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CHARACTERIZATION OF *G10H* PROMOTER AND ISOLATION OF WRKY TRANSCRIPTION FACTORS INVOLVED IN *CATHARANTHUS* TERPENOID INDOLE ALKALOID BIOSYNTHESIS PATHWAY

DISSERTATION

A dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in
the College of Agriculture
at the University of Kentucky

By

Nitima Suttpanta

Lexington, Kentucky

Director: Dr. Ling Yuan, Associate Professor of Plant and Soil Sciences

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

CHARACTERIZATION OF G10H PROMOTERAND ISOLATION OF WRKY TRANSCRIPTION FACTORS INVOLVED IN CATHARANTHUS TERPENOID INDOLE ALKALOID BIOSYNTHESIS PATHWAY

Catharanthus roseus produces a large array of terpenoid indole alkaloids (TIAs) that are an important source of natural or semi-synthetic anticancer drugs. Biosynthesis of TIAs is tissue-specific and induced by certain phytohormones and fungal elicitors, indicating the involvement of a complex transcriptional control network. However, the transcriptional regulation of the TIA pathway is poorly understood. This study reports the isolation and characterization of the *G10H* promoter and two WRKY transcription factors regulating TIA biosynthesis.

Geraniol 10-hydroxylase (G10H) controls the first committed step in the biosynthesis of terpenoid indole alkaloids (TIA). The *C. roseus G10H* promoter sequence was isolated by a PCR-based genome walking method. Sequence analysis revealed that the *G10H* promoter contains several potential eukaryotic regulatory elements involved in regulation of gene expression. For functional characterization, fusion constructs of *G10H* promoter fragments with the *GUS* reporter gene were generated and expression was analyzed in a tobacco protoplast transient expression assay. Gain-of-function experiments revealed the presence of three potential transcriptional enhancers located in regions between -191 and -147, -266 and -188, and -318 and -266, respectively. The *G10H* promoter was capable of conferring stable GUS expression in transgenic tobacco plants and *C. roseus* hairy roots. In transgenic tobacco seedlings, GUS expression was tissue-specific, restricted to the leaf and actively growing cells around the root tip. GUS expression was not detected in the hypocotyls, root cap and older developing areas of the root. The GUS expression in both transgenic *C. roseus* hairy roots and tobacco seedlings were responsive to fungal elicitors and methyljasmonate. Compared to other known promoters of TIA pathway genes, the G10H promoter contains unique binding sites for several transcription factors, suggesting that the G10H promoter may be regulated by a different transcriptional cascade.

The majority of TIA pathway gene promoters contain typical W-box elements, which are frequently found to be the binding sites of WRKY transcription factors. CrWRKY1 and CrWRKY2 transcription factors were isolated using a degenerate PCR method. The *C. roseus* WRKY transcription factor, *CrWRKY1* is preferentially expressed in roots and induced by phytohormones, jasmonate, gibberellic acid and ethylene. Overexpression of *CrWRKY1* in *C. roseus* hairy roots up-regulated several key TIA pathway genes, especially *tryptophan decarboxylase* (*TDC*), as well as transcriptional repressors *ZCT1*, *ZCT2* and *ZCT3*. In contrast, CrWRKY1 overexpression repressed the transcriptional activators *ORCA2*, *ORCA3* and *CrMYC2*. Overexpression of a dominant-repressive form of CrWRKY1, created by fusing the

SRDX-repressor domain to CrWRKY1, resulted in down-regulation of *TDC* and *ZCTs* but up-regulation of *ORCA3* and *CrMYC2*. CrWRKY1 bound to the W-box elements of the *TDC* promoter in electrophoretic mobility shift, yeast one-hybrid and *C. roseus* protoplast assays. In CrWRKY1 hairy roots, up-regulation of *TDC* increased TDC activity, tryptamine concentration and resistance to 4-methyl tryptophan inhibition. Compared to control roots, CrWRKY1 hairy roots accumulated up to 3-fold higher levels of serpentine. The preferential expression of CrWRKY1 in roots and its interaction with transcription factors, including *ORCA3*, *CrMYC2* and *ZCTs*, may play a key role in determining the root-specific accumulation of serpentine in *C. roseus* plants.

CrWRKY2 is induced by methyljasmonate induction. In plant, *CrWRKY2* expression is mainly found in young leaves and the stem. The stable transformation of *CrWRKY2* in *C. roseus* hairy roots up-regulated many pathway genes, especially the genes in vindoline biosynthesis. The accumulation of vindoline was observed in CrWRKY2 hairy roots.

KEYWORDS: Terpenoid indole alkaloid biosynthesis, *Catharanthus roseus*, WRKY transcription factors, G10H promoter analysis, Isolation and characterization of transcription factor.

Nitima Suttipanta

November 30, 2011

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Chapter 1

Literature Review

1.1 Introduction

Plants produce secondary metabolites to protect themselves against microbial and herbivore attack or UV irradiation. Certain classes of metabolites also function in beneficial interactions with other organisms, such as anthocyanin pigments and terpenoid essential oils to attract pollinating insects. Secondary metabolites are also economically important to man. Flavonoids and terpenoids, for example, have health-promoting activities as food ingredients, and several alkaloids have pharmacological activities. However, depending on the physiological and developmental stage of the plant cell, production of secondary metabolites has frequently been found to be low (Bourgaud et al.,2001; Wink,2003).

Terpenoid indole alkaloids (TIAs) are elegantly represented in *Catharanthus roseus* L.G. Don (Madagascar periwinkle), a member of the Apocynacease family. *C. roseus* produces more than 130 natural products, including the most important bisindole antitumor agents, vinblastine and vincristine, as well as other TIA antihypertensive agents such as ajmalicine and serpentine. The market prices for vinblastine and vincristine are estimated at $2 million USD /kg and $15 million USD /kg, respectively. *C. roseus* produces vinblastine at 0. 01% of its dry weight and even lower amounts of vincristine in trace amounts (0.0003%) (Zhao and Verpoorte,2007).

Due to the structural complexity of TIAs, as well as complicated reaction steps involving stereochemical constraints, the total syntheses of vinblastine and vincristine have proved difficult (Miyazaki,2007). A better understanding of the structure-activity relationship of these TIA anti-tumor agents may provide new strategies to synthesize valuable natural products or even potentially better derivatives (Harvey et al.,2008). Total synthesis of TIAs within bacteria or yeast is not feasible because not all the enzymes within the pathway have been identified. Moreover, the pathways are too long to be introduced into a single microorganism (Oksman-Caldentey and Inzé,2004). At present, the commercial production of vinblastine and vincristine relies on the semi-synthesis of dimeric compounds from monomers such as vindoline and catharanthine. The conversion of vindoline and catharanthine to vinblastine is carried out through an efficient stereospecific chemical or enzymatic coupling reaction. Vindoline and catharanthine, in the presence of flavin mononucleotide, couple to produce the dihydeopyridinium intermediate (DHPI) under near-UV light radiation (λ_{max} = 370 nm). Subsequently, DHPI can be reduced to

anhydrovinblastine (AVLB) with an overall yield of 50% of the initial amount of vindoline substrate (Hirata et al.,1990; Duangteraprecha et al.,1997). In the presence of triphenylpyrilium hydrogen sulfate, the photo-oxidation product, AVLB, can be increased to yield 77% of the starting compound. Finally, AVLB is converted to vinblastine by treatment with NAD(P)H (Hirata et al.,1990). The enzymatic semi-synthesis of vincristine is catalyzed by the horse radish peroxidase enzyme under basic conditions. The highest reported yield of vincristine is 20% catharanthine precursor and the ability to achieve this depends on the quality of catharanthine (Verma et al.,2007). The pharmaceutical company, Lilly, produces the more valuable vincristine by a semi-synthesis process from vinblastine. Two routes of chemical synthesis are employed. The first route is the isolation of *N*-deformyl vinblastine, which is converted to vincristine by formylation (van der Heijden et al.,2004). In the second route, vinblastine can be converted to vincristine by controlled chromic acid oxidation, or by chemical formylation of demethylvinblastine, which can be obtained from vinblastine via a microbiological *N*-demethylation using *Streptomyces albogiseolous* (Kuboyama et al.,2004).

Plant tissue culture technology has been applied in attempts to produce these useful anticancer drugs more efficiently. The ultimate aim was high level production of TIAs through large scale plant cell cultures. At present, there is no commercial biotechnological production of these alkaloids (van der Heijden et al.,2004). *C. roseus* cells can be cultured at large scales; however, the levels of alkaloid accumulation are too low for commercial purposes. Even the production of ajmalicine and serpentine, the major products from *C. roseus* cell cultures, are lower than the field cultivation plant. In addition, vinblastine and vincristine fail to accumulate in cell cultures in vitro due to the absence of the biosynthesis of vindoline (Zhao and Verpoorte,2007).

After the realization that *C. roseus* cell cultures are unable to produce bisindole alkaloids at industrial scales, it is hoped that metabolic engineering of biosynthetic pathways will provide a more promising approach to economically enhance the production of vinblastine and vincristine through the creation of new high-alkaloid yield cell lines (Verpoorte et al.,2000; Kutchan et al.,2008). The idea is to create high-indole alkaloid yield cell lines by stable genetic overexpression of expected biosynthesis pathways or inhibition of competitive pathways through biotechnological approaches (Verpoorte et al.,2000). A specific enzyme can be selected as a target for cloning to influence the metabolic flux to synthesize the desired secondary metabolite product, thus improving the productivity of desired compounds through plant cell culture (Capell and Christou,2004). In addition, increasing the productivity using cell cultures by metabolic engineering can be achieved by redirecting metabolic flux, suppressing competitive pathways that

use the same precursors, inhibiting the catabolic pathway of the product of interest, or the combination of these (Sweetlove et al.,2003). As in whole plants, however, the outcomes of chosen metabolic engineering strategies in plant tissue and cell lines are often difficult to predict (Grotewold,2008). Effects that result from changing the activity of a single enzyme, or unidentified endogenous feedback and/or feedforward controls, may constrain metabolic pathways in manners that are poorly defined (Taylor,1998). For instance, the overexpression of *strictosidine synthase* (*STR*) in *C. roseus* cell lines improved the levels of ajmalicine, serpentine, catharanthine and tabersonine; however, the high-producing lines were unstable and eventually decreased in productivity (Shanks et al.,1998). In another case, despite increased levels of tryptophan and tryptamine precursors, the induction of *tryptophan decarboxylase* (*TDC*) in *C. roseus* hairy roots did not significantly increase downstream TIA accumulation (Whitmer et al.,2002; Hughes et al.,2004). It was later discovered that the conversion of anthranilate to tryptophan, catalyzed by ASα, could be feedback inhibited by downstream products (Hong et al.,2006).

Successful metabolic engineering depends on the detailed understanding of the enzymatic reactions within a given pathway (Verpoorte et al.,2000). In reality, not all enzymes of the pathway have been characterized and identification of rate-limiting enzymes are difficult to ascertain (Leonard et al.,2009). Therefore, transcription factors that are able to regulate the expression of a set of genes represent a valuable tool for metabolic engineering (Pierre,2004; Taylor and Grotewold,2005; Trantas et al.,2009). By manipulating the expression of a single transcription factor, it is theoretically possible to affect the expression of several coordinately regulated biosynthetic enzymes (Grotewold,2008). Furthermore, the far-reaching effects of transcription factors on cellular activities associated with metabolism include the likelihood of impacting the transport and deposition of metabolites, in addition to pathway flux (Grotewold et al.,1998; Roytrakul and Verpoorte,2007). This provides a potential advantage over the manipulation of pathway genes for metabolic engineering because one or more of these metabolite-processing steps may limit the accumulation process (Taylor,1998; Broun et al.,2006). The feasibility of using regulatory genes has been demonstrated by the activation of anthocyanin production in *Arabidopsis* and maize cells through transcriptional activators R and C (Grotewold et al.,1998; Taylor and Grotewold,2005).

1.2 TIA biosynthesis pathway

Terpenoid indole alkaloids (TIAs) comprise a major group of alkaloids found in a limited number of plant species belonging to plant families *Apocynaceae*, *Loganiaceae*, *Rubiaceae* and *Nyssaceae*. Significant progress in TIA biosynthesis pathway characterization has been made in *C roseus*. From a holistic perspective, the biosynthesis of TIAs in *C. roseus* are coordinately regulated by 30 enzymatic steps involving at least 35 known intermediates (van der Heijden et al.,2004; Facchini and De Luca,2008). All TIAs are biosynthesized from a central precursor molecule, strictosidine, which is produced by the condensation of tryptamine, a nitrogen-containing indole moiety derived from tryptophan, with secologanin, a monoterpenoid component derived from iridoid glucoside.

