Characterization of two ORCA-regulated genes, encoding enzymes belonging to the α/β hydrolase and carboxylesterase superfamilies, respectively

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Abstract

*Catharanthus roseus* is a medicinal plant which synthesizes a class of secondary metabolites known as terpenoid indole alkaloids (TIA). The monomeric alkaloids ajmalicine and serpentine are used in the treatment of cardiac and circulatory diseases, and the dimeric alkaloids vinblastine and vincristine are potent anti-tumour drugs. Starting from the primary metabolites tryptophan and geraniol the biosynthesis of bisindole alkaloids in *C. roseus* is thought to involve at least 35 intermediates and a similar number of enzymes. Fourteen enzyme-encoding genes have been isolated to date. Two transcription factors called ORCA2 and ORCA3 which regulate the MeJA-responsive expression of at least half of the isolated genes have been described. A genome-wide screen using the cDNA-AFLP technique for ORCA target genes described in Chapter 2 has resulted in the identification of several dozens of new genes, many of which are predicted to encode enzymes. In this chapter we report the isolation of full-length cDNAs for two tags, CR-93 and CR-65, which upon database searching were found to encode enzymes that belong to the α/β hydrolase and carboxylesterase superfamilies, respectively.

Introduction

*Catharanthus roseus* or Madagascar periwinkle produces a wide range of terpenoid indole alkaloids (TIAs) such as the antihypertensive compound ajmalicine and the antitumor agents vinblastine and vincristine. The commercial importance of TIAs attracted a lot of research interest to dissect their pharmacological activities and to uncover their biosynthesis route. Although a lot is known about the pharmacological effects of many TIAs, relatively little is known about how plants synthesize them. This is mainly due to the complexity of the pathway and the complex regulatory mechanisms controlling the synthesis of these secondary metabolites.

The central intermediate in TIA biosynthesis is strictosidine. It is formed by the coupling of the iridoid glycoside secologanin, a product of the methyl-erythritol phosphate (MEP) pathway (Contin et al., 1998), and tryptamine, a product of the shikimate pathway. Starting from the amino acid tryptophan and the monoterpenoid geraniol, the biosynthesis of bisindole alkaloids in *C. roseus* is thought to involve at least 35 intermediates and a similar number of enzymes (St-Pierre et al., 1999; van der Heijden et al., 2004). Fourteen enzyme-encoding genes have been isolated (Table 1). Hence the large majority of the genes involved in TIA biosynthesis are still unknown.

The classical strategy to clone biosynthetic enzymes is to purify them to homogeneity through several chromatographic steps from alkaloid-producing plant tissue or cell cultures guided by an enzyme assay, and then to generate antibodies or determine N-terminal or internal peptide sequences as tools to clone the corresponding genes. Many of the enzymes amenable to purification have already been cloned in this way, but the remaining enzymes in the TIA pathway may be recalcitrant to conventional purification strategies. A more recent trend is to avoid biochemical purification and to directly isolate candidate clones by combination of expression pattern analysis and homology-based screening (Hashimoto and Yamada, 2003). This homology-based cloning and functional testing approach has been successful for obtaining several new cDNAs encoding TIA biosynthetic enzymes (Table 1). Recently a TIA pathway gene candidate was identified by EST
sequencing of a leaf epidermal cell-enriched cDNA population, and was found to correspond to loganic acid methyl transferase (LAMT) (Murata et al., 2008). A variation of this approach is to look for genes with altered expression in regulatory mutants affected in TIA biosynthesis. However such mutants do not exist for *C. roseus*. Luckily two regulators of the TIA pathway have been described from *C. roseus*. The transcription factors ORCA2 and ORCA3 regulate the MeJA-responsive expression of at least half of the known genes (Chapter 2).

We used a different approach for the isolation of new TIA pathway genes, in which ORCA2 and ORCA3 were inducibly over-expressed in *C. roseus* cell cultures and their target genes were traced by cDNA-AFLP technology. Genome-wide expression analysis by cDNA-AFLP resulted in several dozens of new genes, many of which are predicted to encode enzymes. In this chapter we report the isolation of full-length cDNAs for two tags, CR-93 and CR-65, which upon database searching were found to encode enzymes that belong to the α/β hydrolase and carboxylesterase superfamilies, respectively.

