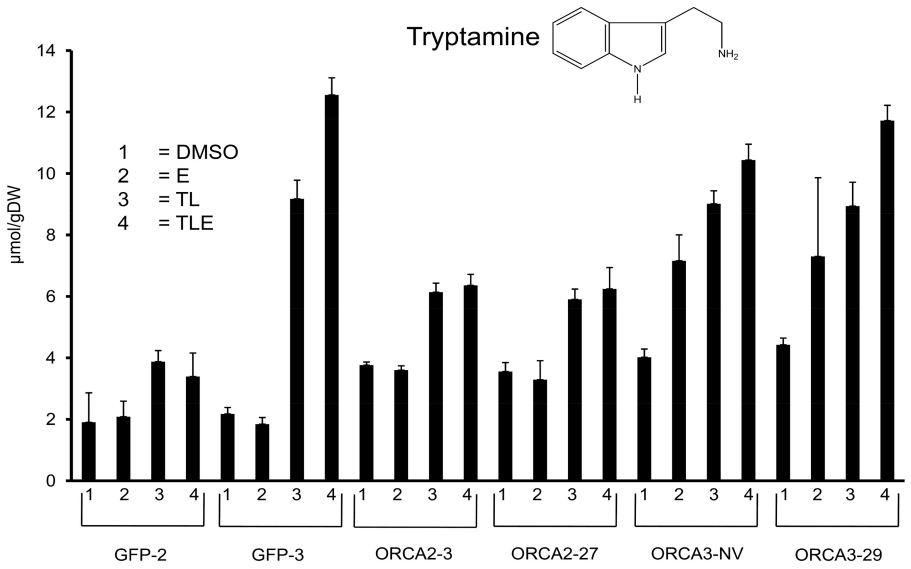

Figure 9. Tryptamine levels in transgenic *C. roseus* cell lines. See legend of Fig.2 for explanation.

lines metabolized loganin at similar levels, except the GFP3 cell line in which the concentration was 19.85 ± 0.21 µmol g^{-1} DW as compared to 4.57 ± 0.66 µmol g^{-1} DW in GFP2 cell line. The concentration of loganin in ORCA2 cell lines, was not different from the GFP2 cell line, and was not affected by estradiol induction. In ORCA3 cell lines the induction by estradiol resulted in a 2-fold reduction in loganin levels.

As evident from Figure 9, tryptamine was present in all non-fed cell lines and the concentration was significantly higher in ORCA2 and ORCA3 cell lines as compared to GFP lines. Estradiol induction did not affect the tryptamine concentration in ORCA2 cell lines, while in ORCA3 cell lines estradiol led to a further increase. Feeding of tryptamine increased the cellular tryptamine concentration only 1.5 to 2-fold except in the GFP3 cell line which showed a 4-fold increase. Tryptophan concentration was also not affected by ORCA2 or ORCA3 overexpression. Both estradiol induction and precursor feeding had no effect on tryptophan accumulation in all cell lines. The overall level of tryptophan was high in ORCA2 cell lines compared to ORCA3 cell lines. Among the two GFP lines the tryptophan concentration was high in the GFP2 cell line as compared to the GFP3 cell line.

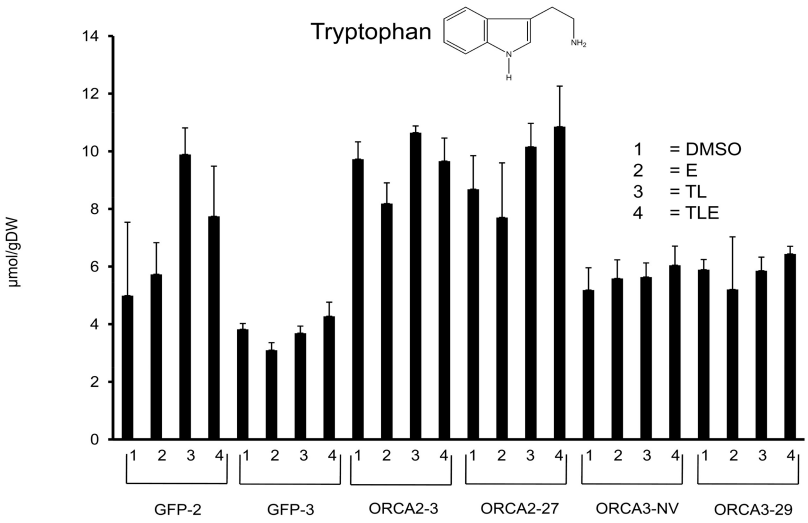

Figure 10. Tryptophan levels in transgenic *C. roseus* cell lines. See legend of Fig. 2 for explanation.