The biosynthesis of secologanin (Figure 1.1), which contributes the terpenoid moiety to the TIA skeleton, proceeds via the 2-*C*-methyl-*D*-erythriol-4-phosphate (MEP) pathway (Oudin et al.,2007). The first monoterpenoid skeleton, geranyl pyrophosphate (GPP), is produced by GPP synthase (GPPS) which catalyzes the head-to-tail condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAP) to form a C-10 structure (Croteau,1995). This synthase has been characterized in many plant species, including *Mentha piperita* (Dudareva et al.,2004; Bouvier et al.,2005) To date, there has been no reported GPPS identified from *C. roseus*. GPP, a universal precursor for all monoterpenoid compounds, is subsequently dephosphorylated to geraniol (Bouvier et al.,2005; Hedhili et al.,2007). The multi-enzymatic steps involved in the conversion of geraniol to secologanin are not well-understood. Only five cDNAs, encoding P450 dependent geraniol 10-hydroxylase (G10H), acyclic monoterpene primary alcohol dehydrogenase (ADH), monoterpene cyclase (MC), *S*-adenosyl-L-methionine: loganic acid methyltransferase (LAMT) and P450-dependent secologanin synthase (SLS), have been characterized (Ikeda et al.,1991; Collu et al.,2001; Collu et al.,2002; Murata et al.,2008). However, at least 11 enzymatic steps during geraniol to secologanin formation via loganic acid and loganin have been proposed (Loyola-Vargas et al.,2007). The most important step of secologanin biosynthesis is the oxygenation at the C-2 position of geraniol by G10H to form 10-hydroxyl geraniol. To facilitate this enzymatic reaction, G10H also requires a cytochrome P450 reductase (CPR) for its function (Meijer et al.,1993; Canto-Canché and Loyola-Vargas,2001). The up-regulation of *G10H* has been induced by jasmonic acid, cytokinin and ethylene and feedback inhibited by catharanthine, vindoline and vinblastine (Meijer et al.,1993; Collu et al.,2002; Papon et al.,2005). 10-hydroxyl geraniol is subsequently catalyzed by 10-geraniol oxidase (10HGO), in the presence of NADPH oxidoreductase, to produce 10-oxogeraniol which is cyclized into iridodiol by iridodial cyclase

(De Luca,2011). The last step of secologanin biosynthesis from loganin is the oxidative rupture to the methylcyclopentane ring, catalyzed by secologanin synthase (SLS). The cloning and functional characterization of SLS has been reported. SLS, heterologously expressed in *Escherichia coli*, converts loganin into secologanin (Irmler et al.,2000).

The indole moiety of terpenoid indole alkaloids is derived from the shikimate pathway, ending with chorismate (Figure 1.2). In *C. roseus*, anthranilate synthase (AS) catalyzes the conversion of chorismate to anthranilate, however the enzymatic activity of AS is feedback-inhibited by tryptophan which binds to an allosteric site on the α subunit of AS (Poulsen et al.,1994; Radwanski et al.,1996). Thus far, the enzymatic reaction from anthranilate to tryptophan in *C. roseus* remains unclear. In the last step of the shikimate pathway, tryptophan is decarboxylated from tryptamine by tryptophan decarboxylase; TDC (Goddijn et al.,1994). TDC, encoded by single gene localized in the cytosol (De Luca and Cutler,1987), is feedback-inhibited by tryptamine (Meijer et al.,1993). The *TDC* gene exhibits both developmental and inducible regulation by plant hormones and external stress signals, such as fungal elicitors and UV light (Eilert et al.,1987; Pasquali et al.,1992; Ouwerkerk et al.,1999). Hence, it is an attractive target for metabolic engineering of the TIA pathway in order to increase alkaloid production. Overexpression of the *TDC* gene in cell culture and hairy roots could not increase alkaloid production (Canel et al.,1998); however this can be explained by insufficient tryptophan precursor or rapid utilization of tryptamine by other pathways which subsequently transport tryptamine out of tonoplast (Whitmer et al.,1998; Whitmer et al.,2003). In the vacuole, tryptamine is further coupled to secologanin by a highly substrate specific enzyme, strictosidine synthase (STR), to produce gluco-alkaloid strictosidine (Figure 1.3) (De Luca and Cutler,1987; Dewaal et al.,1995). In *C. roseus*, at least seven STR isoforms have been identified (de Waal et al.,1995). As STR is encoded by a single gene, its isoforms are likely a result of post-translational modification of the enzyme (McKnight et al.,1991; Pasquali et al.,1999). The expression of the *STR* gene is down-regulated by auxin (Pasquali et al.,1992) and up-regulated by a fungal elicitor and methyl jasmonate (MeJA) (Menke et al.,1999). Strictosidine, a precursor of all TIAs, is subsequently reacted upon by strictosidine *β*-glucoside (SGD) to yield the highly reactive ring-open dialdehyde intermediate (Geerlings et al.,2000; Barleben et al.,2005). The unstable strictosidine aglycone undergoes spontaneous conversions to yield either cathenamine or equally equilibrated to 4,21-dehydrogeissoshizine that can also be routed toward catharanthine and vindoline biosynthesis (Luijendijk et al.,1996; Facchini and St-Pierre,2005). In *C. roseus*,

SGD is encoded by a single gene and *SGD* gene expression is induced by MeJA with kinetics similar to *TDC* and *STR* (Luijendijk et al.,1998; St-Pierre et al.,1998).

The biosynthesis route from strictosidine to serpentine has not been completely elucidated Felix et al.,1981 proposed cathenamine is converted to ajmalicine (Figure 1.2). However the reported ajmalicine formation was demonstrated in a cell free extract experiment, in which crude extracts of cathenamine reductase from *C. roseus* suspension culture catalyzed the formation of ajmalicine from cathenamine using NADPH as a cofactor (Hemscheidt and Zenk,1985). Moreover, ajmalicine was proven to be formed via epi-cathenamine, an intermediate of the SGD reaction (Luijendijk et al.,1998). Ajmalicine is subsequently oxidized to serpentine in the vacuole by peroxidase (Blom et al.,1991). Serpentine cannot travel across tonoplast membrane, hence it accumulates in vacuoles (Guirimand et al.,2011).

The branches toward the synthesis of catharanthine and tabersonine, (Figure 1.2) were proposed to go from strictosidine via 4, 21-dehydrogeissochizine, stemmadenine and dehydrosecodine (Battersby et al.,1969). The feeding of stemmadenine precursor into *C. roseus* suspension was catalyzed to catharanthine and tabersonine (El-Sayed et al.,2004). While biosynthesis of the catharanthine is still undefined, the route towards vindoline has been intensively studied at the gene and associated enzymes levels (St-Pierre et al.,1999; Laflamme et al.,2001; Loyola-Vargas et al.,2007). Diversion of flux from cathenamine intermediate to vindoline is facilitated by six enzymatic steps in aerial tissue of plant (Figure 1.4). The sequence of reactions include the conversion of tabersonine to 16-hydroxyl tabersoine by cytochrome P-450 dependent tabersonine-16-hydroxylase (T16H), installs a hydroxyl group at 16-position of tabersonine, which is subsequently methylated to 16-hydroxytabersonine by 16-*O*-methyltransferase; 16OMT (Schröder et al.,1999). The conversion of 16-methoxytabersonine to 16-methoxy-2,3-dihydro-3-hydroxytabesonine occurs via an uncharacterized hydratase. The subsequent step requires a chloroplast thylakoid membrane-associated *N*-methyltransferase (NMT) to convert 16-methoxy-2,3-dihydro-3-hydroxytabesonine to 16-methoxy-2,3-dihydro-3-hydroxy-*N*-methyltabesonine (Dethier and De Luca,1993; Liscombe et al.,2010). The two terminal steps of vindoline biosynthesis are catalyzed by a light-regulated deactetylvindoline-4-hydroxylase; D4H (Vazquez-Flota et al.,1997) and deacetylvindoline-4-*O*-actyltransferase; DAT (St-Pierre et al.,1998), respectively, that are expressed only in idioblast and laticifer cells (De Luca and Cutler,1987; De Carolis et al.,1990; St-Pierre et al.,1999; Shukla et al.,2006; Campos-Tamayo et al.,2008; Guirimand et al.,2011). Furthermore, overexpression of *DAT* in hairy root culture did not

increase vindoline production but improved the accumulation of another root specific monoterpenoid indole alkaloid, horhammericine (Magnotta et al.,2007).

In Contrast to aerial tissues, the conversion of tabersonine to lochnericine (Figure 1.5) in roots is catalyzed by a NADPH and O_2-dependent tabersonine-6,7-peroxidase; T6, 7E; (Rodriguez et al.,2003). Lochnericine can be catalyzed to horhammericine by tabersonine 19-hydroxylase, T19H. Alternatively, tabersonine is routed towards horhamericine by T6, 7E. Minovincinine and/or horhamericine can be acetylated to yield 6, 7-dehydro-echitovenine and/or 19-*O*-acetyl-horhamericine by minovincinine-10-hydorxy-*O*-acetyl transferase (MAT), which is localized in cortical cells of the growing root tip. MAT is responsible for the synthesis of 6, 7- dehydro-echitoventine and/or 19-*O*-acetylhorhamericine containing 78% amino acid sequence homology to DAT. Co-expression of *TDC* and *STR* along with *MAT* within cortical tissue of *C. roseus* hairy root also point towards a likelihood that the entire TIA pathway for tabersonine synthesis occurs in roots (Laflamme et al.,2001). The ability of MAT to catalyze the 4-*O*-acetylation of deacetylvindoline at low efficiency might also explain the presence of trace amounts of vindoline in *C. roseus* hairy roots previously reported (O'Keefe et al.,1997; Shanks et al.,1998).

The final step (Figure 1.3), the dimerization of vindoline and catharanthine, catalyzed by α-3', 4-anhydro vinblastine synthase (AVLBS), producing vinblastine and vincristine, indicates the completion of TIAs biosynthesis in *C. roseus* (Roytrakul and Verpoorte,2007; Costa et al.,2008; Kumar et al.,2011). The dimerization reaction of vindoline and catharanthine has been proposed that an unstable imminium ion intermediated may be formed prior, then reversibly re-arranged to 3,4-anhydrovinblastine or converted to vinblastine (Sottomayor and Ros Barceló,2003). AVLBS, commonly known as PRX1, is a member of class III basic peroxidase enzymes (Sottomayor et al.,1998). However, at least five isozymes of peroxidase have been implicated in this reaction (Sottomayor et al.,1998; Sottomayor and Ros Barceló,2003; Sottomayor et al.,2004). The expression of *PRX1* in *C. roseus* can be investigated after 6 days of seed germination and is found to localize solely in aerial tissue, but not in roots. Recently, three novel members of class III basic peroxidases have been characterized in *C. roseus* (Kumar et al.,2007; Jaggi et al.,2011; Kumar et al.,2011). Unlike vacuolar CrPRX1 (AVLBS), one of these peroxidase enzymes, named CrPRX, was found to be apoplastic in nature and ubiquitous in all plant tissues with the exception of very young leaves. Moreover *CrPRX* displayed equally expression levels in roots, further backdrop establishing a role in production of root-specific TIAs in *C. roseus* hairy root cultures (Jaggi et al.,2011).

1.3 Intercellular compartmentation of TIA pathway

TIA biosynthesis pathways are under strict developmental regulation. This causes major problems in the exploitation of *C. roseus* cell cultures for the production of valuable alkaloids (Ficchini,2001). To date, significant amounts of dimeric monoterpenoid indole alkaloids, such as vinblastine and vincristine, have been produced in either cell cultures or hairy root cultures. Only accumulation of monomeric terpenoid indole alkaloids, such as tabersonine and ajmalicine, have been observed in *C. roseus* cell cultures. The explanation for the discrepancies of dimeric monoterpenoid indole alkaloid production in *C. roseus* cell cultures relies on the specificity of spatial and temporal regulation of TIA biosynthesis pathway (Sato and Yamada,2008)

Much progress about spatial organization and transportation of TIAs in *C. roseus* have been made through in situ hybridization and immunocytochemical localization studies (Mahroug et al.,2007). The use of in situ RNA hybridization and immunolocalization have revealed that TIA biosynthesis is a highly dynamic, complex and compartmentalized process in *C. roseus* (Kutchan,2005). TIA enzymes are localized in different cell types in leaves and root tips, as well as in different cellular compartments of the cell can be detected (Murata and Luca,2005). Compartmentation and localization of the TIA pathway are considered as regulatory mechanism, since these localizations require the transport of different metabolites from one location to another, where the metabolite are further transformed. Different types of transporter have suggested to be involved in the trafficking of intermediates (Yazaki et al.,2006).

In situ RNA hybridization studies revealed that MEP pathway genes, *DXS*, *DXR*, *MECS* and *G10H*, encoding the first committed enzymes are predominantly expressed in the internal phloem-associated parenchyma (IPAP) of the leaf vasculature (Burlat et al.,2004). Furthermore, immunocytochemistry studies have provided quantitative information support MEP pathway enzymes enrichment in IPAP cells, but rarely in other cell types (Oudin et al.,2007). Expression of *SLS* (CYP72A1), *TDC* and *STR* are observed in the epidermis, while *D4H* and *DAT* are significantly accumulated in the idioblast and laticifer cells embedded in the palisade tissue of leaves. The expression of these TIA genes in leaves follow an expression gradient that presents the highest expression, in young leaf cells near the point of attachment with nodes, and gradually to the lowest, in mature tissue toward the leaf tip (St-Pierre et al.,1999; Irmler et al.,2000). In underground tissue, *TDC*, *STR* and *MAT* transcripts are expressed in the apoplastic region of the root apical meristem (Laflamme et al.,2001; Moreno-Valenzuela et al.,2003; Rodriguez et al.,2003).