Table 1. Cloned enzymes involved in the biosynthesis of terpenoid indole alkaloids in *C. roseus*.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme class</th>
<th>Induction by jasmonate</th>
<th>Cloning method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 reductase (CPR)</td>
<td>P450-dependent Oxidoreductase</td>
<td>Yes</td>
<td>PP</td>
<td>Meijer et al., 1993</td>
</tr>
<tr>
<td>Geraniol 10-hydroxylase (G10H)</td>
<td>P450-dependent monoxygenase</td>
<td>Yes</td>
<td>PP</td>
<td>Collu et al., 2001</td>
</tr>
<tr>
<td>10-hydroxygeraniol oxidoreductase (10HGO)</td>
<td>Alcohol dehydrogenase</td>
<td>NO</td>
<td>PP</td>
<td>unpublished</td>
</tr>
<tr>
<td>Secologanin synthase (SLS)</td>
<td>P450-dependent monoxygenase</td>
<td>Yes</td>
<td>HB</td>
<td>Krämer et al., 2000</td>
</tr>
<tr>
<td>Tryptophan decarboxylase (TDC)</td>
<td>Aromatic L-aminio acid decarboxylase</td>
<td>Yes</td>
<td>PP</td>
<td>De Luca et al., 1989</td>
</tr>
<tr>
<td>Strictosidine synthase (STR)</td>
<td>Strictosidine synthase family</td>
<td>Yes</td>
<td>PP</td>
<td>Pasquali et al., 1992</td>
</tr>
<tr>
<td>Strictosidine β-D-glucosidase (SGD)</td>
<td>β-Glucosidase</td>
<td>Yes</td>
<td>HB</td>
<td>Geerlings et al., 2000</td>
</tr>
<tr>
<td>Tabersonine 16-hydroxylase (T16H)</td>
<td>P450-dependent monoxygenase</td>
<td>Yes</td>
<td>HB</td>
<td>Schröder et al., 1999</td>
</tr>
<tr>
<td>Desacetoxylvindoline 4-hydroxylase (D4H)</td>
<td>2-Oxoglutarate-dependent dioxygenase</td>
<td>Yes</td>
<td>PP</td>
<td>Vaquez et al., 1997</td>
</tr>
<tr>
<td>Acetyl-CoA-4-O-deacetylvinodoline 4-O-acetyltransferase (DAT)</td>
<td>acetyltransferase</td>
<td>Yes</td>
<td>PP</td>
<td>St-Pierre et al., 1998</td>
</tr>
<tr>
<td>Peroxidase 1 (PRX1)</td>
<td>Peroxidase</td>
<td>Yes</td>
<td>PP</td>
<td>Costa et al., 2008</td>
</tr>
<tr>
<td>16-hydroxytabersonine-16-O-methyltransferase (16OMT)</td>
<td>O-Methyltransferase</td>
<td>Yes</td>
<td>PP</td>
<td>Levac et al., 2008</td>
</tr>
<tr>
<td>Loganic acid methyltransferase (LAMT)</td>
<td>O-methyltransferase</td>
<td>Yes</td>
<td>HB</td>
<td>Murata et al., 2008</td>
</tr>
<tr>
<td>Minovincine-19-Hydroxy-O-Acetyltransferase (MAT)</td>
<td>Acetyltransferase</td>
<td>Yes</td>
<td>HB</td>
<td>Lafhamme et al., 2001</td>
</tr>
</tbody>
</table>

| a mRNA level increased when MeJA is applied to *C. roseus* cells (according to Chapter 2). | b The cloning method used to obtain the cDNA clones is given: PP, protein purification; HB, homology-based candidate isolation followed by functional expression. |

**Material and Methods**

**Cell cultures, treatments, RNA extraction and Northern blotting**

*C. roseus* MP183L wild type and transgenic cell lines were maintained as described in Chapter 2. Treatments were performed 4 d after transfer. MeJA (Bedoukian) was diluted in DMSO and added to a final concentration of 10 µM. Yeast extract (Difco) was dissolved in water, autoclaved, passed through an ultra-filter with a molecular weight cut-off of 3 kDa (Millipore) to remove chitin, and
was added at a final concentration of 400 µg/ml to cells. For the induction of transgenes 10 µM estradiol (dissolved in DMSO) was added. Induced cells were harvested at 24 h after induction. Control cultures were treated with DMSO at a final concentration of 0.1% (v/v). Harvested samples were frozen in liquid nitrogen and stored at -80 ºC. RNA extraction and Northern blot hybridization was performed as described in Chapter 2.