Discussion

In this chapter we reported the effects of the transcription factors ORCA2 and ORCA3 on alkaloid metabolism. We used an inducible system in which ORCA expression is controlled by the estradiol-responsive artificial transcription factor XVE. Control cell lines expressed GFP in an inducible manner. To study the influence of ORCA2 and ORCA3 overexpression on alkaloid metabolism we started with a non-targeted approach. Extracts from cell lines treated with estradiol or DMSO were subjected to NMR spectroscopy. The NMR data were processed by Multivariate Data Analysis, which failed to generate any separation between samples on PCA plots (data not shown). This observation led us to conclude that over-expression of ORCA2 and ORCA3 is not sufficient to change cellular metabolism. For alkaloid production the possible bottleneck is the terpenoid moiety of TIA since the key gene *G10H* is not regulated by either ORCA2 or ORCA3 (Chapter 2). We decided to feed one group of cells with tryptamine and loganin. Precursor feeding also did not result in separation on PCA plots. Therefore we used a targeted approach to specifically analyze the alkaloids using HPLC, which is a much more sensitive technique than NMR.

We observed high levels of strictosidine accumulation when cells were fed with precursors consistent with previous reports (El-Sayed and Verpoorte, 2002; Whitmer et al., 1998). The accumulation of strictosidine was not significantly affected by treatment with estradiol except in ORCA3 cell lines where strictosidine levels slightly decreased. Apparently the levels of SLS and STR enzyme activities in the cell cultures are sufficiently high for efficient strictosidine production without the need for enhanced gene expression via ORCAs. The conversion to downstream alkaloids such as ajmalicine was very low. This is remarkable since *C. roseus* cells have a high constitutive level of SGD enzyme activity (Geerlings et al., 2000) due to high constitutive expression of the *SGD*

gene in suspension cells (Chapter 2). We also observed that protein extracts from suspension cells had a high capacity to convert strictosidine to cathenamine in vitro (Chapter 4). It is possible that in vivo an intracellular strictosidine transport step is limiting, since STR is localized in the vacuole (McKnight et al., 1991; Stevens et al., 1993) and SGD is present in the ER (Geerlings et al., 2000).

In precursor-fed ORCA2 and ORCA3 cell lines an elevated level of ajmalicine was found compared to GFP cell lines in the absence of estradiol induction. This may be caused by leaky expression of ORCA2 and ORCA3 from the XVE module. When treated with estradiol ajmalicine accumulation was high in ORCA3 cell lines compared to ORCA2 cell lines. Constitutive overexpression of ORCA3 in cell suspensions also resulted in ajmalicine accumulation after loganin feeding (van der Fits and Memelink, 2000). El-Sayed and Verpoorte (2002) also reported the accumulation of ajmalicine when cells were fed with tryptamine and loganin, which was further increased when cells were treated with MeJA. From our results it can be concluded that the gene encoding cathenamine reductase responsible for the conversion of cathenamine to ajmalicine must be regulated by both ORCA2 and ORCA3. In addition the gene encoding the putative strictosidine transporter protein must also be regulated by both ORCAs. Both of these genes should be present in the gene set corresponding to transcript tags regulated by both ORCAs described in Chapter 2.

In contrast to ajmalicine catharanthine accumulation was only observed in ORCA3 cell lines. Estradiol-induced precursor-fed ORCA3 cell lines accumulated high levels of catharanthine, which was not the case in ORCA2 and GFP cell lines. In the absence of estradiol induction precursor-fed ORCA3 cell lines also had elevated catharanthine levels, presumably due to leaky ORCA3 expression from the XVE module. The pathway leading from cathenamine to catharanthine is poorly understood and is proposed to involve multiple enzymatic steps none of which have been characterized (Loyola-Vargas et al., 2007). Vindolinine, which is an isomer of catharanthine, also accumulated in precursor-fed estradiol-induced ORCA3 cell lines. Although there are many (phyto) chemical studies on vindolinine, it has never been positioned in the TIA metabolite network. The fact that vindolinine showed the same cell line and treatment-specific accumulation pattern as catharanthine suggests that these two compounds are part of the same biosynthetic route. Genes encoding enzymes involved in vindolinine and catharanthine biosynthesis should be present in the ORCA3-specific set of transcript tags described in Chapter 2.

Precursor feeding increased the tabersonine concentration in all cell lines, but to significantly higher levels in ORCA2 and ORCA3 cell lines than in GFP cell lines, presumably due to leaky ORCA expression. Surprisingly the tabersonine concentration did not or only marginally increase further after estradiol induction. Overall the concentration of accumulated tabersonine was low with a maximum level of 0.38 μmol g^{-1} DW. The tabersonine concentration was somewhat lower in ORCA3 cell lines compared to ORCA2 cell lines. Genes encoding enzymes involved in tabersonine biosynthesis should be looked for in the gene set corresponding to transcript tags regulated by both ORCAs in the absence of estradiol induction. Unfortunately we did not sequence this set of cDNA-AFLP tags in the study described in Chapter 2.

Loganin was completely absent in all transgenic lines which supports the suggestion that in cell cultures biosynthesis of the terpenoid moiety is the most rate-limiting factor (Hedhili et

al., 2007). When we fed loganin to the cell suspension at 1 mM concentration, most of it was metabolized to downstream alkaloids and we could detect only 7.0 μmol g^{-1} DW on average in all cell lines except in GFP3 cell line where a 3 fold higher level of loganin was present.