Previous studies of the TIA pathway in *C. roseus* hairy roots showed the accumulation of tabersonine and catharanthine along with their substituted derivatives lochnericine, horhammericine and catharanthine in cortical cells of roots, which indicate to root specificity involved in the regulation of TIA biosynthesis. However, it has not been determined whether biosynthesis of catharanthine occurs independently in leaf epidermis or whether alkaloid is produced in roots and then transported to aerial tissues (Facchini and De Luca,2008). The biosynthetic competence in rootless shoot culture to produce both catharanthine and vindoline has been reported, however, the leaf epidermis might support the formation of both alkaloids (Hirata et al.,1990; Vazquez-Flota and De Luca,1998; Shukla et al.,2010)

Many puzzles of the TIA pathway have been resolved by using laser capture microdissection (LCM) and carborundum abrasion (CA) (Murata and Luca,2005; Levac et al.,2008; Murata et al.,2008; Roepke et al.,2010). The LCM technique allows precise harvesting of different cell types from plant tissues followed by the isolation of their mRNA (Murata and Luca,2005). The analysis of TIA pathway genes assisted by LCM-based RT-PCR in *C. rosus* leaves showed that *TDC*, *STR*, *D4H* and *DAT* followed an expression pattern consistent with those obtained from *in situ* hybridization experiments (St-Pierre et al.,1999). While TDC and STR were harbored in epidermal cells, D4H localized in epidermis, idioblast and laticifer and DAT was only found in laticifer. Furthermore, T16H and SGD were mainly expressed in the epidermis, whereas ORCA3 was found in all four cell types. The expression of G10H, earlier suggested to be confined in vascular tissue, was present in epidermis and laticifer (Murata and Luca,2005).

The CA technique was employed as a complementary approach to obtain epidermis-enriched leaf extracts in order to monitor enzyme, gene expression profiles and alkaloid quantification (Levac et al.,2008). The accumulation of vindoline and catharanthine in CA extracts of upper and lower epidermis were significantly lower compared to whole leaf. However the level both compound was highly present in the central of leaf which is located with palisade-, laticifer-, idioblast- and vasculature cell. At the same time, tabersonine and 16-methoxytabersonine contents were significantly higher in epidermis, than in the whole leaf (Murata and Luca,2005). The maximum activity of TDC was observed in epidermal cells, while DAT activity was detected in the whole leaf. In addition, 16OMT activity was also found in abaxial and adaxial epidermal cells, whereas NMT activity was detected only in the whole leaf, this observation support the localization of NMT in chloroplast thylakoids. RT-PCR analysis of *TDC*, *G10H*, *SLS*, *SGD*, *T16H*, *D4H*, *DAT* and *ORCA3* in abaxial and adaxial epidermal cells was also consistent with the enzymatic activity profile. The presence of *STR* and *SGD* transcripts in the epidermis indicate both the synthesis of

strictosidine and its deglycosylation reaction present in the same cell type. As mentioned earlier, G10H was previously shown to be localized in vascular cells (Burlat et al.,2004). The presence of G10H and other MVA transcripts in leaf epidermis indicated it as another site of secologanin biosynthesis (Murata et al.,2008). The CA approach also led to the generation of a leaf epidermone-enriched cDNA Library that facilitated functional isolation and characterization of several genes associated with the TIA pathway from leaf epidermis (Figure 1.6). The 16OMT, predominantly expressed in leaf epidermis, was isolated from the cognate cDNA, however the remaining uncharacterized biosynthetic pathway will be found in the epidermis-enriched EST database (Levac et al.,2008). A homology-based cloning strategy was used to identify LAMT which catalyzes the second-to-last step of secologanin biosynthesis. As expected, LAMT activity was enriched in leaf epidermis compared with whole-leaf extract. The abundance of LAMT transcript along with one EST of G10H also suggests the complete secologanin biosynthesis occurs in epidermal cells (Murata et al. 2008). Recent studies of TIA accumulation in *C. roseus* leaves showed that catharanthine is mainly present in the waxy layer of the leaf surface. This may serve to restrict the formation of cytotoxic/antimitotic bisindole alkaloids, vinblastine and vincristine, from catharanthine and vindoline, unless required by the plant. The catharanthine present in the wax layer of the leaf could serve as a protection mechanism from insect herbivores or fungus infection (Roepke et al.,2010).

1.4 Subcellular compartmentation of TIA pathway genes and enzymes

The subcellular organization of the TIA biosynthetic pathway is complex and appears to involve numerous distinct subcellular compartments, as well as the necessity of inter-organelle trafficking for some intermediates (van der Heijden et al.,2004; Facchini and St-Pierre,2005; Mahroug et al.,2006). An understanding of the subcellular compartmentation of the TIA pathway will provide insight into whether various enzyme characteristics observed in vitro, such as inhibition by pathway intermediates, are representative of true regulatory function in vivo (St-Pierre et al.,1999; Ficchini,2001; Burlat et al.,2004; Kutchan,2005). Only a few numbers of enzymes have been studied and the earlier information regarding subcellular localization was mainly obtained from density gradient analysis. Moreover, this kind of analysis led to contradictory predictions of localization for some enzymes such as STR that has been successively predicted to be localized to the cytoplasm (De Luca and Cutler,1987) and to the vacuole (Stevens et al.,1993).

During the past few years, immunogold gold staining, GFP-fusion imaging, *in situ* hybridization, *in silico* modeling and transient transformation approaches have been used to assign the

molecular structure and subcellular localization of TIA enzymes (Figure 1.7) (Costa et al.,2008; Guirimand et al.,2009; Guirimand et al.,2011; Guirimand et al.,2011). For secologanin biosynthesis, localization of only two MEP enzymes have been studied. The presence of isopentenyl dimethylallyl diphosphate isomerase (Sapir-Mir et al.,2008) and DXR (Hans et al.,2004) in plastids suggests that geraniol is synthesized in the plastid and transported to the vacuole, where it is hydroxylated to 10-hydroxyl geraniol by G10H. G10H was previously believed to be a pro-vacuolar membrane enzyme (Madyastha et al.,1977); however the latest results have conclusively assigned its subcellular localization in the endoplasmic reticulum (ER). ER localization of G10H was supported by the presence of a transmembrane amino acid signal at the N-terminal end of G10H which is necessary for ER anchoring and exposure of its carboxyl terminal tail towards the cytosol. This model of G10H harbored in ER is consistent with the subcellular localization of cytochrome P450 reductase (CPR), which provides electron to G10H in redox reaction, in ER (Guirimand et al.,2009). The penultimate step of the terpenoid precursor synthesis is achieved by a cytosol-sequestrated LAMT-homodimer. The resulting loganin is next converted into secologanin by SLS. Due to the absence of a C-terminal ER membrane anchoring signal and the presence of loganin and secologanin, SLS has been proposed to operate in the vacuole. However, the results obtained by RNA in situ hybridization in combination with binocular florescence complementation assays and yeast two hybrid analysis clearly established that SLS is targeted to the ER. This is in agreement with the identification of a putative 23-residue transmembrane helix at the N-terminus of the protein (Guirimand et al.,2011). On the other hand, the TDC enzyme operates in the cytosol. Thus, tryptophan, which is synthesized via the shikimate pathway, has to be transported to the cytosol for decarboxylation by TDC to yield tryptamine (De Luca and Cutler 1987; Vazquez-Flota et al. 1997). STR was reported earlier to be localized either to the cytoplasm or vacuole (De Luca and Cutler 1987; McKnight et al. 1991; Stevens et al. 1993). However, localization of STR in vacuole has been demonstrated by a GFP fusion transient transformation experiment and is in agreement with its N-terminal signal peptide followed by a vacuolar sorting-like sequence (Guirimand et al.,2011). Secologanin and tryptamine precursors are then transported, by as yet uncharacterized mechanisms, into the vacuole where condensation of both precursors is carried out by STR to produce strictosidine. Strictosidine is then translocated outside the vacuole by unknown transport systems that allow it be glycosylated by a multimerized complex of SGD in the nucleus. This unknown transportation step appears to be a highly rate-limiting control point, leading to an important increase of the strictosidine pool in vacuoles. The unusual localization of SGD in the nucleus may be essential for its physical difference from the accumulated strictosidine pool in the vacuolar organelle under

normal physiological conditions. A massive activation of strictosidine may occur upon organelle disruption during herbivore feeding or necrotrophic pathogen attack. Induced protein cross-linking and precipitation by strictosidine aglycone could serve/function to deter some herbivores from their feeding habit. The exact role/s of protein cross-linking and precipitation in defense against pathogens is not fully understood. However, it has been hypothesized that protein cross-linking and precipitation could be either deleterious for the herbivore/necrotrophic pathogen enzymatic activities and/or leads to decrease of nutritive value as a food source (Guirimand et al.,2010).

The subcellular localization of pathway enzymes involved with vindoline biosynthesis and dimerization with catharanthine to form vinblastine and vincristine are poorly understood. The biosynthesis of vindoline from tabersonine sequentially takes place in at least in two cell types. T16H is anchored in the ER (St-Pierre et al.,1999), whereas 16OMT forms homodimers in the cytoplasm to facilitate uptake of the T16H conversion product. NMT is associated with the thylakoid membrane of chloroplasts (Dethier and De Luca,1993). D4H and DAT were previously believed to be localized in the cytosol of idioblast and laticifer cells, but has now been confirmed as a small monomer which passively diffuses to the nucleus and resides in nucleocytoplasmic compartment. The formation of a metabolon of four enzymes, consisting of T16H, 16OMT, D4H and DAT, has not been observed. Finally, dimerization of catharanthine and vindoline by AVLBS/PRX1 in vacuolar have been established(Guirimand et al.,2011).

The subcellular compartmentation of TIA biosynthesis and accumulation implicates delivery of alkaloids by a specific transporter to their proper accumulation sites. The subcellular trafficking of pathway intermediates also creates an important level of metabolic regulation that could not occur if enzymes and substrates diffused freely within the cytosol. The H^+ antiporter, ABC transporters have been suggested to be involved in transportation of strictosidine, ajmalicine, catharanthine and vindoline across the tonoplast (Roytrakul and Verpoorte,2007). Up-to-date information related to transport metabolism of TIA enzymes in *C. roseus* is limited. However, an ATP-binding-cassette (ABC) multi drug-resistant transporter, CjMDR1 of *Coptis japonic*a, localized in the plasma membrane of rhizome parenchyma, has been isolated and found to be involved in berberine accumulation in tonoplast (Goossens et al.,2003). Although, the function of most eukaryotic ABC transporters have been well demonstrated to be efflux carriers, functional analyses of CjMDR1 using *Xenopus* oocytes showed that this protein recognizes berberine as its substrate and transports it in an inward direction (Shitan et al.,2003). Thus the accumulation of berberine in rhizome could be explained by translocation of berberine alkaloid biosynthesized in

root tissue, to rhizome where it is trapped by the plasma-membrane-localized CjMDR1 (Yasaki, 2005). The heterologous transformation of *CjMDR1* in *C. roseus* cell cultures significantly enhanced the accumulation of ajmalicine and tetrahydroalastonine (Pomahačová et al.,2009)

1.5 The regulation of terpenoid indole alkaloids in *Catharanthus roseus*

The TIA pathway is regulated by a number of different signaling events within *C. roseus*. Expression of TIA enzymes is regulated by developmental, exogenous and biotic or abiotic stress signals. Studies of germinating seedlings and mature plants showed that alkaloid biosynthesis and accumulation are under developmental control (Aerts et al.,1992). Stimulation of serpentine accumulation in cells and tissue culture, and oxidation of ajmalicine to serpentine are light-dependent (St-Pierre and De Luca 1995). Biosynthesis of the vindoline pathway is also regulated by light. Light exposure of etiolated seedlings activates *T16H*, *D4H*, and *DAT* expressions (St-Pierre and Luca,1995; St-Pierre et al.,1998; Vazquez-Flota and De Luca,1998; Vázquez-Flota and Luca,1998; St-Pierre et al.,1999) Bhadra and Shanks,1997 established a light-adapted *C. roseus* hairy root culture that contain green pigmentation but with lower levels of tabersonine, horhammericine and locnericine accumulation and with increased specific yield of ajmalicine and serpentine, as compared to dark grown cultures .

The role of plant growth regulators (PGRs) in the regulation of *C. roseus* indole alkaloid biosynthesis has been extensively researched (Decendit et al.,1992; El-Sayed and Verpoorte,2004 ;(Pasquali et al.,1992; Zhao and Verpoorte,2007). They affect both growth and secondary metabolite production. Auxin showed a negative influence to TIA alkaloid biosynthesis by decreasing *TDC* and *STR* transcripts; however, exogenous MeJA restored the ability of cells to produce alkaloids. MeJA caused the accumulation of TIAs in cell cultures grown in auxin-free medium. Alkaloid production was reduced in auxin-starved cells treated with octadecanoid pathway inhibitors (Arvy et al.,1994). These results indicate that jasmonic acid (JA) is produced in response to auxin depletion and functions in coordinating biochemical events that lead to TIA biosynthesis (Ficchini,2001). Cytokinins are important growth regulators which regulate many aspects of cell growth and differentiation. On the other hand, addition of zeatin to *C. roseus* cell culture increases accumulation of alkaloids (Garnier et al.,1996; Yahia et al.,1998). Zeatin increases G10H activity and bio-conversion of secologanin to ajmalicine (Decendit et al.,1992). Furthermore, synergistic interactions, between cytokinins and ethylene transduction, induced *G10H* expression (Papon et al.,2005) Gibberellins and cytokinins have

been found to have antagonistic effects on TIA biosynthesis and result the impact on element of signal transduction in *C. roseus* cell culture (Amini et al.,2009).