**Isolation of the CR-93 and CR-65 cDNAs and plasmid constructions**

Transcript tag CR-93 was 92 bp long (Chapter 2). Based on the CR-93 sequence a forward primer (5` TGA ATT AGC GAA AAT GTT GTG C -3`) and a reverse primer (5` GAC CGA ACT TTT GTG TTG T AA ACT TC -3`) were designed to isolate 5` sequences and 3` sequences by PCR with a gene-specific primer and a vector primer using a pACTII cDNA library of YE-treated MP183L cells (Menke et al., 1999). The CR-93 open reading frame (ORF) was PCR amplified with the primers 5` GAA TTC AAT GGA TCA TAT TGT TGG AAA TGG AGA GC -3` and 5` CTC GAG TTA TAA TTA TGG GCT ATG TCA ATA AG -3` using the pACTII library as a template and cloned in the pGEM-T Easy vector (Promega). The ORF was excised from pGEM-T Easy with EcoRI/XhoI and cloned in the protein expression vector pASK-IBA45plus (IBA, Göttingen, Germany) digested with EcoRI/XhoI. Thus when expressed in *E. coli* the recombinant protein contains a Strep tag at the N-terminus and a hexahistidine tag at the C-terminus.

For the isolation of the CR-65 full-length cDNA first we searched for *C. roseus* ESTs in the NCBI DNA database which gave 11 EST hits. Those EST hits which had 100 % sequence identity with CR-65 were combined to form a 961 bp cDNA containing a putative ATG start codon. Based on the assembled EST sequence a forward primer (5` ATT TCA TGG GTT CCT CAG ATG AGA CTA TTT TTG -3`) was designed to isolate 3` sequences by PCR with a gene-specific primer and a vector primer using the pACTII cDNA library.

**Bioinformatics**

Multiple sequence alignments were done with clustalX2.0.11 ([http://www.ddbj.nig.ac.jp](http://www.ddbj.nig.ac.jp)) using the default settings (Thompson et al., 1997) and homology searches were done with the BLAST program ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) in the National Center for Biotechnology Information database. For phylogenetic analysis the 29 GeneBank protein sequences (Table 2) were aligned with the deduced amino acid sequence of CR-65. The tree was constructed by use of the majority rule and strict consensus algorithm implanted in PHYLIP (Felsenstein, 1989). Terminal gaps were removed prior to running the analysis, while the internal alignment gaps were left and analysis conducted scoring gaps as characters or as missing characters. Distances were calculated using the Dayhoff matrix (PROTDIST), and the neighbor joining method was used to create the tree. Bootstrap analysis was conducted using 1000 bootstrap replicates (Felsenstein, 1989). TreeView (v.1.6.6) was used to display resulting trees (Page, 1996).
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Results

Full-length CR-93 cDNA encodes a protein belonging to the α/β hydrolase superfamily

The genome-wide screen using the cDNA-AFLP technique for ORCA target genes described in Chapter 2 has resulted in the identification of several dozens of new genes, many of which are predicted to encode enzymes. One of the tags, CR-93, was upregulated both in ORCA2 and ORCA3 overexpressing cell lines when induced by estradiol. Isolation of this band from the cDNA-AFLP gel and subsequent sequencing resulted in a 92 bp DNA sequence which when subjected to database searching using the TBLASTX algorithm gave the best hit to polynuridine aldehyde esterase (PNAE) from *Rauvolfia serpentina*, which is a key enzyme in the biosynthesis of ajmaline (Dogru et al., 2000), a TIA which does not occur in *C. roseus*. The regulation of CR-93 abundance by ORCAs and the homology of the encoded peptide to a TIA biosynthetic enzyme indicated that this gene may...
be involved in a metabolic step in TIA biosynthesis. Hence we decided to isolate the full-length cDNA sequence. PCR amplification with CR-93 specific primers and a cDNA library as template resulted in a cDNA sequence of 1128 bp with an open reading frame of 810 bp, which is flanked by 8 nt at the 5’ end and by 310 nt at the 3’ end including a poly-A tail. A similarity search in the sequence database revealed that the encoded protein is a member of α/β hydrolase superfamily, members of which contain a catalytic triad consisting of the conserved amino acids Ser, Asp, and His. CR-93 protein exhibited significant similarity to several members of this superfamily, including plant lyases, esterases, and lipases with the highest amino acid identity of 70% to PNAE from *R. serpentina*. CR-93 showed second highest identity to salicylic acid binding protein 2 (SABP2) (55% identity) from *Nicotiana tabacum* (Kumar and Klessig, 2003). In addition CR-93 showed 47 % identity to ethylene-induced esterase (EIE) from *Citrus sinensis* (Zhong et al., 2001), methyl jasmonate esterase (MJE) from *Solanum lycopersicum* (Stuhlfielder et al., 2004), a putative PNAE from *Ricinus communis* (unpublished) and *C. roseus* Protein S (CrPS) (Lemenager et al., 2005). CR-93 protein also contains the conserved catalytic triad (Ser92, Asp223, and His251) (Fig. 1).