Tryptophan and tryptamine were detectable in all non-fed cell lines. Tryptophan concentration was not affected by overexpression of either ORCA2 or ORCA3. Tryptamine levels were significantly higher in ORCA2 and ORCA3 cell lines than in GFP lines. Estradiol induction increased the tryptamine concentrations in ORCA3 cell lines but not in ORCA2 lines. This is quite surprising in view of the observation that ORCA2 and ORCA3 overexpression were equally effective in switching on *TDC* gene expression (Chapter 2). It is possible that a rate-limiting transport step is regulated specifically by ORCA3, notably transport of tryptophan from the plastids where it is synthesized to the cytoplasm where TDC is localized (De Luca and Cutler, 1987; Stevens et al, 1993). TDC is a pyridoxo-quinoprotein, which has pyrroloquinoline quinone covalently bound to the enzyme and which requires pyridoxal phosphate as a cofactor (Pennings et al., 1989). Therefore it is also possible that ORCA3 regulates a rate-limiting step in the biosynthesis of pyrroloquinoline quinone or pyridoxal phosphate. Finally TDC has been reported to be degraded via the ubiquitin-proteasome pathway (Alvarez-Fernandez and De Luca, 1994). So ORCA3 might also negatively regulate this degradation pathway and thereby stimulate tryptamine biosynthesis. Genes encoding additional positive regulators of TDC activity should be present in the ORCA3-specific set of transcript tags described in Chapter 2. Since we did not analyze negatively regulated tags such genes are missing from our collection.

A model for regulatory control by ORCAs of TIA metabolism based on the measurements described in this chapter are summarized in Fig. 11. Based on the results we propose that specific sub-pathways are regulated either by ORCA3 alone or by both ORCA2 and ORCA3. We have not found metabolic processes specifically regulated by ORCA2. Biosynthesis of ajmalicine and tabersonine is regulated by both ORCA2 and ORCA3. A difference between these two alkaloids is that tabersonine accumulation is not stimulated by estradiol induction. Apparently the low levels of ORCA expression due to leakiness of the XVE expression module are already sufficient for efficient stimulation of tabersonine accumulation. Unfortunately we did not analyze the set of transcript tags upregulated in ORCA lines irrespective of estradiol treatment. Biosynthesis of catharanthine is specifically regulated by ORCA3. In addition to predicting in which set of tags genes encoding enzymes active in sub-pathways should be present, our metabolite analysis also led us to propose the existence of two ORCA-regulated steps acting at the protein or metabolite level. We postulate that a regulator of TDC activity is encoded by an ORCA3-regulated gene, and that a transport protein responsible for strictosidine transport from the vacuole to the ER is encoded by a gene regulated by both ORCAs.

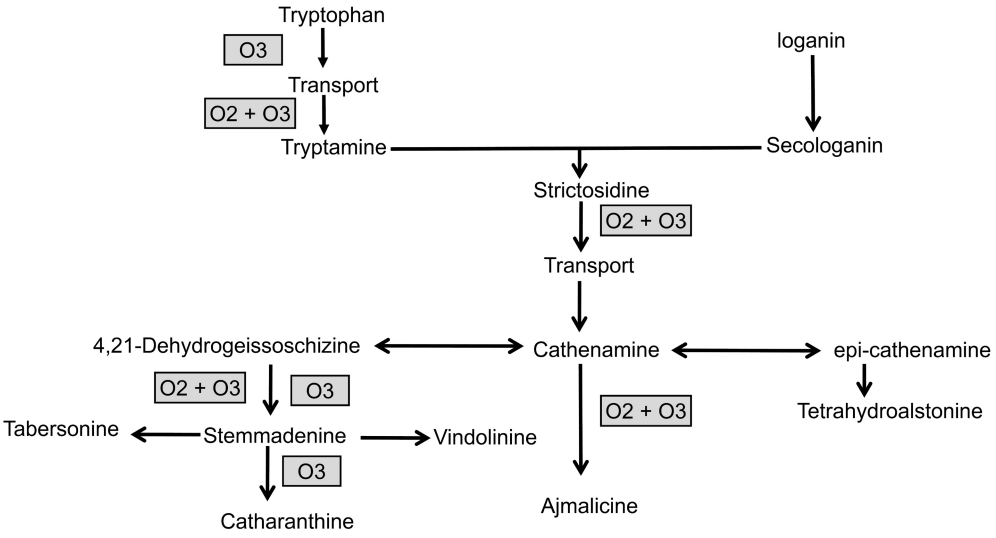

Figure 11. A proposed model for regulation of TIA metabolism by ORCA transcription factors

Acknowledgements

We thank Young Hae Choi for help with NMR analysis, Frank van der Kooy for his help with LCMS analysis, Ward de Winter is acknowledged for expert help with cell suspension cultures. G.H. was partially supported by Institute of Biotechnology and Genetic Engineering NWFP Agricultural University Peshawar, Pakistan and by a van der Leeuw grant from the Netherlands Organization for Scientific Research (NWO) awarded to J.M.

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