Since the major role for secondary metabolites in plants is protection from environmental stresses, it follows that biosynthesis of secondary metabolites is often induced by pathogen challenge and wounding (Wink,2003). The treatment of *C. roseus* cell culture with fungal elicitors increased the accumulation of tryptamine and alkaloids (Eilert et al.,1987). Fungal elicitors rapidly induced *TDC*, *STR* and *SGD* gene expression, suggesting that the elicitor-mediated signal transduction pathway consists of relatively few steps that activate pre-existing transcription factors (Moreno et al.,1993; Geerlings et al.,2000). Fungal elicitors also induce JA biosynthesis in *C. roseus* cell culture (Lee-Parsons et al.,2004). Treatment of *C. roseus* hairy roots with MeJA caused an increase in *Asα*, *DXS*, *G10H*, *TDC*, *STR*, *D4H*, *DAT* and *PRX1* transcript levels and enhanced the accumulation of ajmalicine, serpentine, lochnericine and horhamericine (Vázquez-Flota et al.,1994; Rijhwani and Shanks,1998). JA serves as a second messenger for the elicitor signal transduction pathway (Menke et al.,1999). The elicitor first induces JA biosynthesis, which then induces TIA biosynthetic gene expression. For elicitor-induced JA biosynthesis, protein phosphorylation and Ca^{2+} influx are important intermediate events (Menke et al.,1999; Memelink et al.,2001). Upstream of JA, the elicitor also induces medium alkalinization, the production of reactive oxygen species (ROS), and the activation of a mitogen-activated protein kinase (MAPK) (Pauw et al.,2004). Downstream of JA biosynthesis, protein phoshorylation is an important step (Menke et al.,1999). JA finally activates the ORCA transcription factors, which bind and transcriptionally activate the promoters of the *TDC* and *STR* genes (Menke et al.,1999; van der Fits and Memelink,2000).

The plant hormone ethylene (ET) regulates a wide range of developmental processes, as well as responses of plants to stress and pathogen attack. In *C. roseus* cell culture, ethylene steered the pathway toward ajmalicine, serpentine, tabersonine and vindoline (El-Sayed and Verpoorte,2005; Vázquez-Flota et al.,2009). Overexpression of the ethylene-related-signaling-pathway CrETR1 (*C. roseus* ethylene receptor I) gene enhanced the biosynthesis of TIA alkaloids in *C. roseus* cell culture. Synergistic regulation of ET and other plant hormones have been observed. The addition of cytokinin and ET enhanced *G10H* expression in *C. roseus* cell culture (Papon et al.,2005). Pré et al.,2008 showed that the expression of ORA59, a member of Apetala2/Ethylene responsive factor (AP2/ERF), is induced by JA and ET, and synergistically activated by both hormones. Giberellic acid (GA) is generally considered in plants to have positive interaction with auxin and antagonistic effects of cytokinin (Greenboim-Wainberg et

al.,2005). The effects of GA on TIA biosynthesis in *C. roseus* have been poorly investigated. Experiments performed in mature plants (Jaleel et al.,2007) and seedlings (El-Sayed et al.,2004) showed that GA slightly enhances ajmalicine accumulation, whereas this alkaloid was strongly reduced in cell culture (Carpin et al.,1997). However, in vitro studies utilizing hairy roots from different species such as *Artemesia annua* (Smith et al.,1997) or *Datura quercifolia* (Dupraz et al.,1994), provided evidence of the positive effect of GA on secondary metabolism. Amini et al.,2009 reported the effect of GA on the accumulation of ajmalicine in *C. roseus* cell culture is dose-dependent; the addition of nanomolar concentrations of GA was sufficient to both completely counteract the stimulation effect of cytokinin on ajmalicine accumulation, and also dramatically down-regulate G10H gene expression. Moreover, lower amounts of paclabutrazol, an inhibitor of GA biosynthesis, allowed ajmalicine accumulation in auxin treated cells; thus, the inhibitory effect of auxin in these cells could be attributed to a low level of endogenous GA. Abscisic acid (ABA) regulates many plant growth and development processes, seed maturation and adaptation of abiotic stress (Finkelstein et al.,2002). In *C. roseus*, ABA stimulated the accumulation of catharanthine and vindoline (Smith et al.,1987). However, the treatment of tryptamine and loganin fed *C. roseus* cells with ABA did not induce accumulation of alkaloids, but delayed the catabolism of strictosidine (El-Sayed and Verpoorte,2005). Recent studies showed that salicylic acid (SA) and GA treatment of *C. roseus* seedlings resulted in significant increases in vinblastine, vindoline and catharanthine production, whereas ABA and GA had strong negative effects on the three alkaloids (Srivastava and Srivastava,2007; Pan et al.,2010). The molecular mechanisms of TIA biosynthesis regulated by abiotic and biotic stress remain unknown. However, some evidence for ubiquitin-mediated degradation of TDC during plant development confirmed that abiotic/biotic stress involves transcriptional and post-transcriptional control of TIA enzymes and also defines as regulatory control of TIA biosynthesis(Fernandez and de Luca,1994).

1.6. Transcription factors involved in TIA biosynthesis

The biosynthesis of TIAs in *C. roseus* is under transcriptional control. Transcription factors regulate the expression of specific gene(s) through sequence-specific DNA-binding and protein-protein interactions. They can both activate or repress gene expression, resulting in either increased or decreased messenger RNA (Broun et al.,2006). Transcription factors not only regulate the primary metabolite, but also modulate secondary pathways.

TIA production is induced by JA and fungal elicitors. Both induction pathways may intersect, as elicitors can induce JA production. A promoter involved in jasmonate and elicitor responses (jasmonate and elicitor responsive element, JERE) was identified in the promoter region of the *STR* gene, close to the TATA box. The JERE element consists of a 24 bp sequence with a GCC core (Menke et al.,1999; van der Fits and Memelink,2000). The *STR* promoter also contains a G-box sequence (CACGTG) which is commonly present in abiotic and biotic responsive genes. The BA region contains a quantitative enhancer upstream of the promoter (Pauw et al.,2004). By using the JERE element as bait in yeast one hybrid screening, two members of AP2/ERF transcription factors, named ORCA1 and ORCA2 (octadecanoid-responsive *Catharanthus* AP2/ERF-domain), were isolated. ORCA1 contains a single AP2 domain located in the N-terminal region of protein, whereas the *ORCA2* cDNA encodes an AP2-domain in the C-terminal region. Electrophoretic mobility shift assay (EMSA) demonstrates that both proteins specifically bind the JERE element of the *STR* promoter. MeJA and elicitors, through increased *STR* transcript abundance, induced ORCA2, but not ORCA1, activity. The transient expression also indicated that ORCA2 trans-activated the *STR* promoter via direct binding. The third member of *C. roseus* AP2/ERF transcription factors was subsequently isolated by T-DNA activation tagging (van der Fits et al.,2000; Van der Fits and Memelink,2001). ORCA3 showed high similarity to the AP2/ERF DNA binding domain of ORCA2, but amino acid identity elsewhere on the protein was not observed. Like ORCA2, ORCA3 also responds to JA induction and binds specifically to the JERE element in the *STR* promoter. Overexpression of *ORCA3* in *C. roseus* cell culture increases the expression of *AS*, *DXS*, *CPR*, *SLS*, *TDC*, *STR*, and *D4H*. Electrophoresis mobility shift assay (EMSA) and protoplast transient expression assays confirmed that ORCA3 functions as a DNA-binding protein that is directly involved in transcriptional activation of *TDC*, *STR* and *CPR* gene expression. Thus, ORCA3 acts as an important regulator to direct the metabolic flux of the TIA pathway by regulating the expression of genes involved in primary and secondary metabolites. However, interestingly, two JA-responsive genes, *G10H* and *DAT*, are not susceptible to ORCA3 induction, suggesting that additional jasmonic responsive transcription factors are involved in the regulation of these two genes (Memelink et al.,2001). Although ORCA3 plays an important role in regulating TIA biosynthesis, it is not sufficient by itself to regulate the complete pathway. Thus, one or more transcription factors co-operate in regulation of the pathway.

Using the G-box element (-98 to -93 bp) of the *STR* promoter as bait for yeast one hybrid system, cDNA encoding G-box binding factors, CrGBF1 and CrGBF2, were isolated. CrGBF2 showed high affinity to both the *STR* G-box element and the *TDC* G-box element, whereas CrGBF1 had

high affinity to the *STR* G-box but lower affinity for the *TDC* G-box. In transient assays, CrGBF1 and CrGBF2 repressed expression of the *STR* gene, but did not appear to be involved in stress induction (Siberil et al.,2001). Moreover a finding supported result in transgenic tobacco show that the deletion of G-box in *STR* promoter could not reduce CrGBFs elicitor responsiveness (Pasquali et al.,1992). However, these results suggest that the CrGBFs may be involved in the regulation of several TIA biosynthetic genes. Another G-box binding factor, CrMYC1 was isolated and characterized as a basic helix-loop-helix (bHLH) transcription factor. The CrMYC1 mRNA level was induced by fungal elicitor and MeJA, suggesting that CrMYC1 may be involved in the regulation of gene expression in response to these signals (Chatel et al.,2003).

The BA region of the *STR* promoter also acts as a functional unit in elicitor- or jasmonic – responsive gene expression. Yeast one-hybrid screening identified a MYB-like protein, CrBF1 (*C. roseus* box P-binding factor-1), which plays a role in elicitor responsive, but jasmonate-independent, signal transduction acting downstream of protein phosphorylation and calcium influx. However, Ca^{2+} is required for jasmonate biosynthesis and induces ORCAs transcription factors in the downstream pathway (Menke et al.,1999; Memelink et al.,2001). Thus it can implicate that *STR* can be induced via jasmonic or non-jasmonic dependent pathways (van der Fits et al.,2000). Furthermore, ajmalicine production in JA-induced *C. roseus* culture depends on intracellular Ca^{2+} and the addition of exogenous Ca^{2+}(3mM) enhances MeJA-induced ajmalicine production (Lee-Parsons et al.,2004; Lee-Parsons and Ertürk,2005).

By using an elicitor-responsive region of the TDC promoter as bait for yeast one hybrid screening, three members of the Cys2/His2-type (transcription factor IIA-type), ZCT1, ZCT2 and ZCT3, were isolated from *C. roseus* cell cultures. All three ZCTs contained the LxLxL motif involved in the repression of *STR* and TDC expression. DNA binding experiments showed that the ZCTs bound in a sequence-specific manner to several sites of the STR and TDC promoters, which had previously been identified as binding sites of ORCA2 or ORCA3. EMSA experiments indicated that binding of the ZCTs distinctly overlapped into the ORCA3 binding site; therefore they can reduce the activity of ORCA3 by non-competitive inhibition at the same binding site. Moreover, the expressions of ZCTs were induced by methyl jasmonate (MeJA) and yeast extracts (YE)(Pauw et al.,2004).

Expression of the *ORCA* genes are rapidly induced by MeJA (Menke et al.,1999; van der Fits and Memelink,2000), which implies that *ORCA* genes either auto-regulate their own gene expression

level or, alternatively, are regulated by one or more upstream transcription factors. Functional studies of the *ORCA3* promoter identified an autonomous jasmonate responsive element: JERE (Vom Endt et al.,2002) composed of a quantitative sequence responsible for high level expression and a qualitative sequence that acts as an on/off switch in response to MeJA. The *ORCA3-JERE* does not contain sequence similarity to the *STR-JERE*, and the ORCA proteins did not bind to the *ORCA3* promoter *in vitro*, or trans-activate it in transient expression assays (Vom Endt et al.,2007). Hence, ORCA genes are regulated by one or more upstream transcription factors. Recently, a novel basic Helix-Loop-Helix (bHLH) transcription factor, CrMYC2, has been characterized as the major activator of MeJA-responsive *ORCA3* gene expression. The *CrMYC2* gene is an immediate-early jasmonate-responsive gene. CrMYC2 binds to the qualitative sequence in *ORCA3-JERE* in vitro, and transactivates reporter gene expression via this sequence in transient assays. Knock-down of the *CrMYC2* expression level via RNA interference caused a strong reduction in the level of MeJA-responsive *ORCA3* mRNA accumulation. In addition, MeJA-responsive expression of the related transcription factor gene, *ORCA2*, was significantly reduced. The results show that MeJA-responsive expression of alkaloid biosynthesis genes in *C. roseus* is controlled by a transcription factor cascade consisting of the bHLH protein, CrMYC2, regulating *ORCA* gene expression, and the AP2/ERF-domain transcription factors, ORCA2 and ORCA3, which, in turn, regulate a subset of alkaloid biosynthesis genes (Zhang et al.,2011).