**CR-93 transcripts accumulate in response to ORCA overexpression and MeJA**

It was evident from cDNA-AFLP analysis that the CR-93 transcript tag is regulated by both ORCA2 and ORCA3 (Chapter 2). The expression of CR-93 was verified by Northern blot hybridization in ORCA2, ORCA3 and GFP cell lines and in wild type cell lines (Fig. 2). The CR-93 transcript level was upregulated in ORCA cell lines when treated with estradiol compared to DMSO-treated samples. CR-93 mRNA accumulation was not affected in GFP cell lines treated with estradiol. When the wild type cell line was treated with MeJA the expression was upregulated compared to the DMSO treatment (Fig. 2). Yeast elicitor also induced CR-93 mRNA accumulation. The regulation of the CR-93 mRNA level by ORCA2 and ORCA3 and the responsiveness to MeJA and YE are consistent with a possible role of the encoded protein in the TIA biosynthesis pathway. With a high similarity with PNAE it is possible that CR-93 protein may have a function similar to PNAE, but ajmaline/sarpagine-type alkaloids are not synthesized in *C. roseus*. Reviewing all described metabolic steps in TIA biosynthesis in *C. roseus* we did not find a step requiring esterase activity. Since CR-93 also showed similarity to MJE from *S. lycopersicum* and MeJA cleaving esterase activity was reported in a cell culture of *C. roseus* (Stuhlfielder et al., 2002), we thought that it may be involved in removing the methyl group from MeJA in *C. roseus*. Another common esterase activity found in plants is geranyl acetate esterase (GAE) (Neelam et al., 2007), therefore we also looked into the possibility that CR-93 may function as GAE.

**Recombinant CR-93 protein does not have MJE or GAE activity**

The CR-93 protein was expressed in *E. coli* strain BL21 (DE3) pLysS with a C-terminal His-tag and an N-terminal Strep-tag and purified by consecutive Ni-NTA and Strep-Tactin affinity chromatography steps with a yield of around 2.5 mg of recombinant protein from 1 g of *E. coli* cell pellet. Western blot analysis revealed the presence of a major band around 35 KDa, which was the expected size of the recombinant CR-93 protein (Fig. 3).
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Figure 1. Alignment of the deduced CR-93 amino acid sequence with sequences of other members of the α/β hydrolase super family. Functionally equivalent amino acids are shown against a gray background while identical amino acids are shown against a black background. PNAE, Rauvolfia serpentina polyneuridine aldehyde esterase (AAF22288.1); PPNAE, Ricinus communis Putative polyneuridine aldehyde esterase precursor (XP_002522344.1); MJE/SAE, Arabidopsis thaliana methyl indole-3-acetate esterase/ methyl jasmonate esterase/ methyl salicylate esterase (NP_179943.1); EIE, Citrus sinensis ethylene-induced esterase (AAK58599.1); PS, Catharanthus roseus protein S (AAU95203.1); SABP2, Nicotiana tabacum salicylic acid-binding protein 2 (AAR87711.1). Amino acids forming the conserved catalytic triad (Ser 92, Asp 223, and His 251) are marked with ✫.

Figure 2. CR-93 transcripts accumulate in response to ORCA overexpression, MeJA and YE. Expression pattern of CR-93 mRNA detected by Northern blot hybridization. Wild type (WT) cells were treated with MeJA (M), DMSO (D) or yeast elicitor (YE) for 24 hours. O2 and O3 indicate samples from ORCA2 and ORCA3 cell lines respectively. Samples from negative control lines are indicated with GFP. Transgenic cell lines were treated for 24 hours with estradiol (E) or DMSO (D). The bottom panel shows the ethidium bromide-stained gel prior to blotting.
Recombinant CR-93 protein was checked for its possible activity towards MeJA and geranyl acetate with a TLC-based assay system. Geranyl acetate and MeJA at concentrations of 500 µM were incubated with 1-5 µg of recombinant CR-93 protein in 50 mM K-phosphate buffer (pH 7) at 37 °C for 30 min and the reactions were terminated by adding 10 µl 1 M HCl. The reaction mixtures were extracted with diethyl ether and concentrated. Tracing the formation of product by comparison with authentic JA and geraniol on TLC plates did not show enzyme activity, indicating that MeJA and geranyl acetate are not substrates for CR-93.

Figure 3. Analysis of recombinant CR-93 protein. The purified protein (1 µg) was separated by 10% SDS-PAGE and either stained with Coomassie Brilliant Blue (lane 1), or visualized after Western Blotting using anti-His antibodies (lane 2). Sizes of marker (M) bands are indicated in kDa.

Full-length CR-65 cDNA encodes a protein belonging to the carboxylesterase superfamily

cDNA-AFLP tag CR-65 was upregulated specifically in ORCA2 overexpressing cell lines but not in ORCA3 overexpressing cell lines and GFP cell lines (Chapter 2). Sequencing of the CR-65 tag resulted in a 378 bp sequence which upon TBLASTX database searching showed homology to 2-hydroxyisoflavanone dehydratase (HID) from Glycyrrhiza echinata, an enzyme which is involved in the isoflavonoid biosynthesis pathway (Akashi et al., 2005). The regulation of CR-65 by ORCA2 is interesting as it may be involved in an ORCA2-controlled specific branch of TIA biosynthesis in C. roseus, although our targeted metabolic analysis (Chapter 3) of ORCA2 and ORCA3 overexpressing cell lines did not result in the identification of an ORCA2-controlled TIA branch. Characterization of this ORCA2-specific gene may be helpful to identify a metabolic branch which is controlled by ORCA2 alone.

PCR amplification with CR-65 specific primers using a cDNA library as template resulted in a cDNA sequence of 1316 bp with an open reading frame of 960 bp, which is flanked by 32 nt at the 5’ end and by 324 nt at the 3’ end including a poly-A tail. A conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) suggested that the CR-65 protein is structurally related to the α/β-hydrolase fold protein family (Ollis et al., 1992) and has a conserved motif found
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in carboxylesterases (about 40-180 amino acids from the N terminus). Alignment of the deduced amino acid sequence of CR-65 (Fig. 3) with those of 4 of the best TBLASTX hits revealed that CR-65 has 43 % identity to *G. echinata* HID and 45 % identity to HID from *Glycine max* (Akashi et al., 2005). The other two TBLASTX hits were unpublished cDNA sequences from *R. communis* (XP_002517206) and *Actinidia eriantha* (ABB89023) having conserved carboxylesterase domains and which showed 46 % identity to CR-65.

**Phylogenetic analysis of HIDs and the carboxylesterase gene family**

The conserved domain search on NCBI database revealed that the CR-65 protein belongs to the carboxylesterase family, which was recently reported to consist of 7 clades by phylogenetic analysis of 20 Arabidopsis proteins (representing 6 clades) and other plant carboxylesterase-like proteins (Marshall et al., 2003). We performed the phylogenetic analysis of HID-like carboxylesterases for those to which CR-65 showed significant similarity. We used *S. cerevisiae* carboxylesterase as an out-group for the analysis. The phylogenetic relationships between carboxylesterases are essentially in agreement with the reported classification (Marshall et al., 2003).

*C. roseus* CR-65 located in a small group of proteins in clade IIIB, which contains HIDs from leguminous plants and two putative HIDs from *R. communis* and *A. eriantha*. It is possible that enzymes for (iso)flavonoid biosynthesis and alkaloid biosynthesis have evolved from the same ancestor, assuming that CR-65 is a TIA biosynthesis enzyme.