1.7 The WRKY transcription factor

WRKY proteins form a large family of transcription factors with their major occurrence in plants. They were generally regarded as being plant-specific. However, WRKY-like proteins were isolated from protist *Giardia lamblia* and the slime mold *Dictyostelium discodeum.* Based on available information, it is hypothesized that the early origin of WRKY genes was in Eukaryotes, which immensely amplified afterwards in the plant lineage (Ülker and Somssich,2004). Members of this family are characterized by a WRKY domain, a 60-amino acid stretch containing the highly conserved WRKYGQK peptide sequence and a zinc finger motif ($CX_{4–7}CX_{22–23}HXH/C$) (Eulgem et al.,2000). 74 and 109 members of WRKY TF have been identified in Arabidopsis and rice, respectively (Eulgem and Somssich,2007). The first WRKY-cDNA clone was characterized in 1994 from sweet potato (Ishiguro and Nakamura,1994). WRKY proteins have specific binding affinity for the consensus W-box motif TTGAC(T/C), a *cis*-acting DNA element in the promoter region of genes (Rushton and Somssich,1998; Yang et al.,1999) However, Sun et al.,2003 reported the alternative binding of a barley WRKY factor, SUSIBA2 (sugar signaling in barley), to a newly identified sugar responsive *cis* element named SURE. Moreover, the

characterization of tobacco NtWRKY12 revealed that this WRKY protein contains a variant WRKYGKK amino acid sequence in the WRKY domain rather than the WRKYGQK sequence found in the majority of WRKY proteins. NtWRKY12 is involved in transcriptional activation of the PR-1a promoter and binds specifically to WK-boxes, TTTTCCAC, in this promoter, but is unable to bind to the consensus W-box (van Verk et al.,2008).

Based on their DNA-binding domain structure and the certain feature of a zinc finger motif, WRKY proteins are divided into three major groups. Proteins with two WRKY domains belong to group I. WRKY proteins containing one WRKY domain belong to groups II or III, depending on the type of zinc-finger motif (Eulgem et al.,2000). Thus, group I WRKY gene were defined by the presence of two WRKY domains, whereas, Group II and III WRKY genes contain only a single domain. Both group I and II have a C_2H_2-type zinc finger motif ($CX_{4-5}CX_{22-23}HXH$) with only the C-terminal domain active in DNA binding. Group II is further divided in to subgroups a-e depending upon the additional amino acids present outside the WRKY domain. Group III differ from I and II in its altered C_2H_2 zinc finger motif $CH_7CX_{23}HXC$. Most WRKY proteins contain a basic nuclear localization signal (Eulgem et al.,2000; Cormack et al.,2002).

The NMR solution structure of *Arabidopsis* WRKY4 revealed that the C-terminal WRKY domain consists of a four-stranded ß-sheet, with a zinc binding pocket formed by conserved Cys/His residues located at one end of the ß-sheet, whereas, the WRKYGQK residues corresponding to the most N-terminal ß-strand (strand ß-1) are kinked in the middle of the sequence by the Gly residue. The concave curvature of strand ß-1 induced by this kink is predicted to enable this strand to deeply enter the DNA groove and make contact with bases of the W box element (Yamasaki et al.,2005). Furthermore, the crystal structure of the extended WRKY domain of *Arabidopsis* WRKY1 (AtWRKY1-C) revealed that this domain forms a globular structure with five β strands forming an antiparallel β–sheet with DNA binding residues located in the β2 and β3 strands (Duan et al.,2007). Some members of the family such as PcWRKY4 and PcWRKY5 from parsley (*Petoselinum crispum*), appear to have leucine zipper sequences, $Lx_6Vx_6Lx_6Mx_6L$ and $Lx_6Lx_6Lx_6I$, toward the N-terminus, which would involve a protein-protein interaction process (Cormack et al.,2002).

The WRKY domain of all WRKY proteins studied to date, show binding preference to their cognate *cis*-acting element, the W-box (TTGAACC/T); however, is not clearly understood how specificity to certain promoters is determined (Ülker and Somssich,2004). The flanking sequence of the W-box and the distinct arrangement of such elements play a role in interaction with

selected WRKY factors. The resulting discrete higher order protein-DNA complex, determines the binding of the WRKY protein, and thereby leads to distinct transcriptional outputs (Ciolkowski et al.,2008). It has been demonstrated for group I WRKYs that the C-terminal WRKY domain, not N-terminal domain, is responsible for sequence-specific binding to DNA. The N-terminal region was predicted to participate in binding to DNA by increasing affinity or specificity for their target sites, or to provide an interface for protein-protein interactions (Eulgem et al.,1999). DNA binding activity was abolished by substitution of conserved cysteine and histidine residues to alanine in the C_2H_2-type zinc finger-motif of the WRKY domain indicating that the cysteine and histidine residues coordinate a Zn^{2+} atom to form a domain structure capable of binding to DNA. Moreover, mutation in the conserve WRKYGQK amino acid sequence at the N-terminal side of the zinc finger motif also significantly reduced DNA-binding activity, thus these residues are required for proper folding of the DNA-binding zinc finger (Maeo et al.,2001). Similarly, replacement of tryptamine, arginine, lysine, tyrosine and glycine of the WRKYGQK motif result in a decrease in DNA-binding activity (Duan et al.,2005).

The functions of various WRKY genes have been elucidated using genetic and molecular approaches. Several WRKY proteins from a number of plants are involved in defense against attack and rapidly up-regulated after pathogen infections or phytohormone treatments. In Arabidopsis, only 49 of the 72 *AtWRKY* genes were differentially regulated in plants infected by an avirulent strain of the bacterial pathogen *Pseudomonas syringae* or treated by SA (Dong et al.,2003). Plants ectopically expressing either *AtWRKY18* or *AtWRKY70* display enhanced resistance towards certain virulent pathogens (Chen and Chen,2000; 2002; Li et al.,2004; Li et al.,2006). Overexpression, RNAi and knock-out lines of AtWRKY53 showed accelerated and delayed senescence phenotypes, respectively (Miao et al.,2004). WRKY proteins bind to W boxes which are found in the promoters of many plant defense genes including the well-studied PR genes (Ülker and Somssich,2004). Regulation of the PR1 promoter by AtWRKY proteins appears to be complex and may involve both activation and repression functions (Turck et al.,2004; Rocher et al.,2005). In addition, W boxes are present in clusters within short promoter stretches suggesting that WRKY proteins may act synergistically with other family members or other classes of transcription factors (Maleck et al.,2000; Eulgem,2005). AtWRKY6 positively influenced pathogen defense-associated PR1 promoter activity. Target gene analyses for AtWRKY6 using cDNA-AFLP identified a gene designated senescence induced receptor kinase (SIRK). Interestingly, AtWRKY6 activates the expression of SIRK, which contains W boxes in its promoter, but represses its own expression (Robatzek and Somssich,2001); however, the

mechanism by which it acts as a repressor is not yet known. Many reports suggest the involvement of specific WRKY factors associated with defense-induced mitogen-activated protein kinase (MAPK) signaling cascades (Asai et al.,2002; Wan et al.,2007). Arabidopsis WRKY proteins, AtWRKY22 and AtWRKY29, were activated by the MAP kinase cascade and the expression of AtWRKY29 led to reduced disease symptoms following bacterial and fungal infections (Asai et al.,2002). In situ RNA hybridization experiments revealed that PcWRKY1, a parsley WRKY protein, was rapidly and locally activated in leaf tissue around fungal infection sites (Eulgem et al.,1999). In tobacco, the N gene-mediated resistance to tobacco mosaic virus was compromised by down-regulation of one of the three WRKY genes (Liu et al. 2004). The induction of WRKY genes together with increased W box binding activity was observed during the activation of the MAPK cascade (AtMEKK1, AtMKK4/AtMKK5 and AtMPK3/AtMPK6) and this observation indicates downstream functions of the bacterial flagellin receptor FLS2, a receptor-like leucine-rich repeat serine/threonine kinase (LRR receptor kinase) involved in perception of flagellin. Transient overexpression of truncated AtMEKK1, constitutively active *AtMKK4* and *AtMKK5*, or *AtWRKY22*/*AtWRKY29* confer resistance to the bacterial pathogen *Pseudomonas syringae* or the fungal pathogen *Botrytis cinerea* (Asai et al.,2002). In addition to *Arabidopsis*, the pathogen responsive mechanisms of WRKYs have been reported in other plant species. In canola (*Brassica napus* L.), transcript abundance of 13 *BnWRKYs* were altered after pathogen infection, and all were responsive to one or more hormones including ABA, ET, SA, JA and auxin (Yang et al.,2009). In *Gossypium hirsutum* L, *GhWRKY3* is up-regulated by application of SA, MeJA, ABA, GA, and ET. Furthermore, the transcripts of GhWRKY3 are enhanced after infection with *Rhizoctonia solani*, *Colletotrichum gossypii* and *Fusarium oxysporum* f. sp. *vasinfectum*, respectively. *GhWRKY3* can also be induced by wounding treatment, but not by cytokinin, auxin, drought, NaCl, and cold (Guo et al.,2011). Some WRKY genes act on the crosstalk among multiple signals. For instance, AtWRKY70 acts as an activator of SA-induced genes but a repressor of JA-responsive genes by integrating signals from mutually antagonistic SA/JA dependent pathways (Li et al.,2004). Expression profiling revealed that AtWRKY70 also influences the expression of other *AtWRKY* factors, including *AtWRKY53* (Dong et al.,2003; Li et al.,2004; Miao et al.,2004). The expression of *AtWRKY62* was induced by both SA and JA. The presence of basal SA is required for MeJA-induced *AtWRKY62* expression, and both chemicals exhibit a synergistic effect on *AtWRKY62* induction (Mao et al.,2007). Research has also focused on additional roles of WRKY factors in plant processes. AtWRKY44 is required for proper trichome development (Johnson et al.,2002). High levels of expression of *AtWRKY6* or *AtWRKY18* led to growth retardation and other stress-related

phenotypes (Chen and Chen,2000; Robatzek and Somssich,2001; Chen and Zhu,2004). Additionally, identification of WRKY factors from plants other than *Arabidopsis* further support their involvement in various cellular processes. The rice WRKY protein, OsWRKY71, acts as a transcriptional repressor of gibberellin-responsive genes (Zhang,2004). Barley HvWRKY38, an orthologue of AtWRKY40, oat ABF2, and rice OsWRKY71 were shown to be involved in cold and drought stress responses (Marè et al.,2004). ScWRKY1 (*Solanum chacoense*), an orthologue of AtWRKY33, has been shown to express strongly, but transiently, in fertilized ovules bearing late torpedo-staged embryos, suggesting a specific role during embryogenesis (Lagacé and Matton,2004).

The biosynthesis of secondary metabolites in plants is widely believed to be part of response to biotic or abiotic stress (Hahlbrock et al.,2003). It has been well-documented that endogenous signal compounds, such as JA, are involved in elicitor-induced secondary metabolite biosynthesis. In various plant species, elicitors were shown to induce accumulation of endogenous JA. JA, itself, increases secondary metabolite production and inhibition of JA biosynthesis abolishes elicitor-induced metabolite accumulation and the expression of secondary metabolite synthesis genes (Menke et al.,1999; Zhao et al.,2005; Wasternack,2007). Elicitors also induce many intracellular events, including cytoplasmic calcium concentration, ion transport, production of reactive oxygen species and protein phosphorylation. The induction of JA biosynthesis by the combination of the above-mentioned events is still largely unknown (Zhao et al.,2005). Nonetheless, these results suggest that JA is involved in elicitor-triggered signal transduction pathways leading to the biosynthesis of secondary metabolites. WRKY proteins have been well demonstrated to regulate defense response and secondary metabolite biosynthesis. For instances, the induction of *Medicago truncatula* cell suspension culture with YE and MJ lead to the up-regulation of several *WRKY* factors and overexpression of four *WRKY* genes in tobacco demonstrated their regulatory roles in lignin deposition, *PR* gene expression, and systemic defense responses against Tobacco mosaic virus (Naoumkina et al.,2007). Moreover, in recent years, many WRKY genes have been isolated from medicinal plants. The *Cj*WRKY1 from *Coptis japonica* represents the first discovery of transcriptional regulator of benzyl isoquinoline alkaloid biosynthesis (BIA). Silencing of Cj*WRKY1* transcripts led to a substantial reduction in the expression of several gene transcripts involved in berberine biosynthesis, whereas expression of Cj*WRKY1* in protoplasts of a berberine-producing *C. japonica* cell line increased expression of berberine biosynthetic genes (Kato et al.,2007). A full-length cDNA *AaWRKY1* was isolated from a cDNA library of the glandular secretory trichomes of *Atemesia annua*, in which

artemisinin is synthesized and sequestered. Transient expression of *AaWRKY*1 cDNA in *A. annua* leaves clearly activated the expression of the majority of artemisinin biosynthetic genes, suggesting the involvement of the AaWRKY1 transcription factor in the regulation of artemisinin biosynthesis and indicating that *Amorpha-4,11-diene synthase* (*ADS*) is a target gene of AaWRKY1 in *A. annua* (Ma et al.,2009). The cotton (+)-δ-Cadinine synthase (CAD1), a sesquiterpene cyclase, catalyzes the first committed step in the formation of the phytoalexin gossypol. Its promoter contains a W-box palindrome with two reversely oriented TGAC repeats, which are proposed binding sites of WRKY transcription factors. Thus, GaWRKY1, an orthologous of AtWRKY18, has been isolated and participated in regulation of sesquiterpene biosynthesis in cotton. Coordinated expression of *GaWRKY1* and *CAD1-A* were observed in floral organs and in response to fungal elicitor treatment (Xu et al.,2004).