**CR-65 transcripts accumulate in response to ORCA2 overexpression and MeJA**

It was evident from cDNA-AFLP analysis that the CR-65 transcript tag was upregulated in ORCA2 overexpressing cell lines upon treatment with estradiol, which was not the case in ORCA3 and GFP overexpressing cell lines (Chapter 2). The expression of CR-65 in ORCA2, ORCA3 and GFP cell lines and in wild type cell lines was verified by Northern blot hybridization. Consistent with the cDNA-AFLP results the CR-65 transcript level was upregulated in ORCA2 cell lines when treated with estradiol compared to DMSO-treated samples. The accumulation of CR-65 mRNA was not affected in ORCA3 and GFP cell lines treated with estradiol. When wild type cell lines were treated with MeJA the expression was upregulated compared to the DMSO-treated cells (Fig. 5). Yeast elicitor also induced CR-65 mRNA accumulation.
Figure 4. Alignment of the deduced amino acid sequence of CR-65 with those of other members of the carboxylesterase superfamily. Functionally equivalent amino acids are shown against a gray background while identical amino acids are shown against a black background.

Ge2-HID, *Glycyrrhiza echinata* 2-Hydroxyisoflavanone dehydratase (BAD80839.1); Gm2-HID, *Glycine max* 2-hydroxyisoflavanone dehydratase (BAD80840.1); RcCP, *Ricinus communis* putative catalytic protein (XP_002517206.1); AeCXE, *Actinidia eriantha* carboxylesterase (ABB89023.1).

Figure 6. CR-65 transcripts accumulate in response to ORCA2 overexpression, MeJA and YE. Expression pattern of CR-65 mRNA detected by Northern blot hybridization. Wild type (WT) cells were treated with MeJA (M), DMSO (D) or yeast elicitor (YE) for 24 hours. O2 and O3 indicate samples from ORCA2 and ORCA3 cell lines respectively. Samples from negative control lines are indicated with GFP. Transgenic cell lines were treated for 24 hours with estradiol (E) or DMSO (D). The bottom panel shows the ethidium bromide-stained gel prior to blotting.
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**Discussion**

The elucidation of biosynthetic pathways in higher plants leading to structurally complex alkaloids, or natural products in general, can usually be achieved by isolation of each individual enzyme, catalysing each reaction as part of a multistep process. If the isolation is successful, reaction mechanisms and regulatory aspects of each enzyme can be investigated. Because of the low enzyme concentrations involved in secondary metabolism, it is, however, tedious to obtain sufficient enzyme amounts, which means that from several kilograms of fresh cell material only a few micrograms of pure enzyme might be available. This technical difficulty has so far prevented the accumulation of a broad knowledge of alkaloid biosynthesis pathways. A recent trend is to avoid biochemical purification and to directly isolate candidate clones by combination of expression pattern analysis and homology-based screening. A variation of this approach is to look for genes with altered expression in regulatory mutants of TIA biosynthesis, however such mutants do not exist for *C. roseus*.

Luckily two regulators of the TIA biosynthetic pathway in *C. roseus* have been described (Menke et al., 1999; van der Fits and Memelink, 2000). The transcription factors ORCA2 and ORCA3 regulate the MeJA-responsive expression of at least half of the known genes (Chapter 2). For the identification of new TIA pathway candidate genes in *C. roseus* we inducibly overexpressed ORCA2 and ORCA3 in cell suspension culture and differentially expressed genes were monitored by cDNA-AFLP technology. This approach resulted in sets of transcript tags which were either
up-regulated by both ORCAs or specifically by one of the two (Chapter 2). Combination of cDNA-AFLP data and metabolite analysis led us to identify cathenamine reductase which is responsible for the conversion of cathenamine to ajmalicine (Chapter 4). The collection of tags isolated by cDNA-AFLP analysis most likely represents more missing TIA pathway genes.

Looking at that possibility, one of the candidates is the tag CR-93, which was up-regulated by both ORCA2 and ORCA3. Upon TBLASTX search CR-93 showed a high level of similarity to a TIA pathway gene encoding polyneuridine aldehyde esterase (PNAE). PNAE catalyses the conversion of polyneuridine aldehyde into epi-vellosimine during the formation of ajmaline and sarpagine in the medicinal plant *R. serpentina* (Dogru et al., 2000). The ajmaline/sarpagine type alkaloids are not synthesized in *C. roseus* but a similar enzymatic reaction may be possible in biosynthesis of other TIAs. Therefore we isolated the full-length sequence of CR-93. cDNA library screening by PCR resulted in a 1128 bp sequence having 100 % nucleotide identity to the CR-93 cDNA-AFLP tag.