1.8 Promoter analysis of TIA biosynthesis genes

The basic plant promoter consists of a core promoter region and upstream *cis*-elements, where are considered as TF-binding sites. Core promoter which is the minimal promoter necessary to direct gene transcription orchestrates pre-initiation complex (PIC) assembly. Transcriptional initiation of protein coding genes by RNA polymerase II involves the stepwise assembly of general transcription factors (GTFs) to the core promoter, usually containing the TATA-box and/or an initiator element, to form a stable PIC. A number of promoters responsive to specifically environmental and hormonal stimuli have been characterized. Promoters induced by environmental cues include light-, heat-, cold-, anaerobic stress-, dehydration- and elicitor-responsive promoters, which are usually associated with plant defenses and survival. Hormonal responsive promoter induced by auxin, abcisic acid (ABA), gibberellic acid (GA), ethylene, salicylic acid (SA) and methyl jasmonate play an important role during growth , development , ripening, flowering and certain stress responses (Venter and Botha,2004). Previous studies showed that TIA biosynthesis could be regulated by a number of biotic and abiotic stimuli, as well as, the stage of plant development (Vázquez-Flota et al.,2000; Whitmer et al.,2002; Vázquez-Flota et al.,2009). These studies also showed that specific pathway genes, such as *TDC*, *STR*, *G10H* and *DAT*, could be activated by various plant hormones. Therefore, experiments for isolating regulators of TIA biosynthesis genes has traditionally focused on the corresponding promoter sequences of JA/elicitor responsive pathway genes. To date, many promoters of TIA pathway have been identified. The upstream sequence of *TDC*, from -1,818 to +198 bp relative to the transcription start site (TSS), was analyzed to identify *cis*-acting elements. The *TDC* promoter contains three functional regions. The region between -160 to -99 bp acts as a main

transcriptional enhancer for basal expression. The two other regions, from -99 to -87 and -87 to -37 bp, respectively, are non-redundant elicitor responsive regions. In vitro binding of a nuclear factor region (-572 to -37 bp) has also been described (Ouwerkerk and Memelink,1999). Only UV-B wavelength could induce TIA expression and accumulation in *C. roseus*. Loss-of-function analysis revealed redundant UV-responsive elements in the *TDC* promoter between -99 to +198 bp (Ouwerkerk et al.,1999). The coordinate accumulation of transcripts suggest that *TDC*, *STR* and *CPR* genes are regulated by common transcription factors in response to elicitor treatment and UV light (Pasquali et al.,1992). The elicitor responsive region of the *CPR* promoter is located between -632 to -366 bp and contains strong GT-1 binding sites (Cardoso et al.,1997). The main elicitor responsive region of STR was identified between -339 to -145 bp regions. A short *STR* promoter region called RV, which contains a JA- and elicitor-responsive element (JERE), is responsible for JA- and elicitor responsive gene expression (Menke et al.,1999). The JERE region of the *STR* promoter is the binding site of ORCA2 and ORCA3. The G-box motif, at -105 bp, was shown to bind G-box binding factors (GBFs) in vitro, but was not essential for elicitor induced *STR* expression in vivo. Compared to other known promoters of TIA pathway genes, the *G10H* promoter contains unique binding sites for several transcription factors, suggesting that the *G10H* promoter may be regulated by a different transcriptional cascade (Suttipanta et al.,2007). DAT is a key enzyme for the terminal step of vindoline biosynthesis. In this research, the DAT gene promoter was cloned, sequenced, and analyzed. An approximately 1,773 bp genomic DNA fragment of the *DAT* promoter was obtained. Sequence analysis revealed that the *DAT* promoter contains several potential regulatory elements involved in regulation of gene expression. Three TGACG motifs and one inverted motif (CGTCA), between -808 and -1,086 bp in the *DAT* promoter, were found to be involved in JA signal transduction pathway (Wang et al.,2010).

1.9 Outline of this dissertation

This research focuses on the terpenoid indole alkaloid biosynthetic pathway in *Catharanthus roseus*, aiming to isolate and functionally characterize the important transcriptional regulator of TIA biosynthesis.

Chapter two focuses on the isolation and characterization of the geraniol 10-hydroxylase (G10H) promoter. G10H, one of the key enzymes of the TIA pathway, catalyzes C-10 hydroxylation of geraniol to form 10-hydroxy geraniol. Previous studies reported the up-regulation of G10H strongly positively correlated with TIA production, suggesting that G10H is a bottle-neck of TIA

biosynthesis (McFarlane et al.,1975; Schiel et al.,1987; Collu et al.,2002; Hong et al.,2003; Papon et al.,2005). The expression of *G10H* is rapidly induced by MeJA (Gantet et al.,1998). Ectopic expression of the JA-responsive transcriptional activator, ORCA3, in *C. roseus* cell culture activates the expression of *DXS*, *CPR*, *TDC*, and *STR* but not that of *G10H*. ORCA3 is necessary but not sufficient to control the TIA pathway and other transcription factors are required (van der Fits and Memelink,2000). The availability and characterization of the *G10H* promoter will facilitate the identification of additional regulators interacting with the promoter and advance our understanding of the regulatory mechanisms underlying TIA biosynthesis.

Chapter three describes the isolation of the CrWRKY1 transcription factor from MeJA-induced *C. roseus* seedlings. Expression profile analysis indicated the expression of *CrWRKY1* is tissue-specific and controlled by phytohormones. We demonstrate that *CrWRKY1* is able to bind specifically to W-box elements in the *tryptophan decarboxylase* (*TDC*) promoter. The functional studies of CrWRKY1 are carried out using yeast one hybrid, *C. roseus* protoplast, and stable overexpression or dominant repressive transgenic *C. roseus* hairy root lines. Furthermore, the accumulations of TIAs in *C. roseus* are analyzed.

Chapter four describes the isolation of the CrWRKY2 transcription factor. Tissue specificity and induction of *CrWRKY2* by phytohormones are studied in *C. roseus* seedlings and plants. The expression TIA pathway genes and TIA accumulation in CrWRKY2 hairy roots indicate the regulatory role of CrWRKY2 in TIA pathway.

Chapter five summarizes the findings of the preceding chapters and discusses possible future directions for research related to functional regulation of TIA pathway.

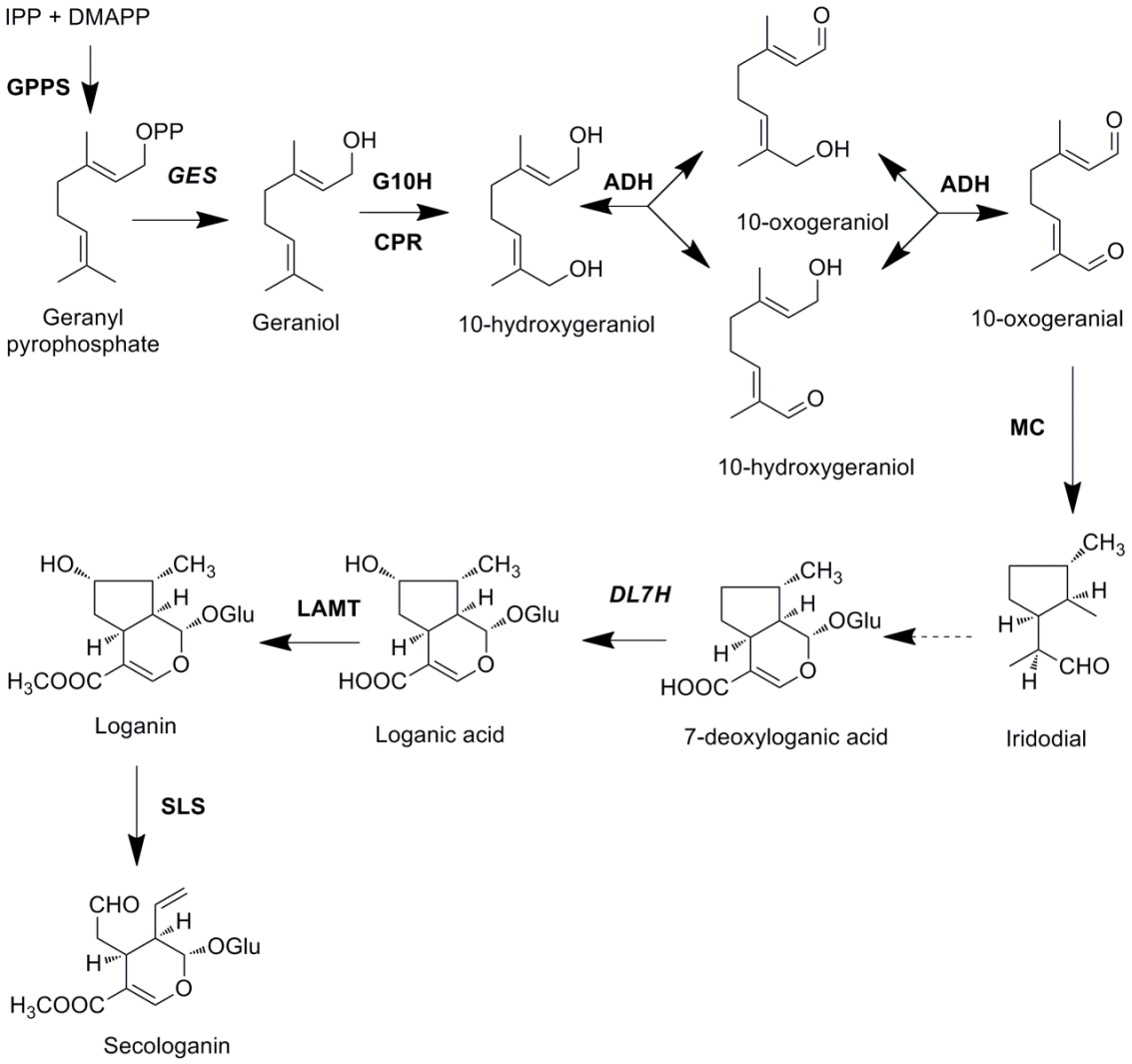

Figure 1.1 Biosynthesis of secologanin in *C. roseus.* Solid arrows represent a single enzymatic step, whereas dash arrows indicate multiple enzymatic steps. The abbreviations of enzymes and uncharacterized enzyme are present in bold and italic respectively. GPPS: geranyl pyrophosphate synthase; GES: geraniol synthase; G10H: geraniol 10-hydroxylase; CPR: cytochrome P450 reductase; ADH: acyclic monoterpene primary alcohol dehydrogenase; DL7H: 7-deoxyloganin 7-hydroxylase; MC: monoterpene cyclase; LAMT: S-adenosyl-L-methionine: loganic acid methyl transferase, SLS: secologanin synthase (Oudin et al., 2007). The reactions between iridodial and 7-deoxyloganin are poorly understood (Imer et al., 200). Adapted from El-sayed and Verproote (2007).

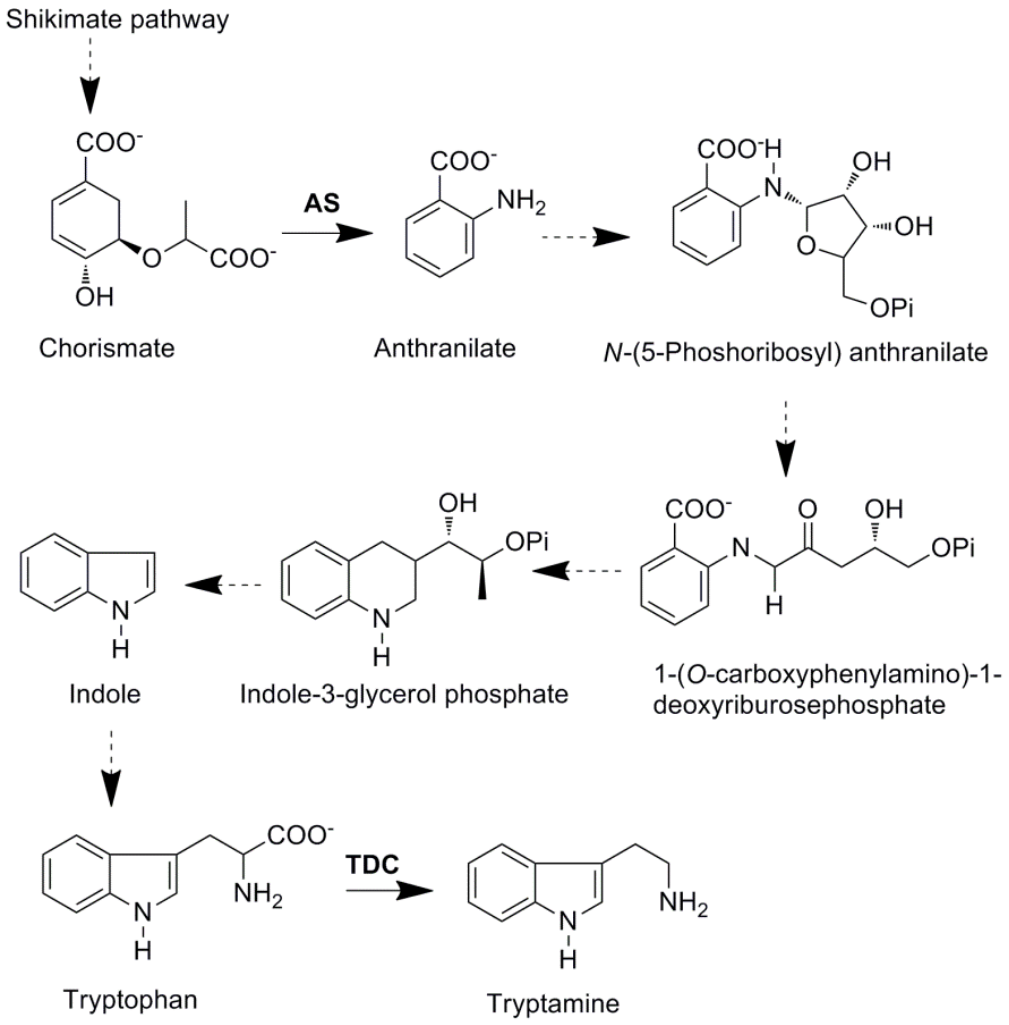

Figure 1.2 Biosynthesis of tyrptamine through the chorismate pathway. Solid arrows represent a single enzymatic step, whereas dash arrows indicate multiple enzymatic steps. The abbreviations of enzymes is present in bold. AS: anthranilate synthase; TDC: trypatophan decarboxylase (Goodigin, 1996, Radwanski, 1996). Adapted from El-sayed and Verproote (2007).