Protein-protein based homology search revealed that CR-93 protein belongs to the extremely divergent superfamily of α/β hydrolases, whose members contain a catalytic triad consisting of the conserved amino acids Ser, Asp, and His. The members of this superfamily display a large range of enzymatic activities (Holmqvist, 2000). The alignment of the deduced amino acid sequence of CR-93 with those of five other members of the α/β hydrolase family showing the highest level of similarity to CR-93 revealed that it also contains the conserved triad Ser93, Asp223, and His251. The highest identity (70 %) was with PNAE. Besides PNAE, the α/β hydrolase superfamily contains proteins involved in plant defence and phytohormone-mediated responses. CR-93 showed second highest identity to salicylic acid binding protein 2 (SABP2) (55% identity) from *N. tabacum*. The tobacco SABP2 protein is required for full local resistance and also for systemic acquired resistance to pathogen infection (Kumar and Klessig, 2003). SABP2 has salicylic acid-stimulated lipase activity and the authors suggested that the protein acts as a salicylic acid receptor involved in the transmission of the defence signal. In addition CR-93 showed 47 % identity to ethylene-induced esterase (EIE) from *C. sinensis* (Zhong et al., 2001), methyl jasmonate esterase (MJE) from *S. lycopersicum* (Stuhlfelder et al., 2004). Ethylene upregulates the expression of the EIE gene in *C. sinensis* (Zhong et al., 2001), whereas methyl jasmonate is the substrate of the MJE protein isolated from tomato cell cultures (Stuhlfelder et al., 2004). MeJA cleaving esterase activity was also reported from a cell culture of *C. roseus* (Stuhlfelder et al., 2002). We looked into the possibility that CR-93 may accept MeJA as substrate using recombinant CR-93 protein expressed in *E. coli*, but did not find enzymatic activity towards MeJA. We also checked whether recombinant CR-93 enzyme accepts geranyl acetate as a substrate because geranyl acetate esterase is a common esterase found in plants (Neelam et al., 2007). Like MeJA, geranyl acetate was also not accepted by CR-93 protein. CR-93 also showed 47 % identity to *C. roseus* CrPS. The accumulation of CrPS mRNA and protein was shown to be associated with the accumulation of TIAs in a *C. roseus* cell suspension culture (Lemenager et al., 2005). The specific function of this protein is unknown.

The regulation of CR-93 mRNA by ORCAs and its responsiveness to MeJA and yeast elicitor indicated that this gene might be involved in TIA biosynthesis in *C. roseus*. In a literature
survey we did not find any described metabolic step dependent on esterase activity which means that it may be involved in an unknown metabolic step. Silencing of this gene in alkaloid-producing cell lines may lead to a clue about its role.

The transcript tag CR-65 was up-regulated specifically by ORCA2 alone. A TBLASTX search found a relationship to the carboxylesterase gene superfamily. Metabolite profiling of ORCA2 and ORCA3 overexpressing cell lines resulted in the identification of an ORCA3-specific sub-pathway, but we did not identify an ORCA2-related sub-pathway (Chapter 3). We think that the characterization of an ORCA2-specific gene may indicate the existence of an ORCA2-controlled TIA sub-pathway. Therefore we isolated the full-length cDNA of CR-65. Northern blot analysis of CR-65 mRNA accumulation validated the expression profile observed in cDNA-AFLP analysis. Phylogenetic analysis revealed that CR-65 falls in a small subgroup of the carboxylesterase family together with genes involved in flavonoid biosynthesis. The similarity of CR-65 to other secondary metabolite biosynthesis genes also points to its involvement in the biosynthesis of secondary metabolites. Interestingly another tag, CR-48, also gave a TBLASTX hit to *G. echinata* 2-Hydroxyisoflavanone dehydratase, but it was regulated by both ORCA 2 and ORCA3 (Chapter 2).

**Acknowledgments**

Ward de Winter is acknowledged for expert help with cell suspension cultures. G.H. was partially supported by the 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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