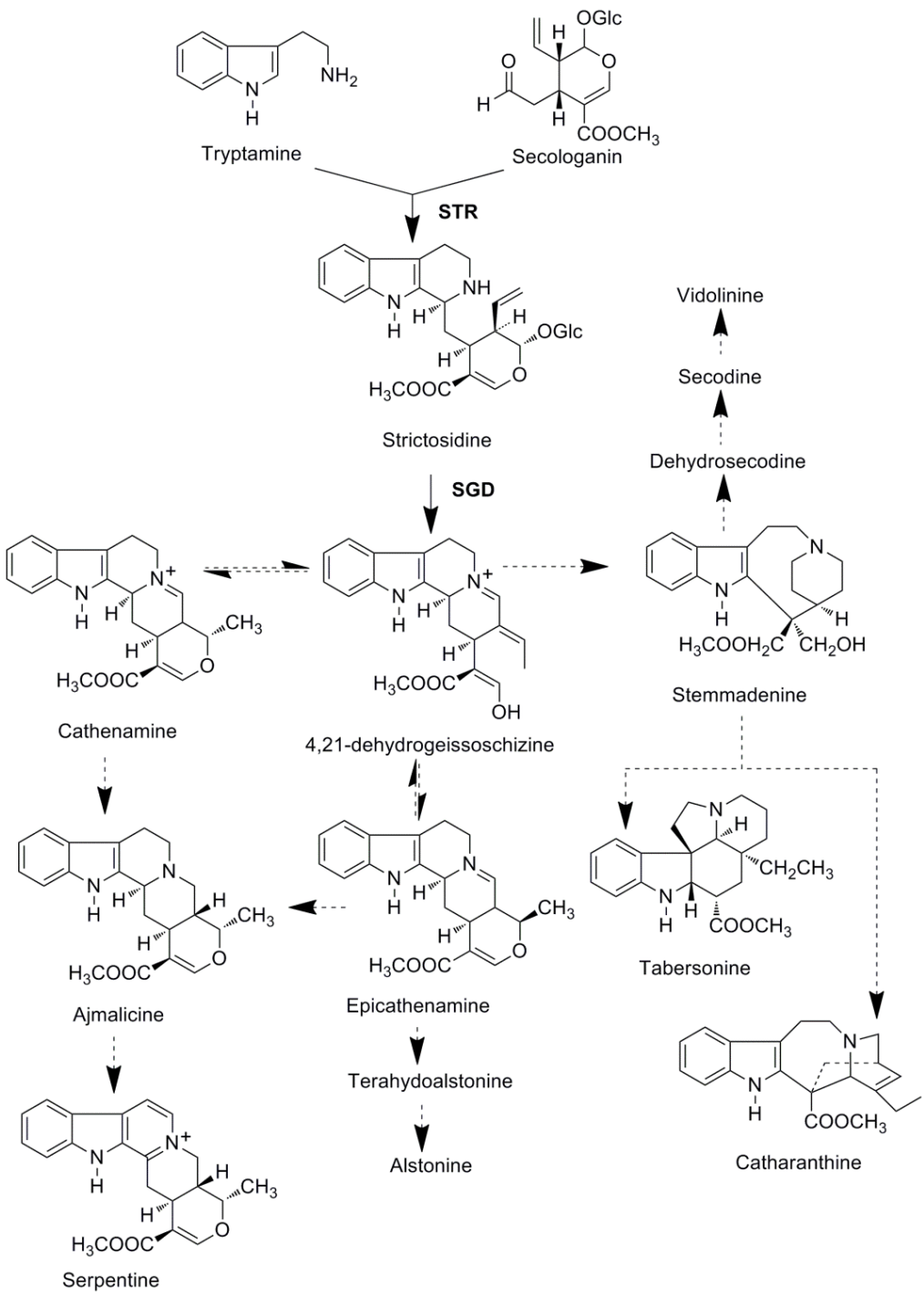

Figure 1.3 Biosynthesis of terpenoid indole alkaloid (TIA). Solid arrows represent a single enzymatic step, whereas dash arrows indicate multiple enzymatic steps. The abbreviations of enzymes and uncharacterized enzyme are present in bold and italic respectively. STR: strictosidine synthase; SGD: strictosidine-β-*D*-glucosidase. Adapted from El-sayed and Verproote (2007).

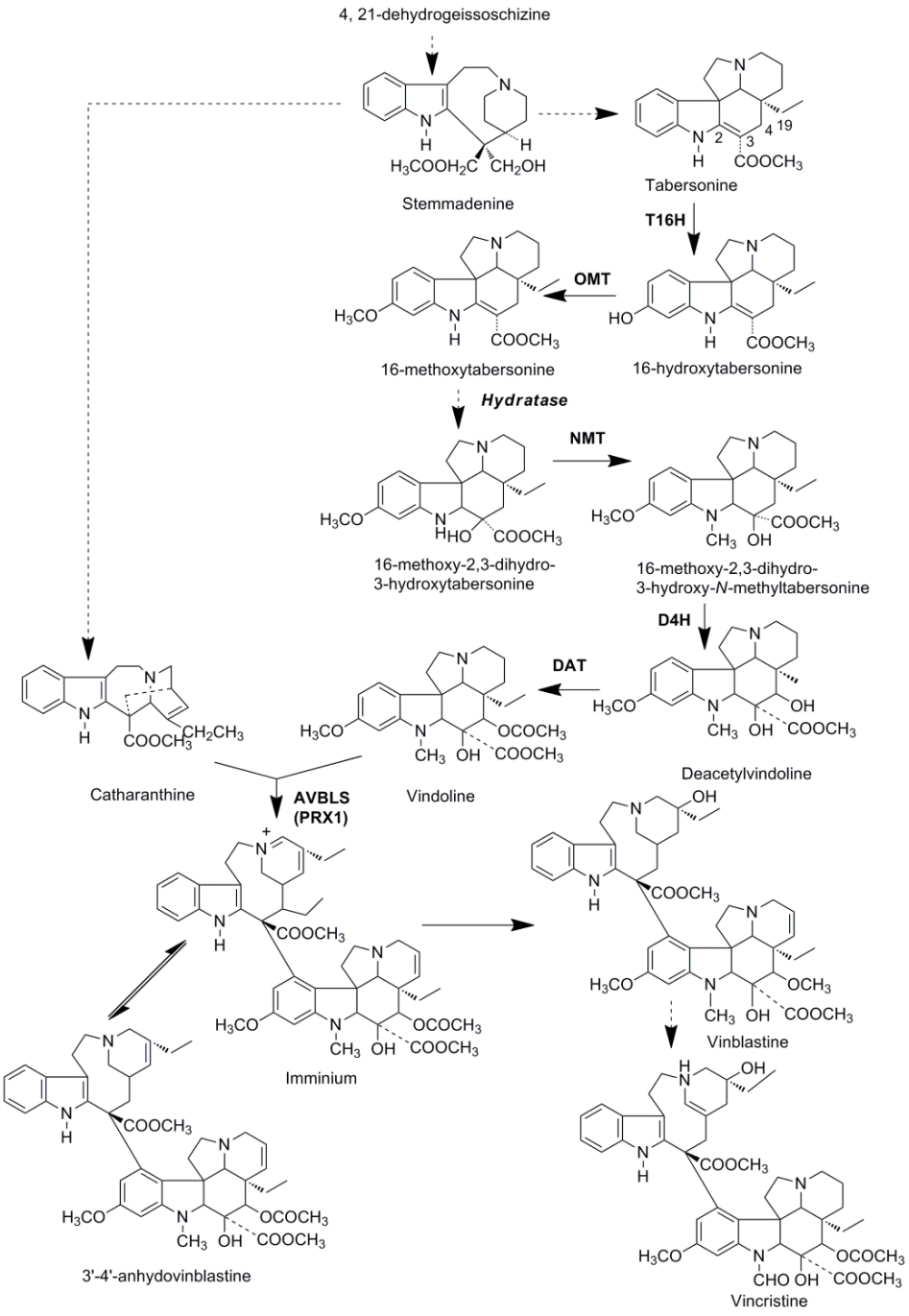

Figure 1.4 Biosynthesis of vinblastine and vincristine in *C. roseus*. Solid lines represent a single enzymatic step, whereas dash lines indicate multiple enzymatic steps. The abbreviations of enzymes and uncharacterized enzyme are present in bold and italic respectively.T16H: tabersonine-16-hydroxylase; 16OMT: 16-*O*-methyltransferase; NMT: N-methyltransferase; D4H: deactetylvindoline-4-hydroxylase, DAT: deacetylvindoline-4-*O*-actyltransferase; AVLBS (PRX1): α-3', 4-anhydro vinblastine synthase. Adapted from El-sayed and Verproote (2007).

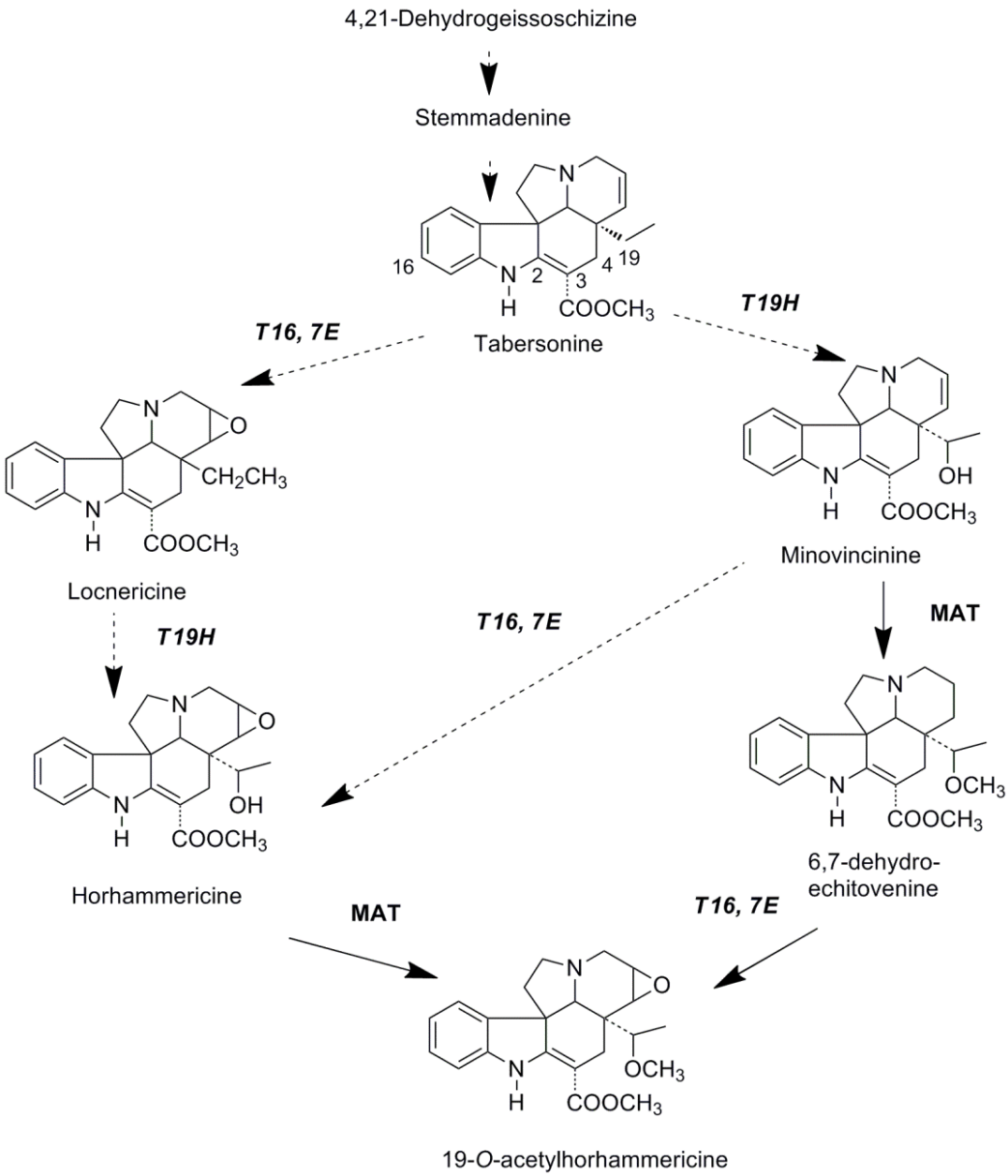

Figure 1.5 Biosynthesis of TIA derived from tabersonine in hairy root culture. Solid lines represent a single enzymatic step, whereas dash lines indicate multiple enzymatic steps. The abbreviations of enzymes and uncharacterized enzyme are present in bold and italic respectively. Tabersonine is converted into lochnericine, horhammericine, and minovincinine via uncharacterized hydroxylations. Horhamericine and minovicinine are converted into their respective products by Minovincinine-19-hydroxy-*O*-acetyltransferase-*O*-acetylation (MAT). Adapted from El-sayed and Verproote (2007).

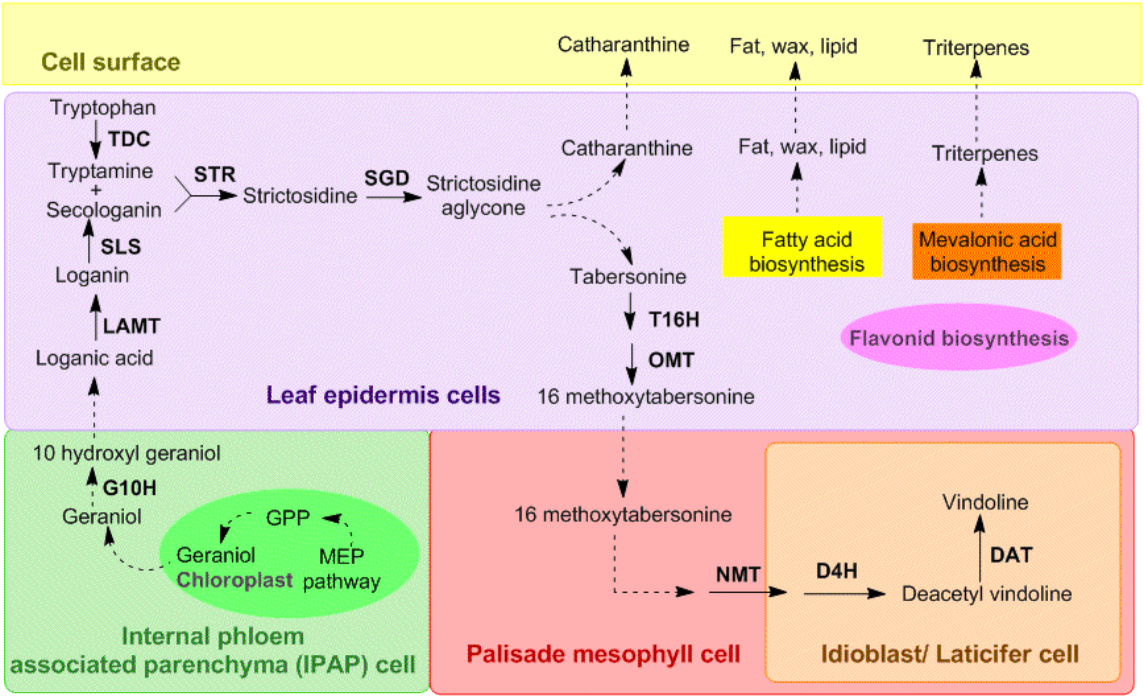

Figure 1.6 Tissue-specific compartmentation of monomeric TIA. The leaf epidermis is specialized for biosynthesis of flavonoid, fatty acid component, terpenes and TIA. The 10 hydroxy geraniol is made in specialized IPAP cell via the methyl-erythriol (MEP) pathway and this metabolite or another intermediates is transported to leaf epidermis, where the loganic acid is catalyzed to secologanin by : *S*-adenosyl-*L*-methionine: loganic acid methyl transferase (LAMT) and secologanin synthase (SLS). In the leaf epidermis, tryptophan is decaboxylated by tryptophan decarboxylase (TDC) to form tryptamine, which is further coupling with secologanin to form strictosidine. The common intermediate, stctisidine aglycone is also formed in leave epidermis by strictosidine-β-*D*-glucosidase (SGD). The strictosidine aglycone is converted to tabersonine or catharanthine by un-characterization enzyme. Catharanthine is transported to cell surface, together with fat, wax, lipid and triterpene compounds. After, tabersonine convert to 16-methoxytabersonine by tabersonine-16-hydroxylase (T16H) and 2,3-dihydro-3-hydroxy-N-methyltabersonine (16OMT) in epidermis, the 16-methoxytabersonine may be secreted to mesophyll, where a further uncharacterized oxidation take place, followed by *N*-methylation by *N*-methyltrasferase (NMT) located in chloroplast thylakoids. The last two reactions in biosynthesis which catalyzed by deactetylvindoline-4-hydroxylase (D4H) and deacetylvindoline-4-O-actyltransferase; DAT appear to occur in special leaf idioblast and laticifer cell. Adapted from Roepke et al. (2010).

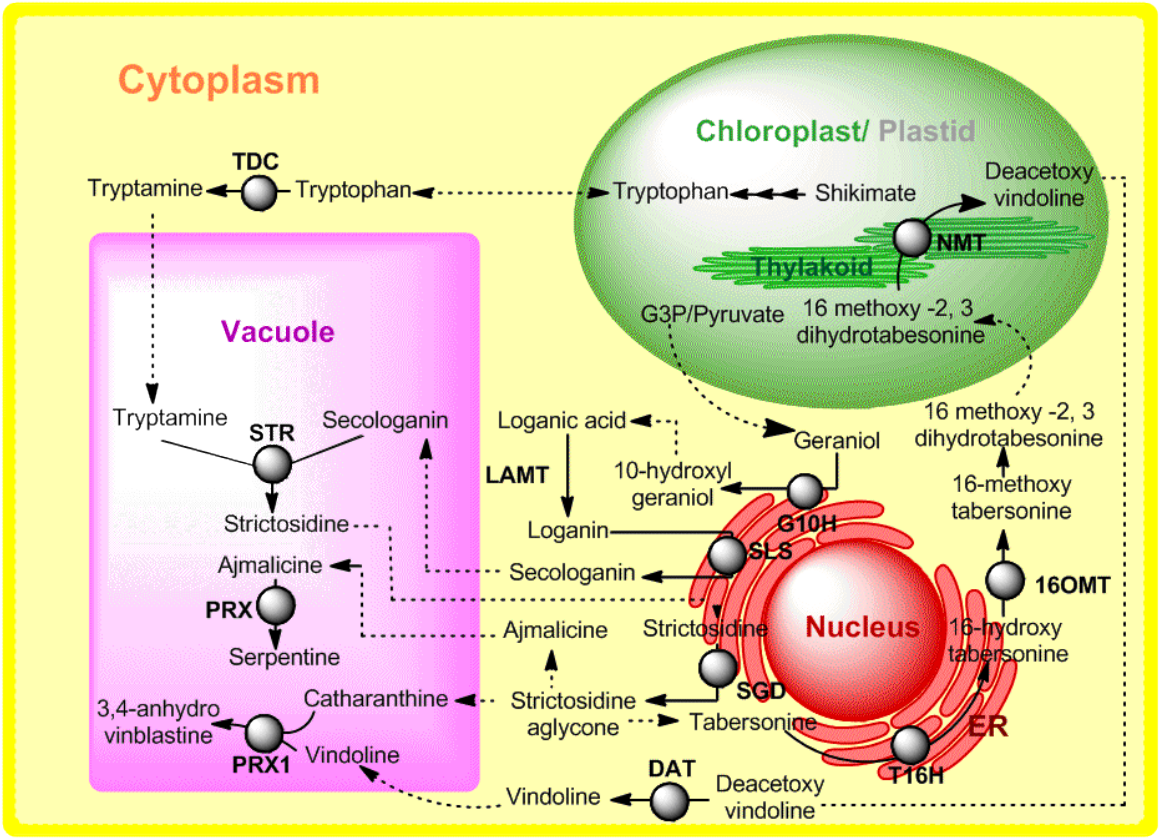

Figure 1.7 Spatial intercellular organization of TIA biosynthesis in *C.roseus*. Solid lines represent a single enzymatic step, whereas dash lines indicate multiple enzymatic steps. The abbreviations of enzymes are presented in bold. G10H: geraniol 10-hydroxylase; LAMT: *S*-adenosyl-L-methionine: loganic acid methyl transferase, SLS: secologanin synthase; TDC: trypatophan decarboxylase; T16H: tabersonine-16-hydroxylase; 16OMT: 16-*O*-methyltransferase; NMT: *N*-methyltransferase; D4H: deactetylvindoline-4-hydroxylase, DAT: deacetylvindoline-4-*O*-actyltransferase; AVLBS (PRX1): α-3', 4-anhydro vinblastine synthase. Adapted from Guirimand et al. (2011).

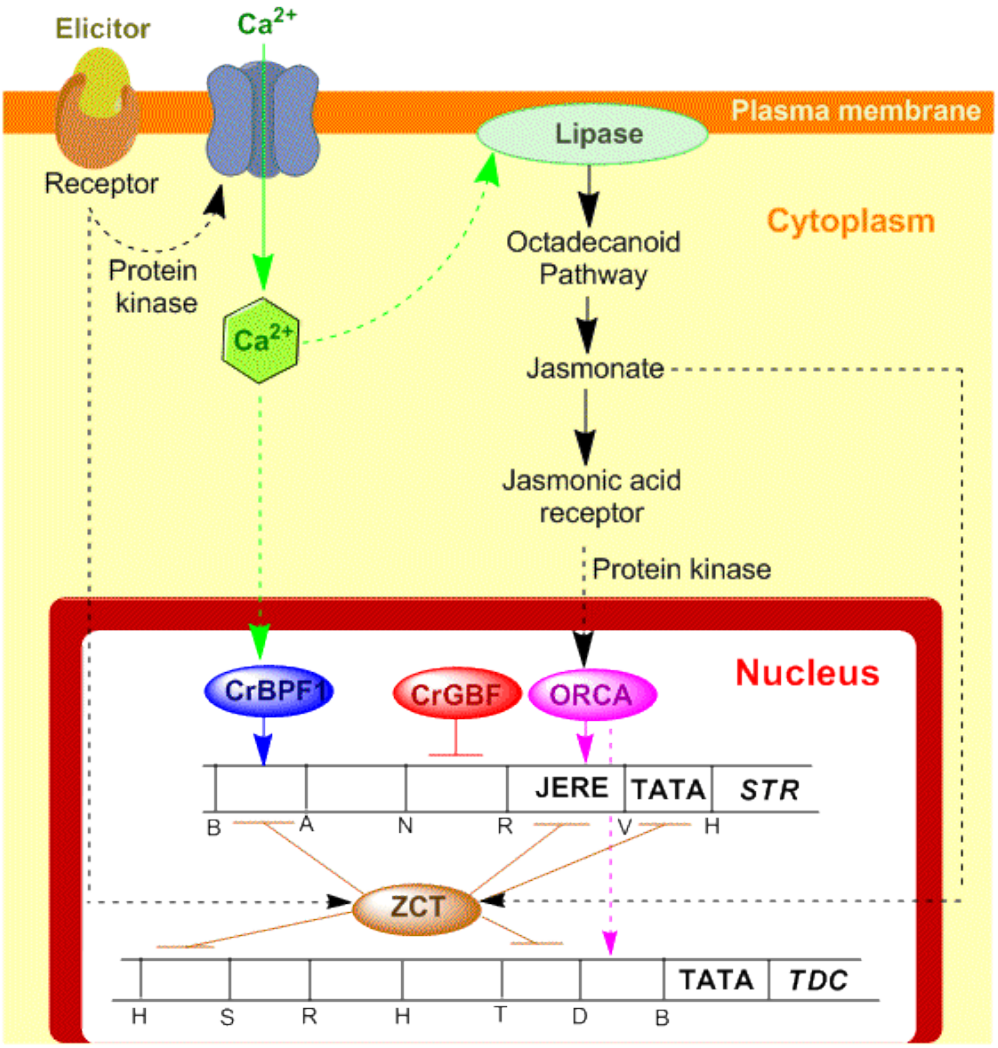

Figure 1.8 Regulation of the STR and TDC promoter in *C. roseus*. The binding of the elicitor activates an influx of Ca^{2+} into the cell. The increase in Ca^{2+} concentration activates the octadecanoid pathway leading to the production of jasmonic acid (JA). Jasmonic acid activates ORCA (Octadecanoid-derivative Responsive *Catharanthus* AP2-domain protein) ZCTs are also increased by an unknown signal cascade from the receptor (Pauw et al., 2004). Ca^{2+} influx causes the increase o 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). Adapted from Pleeble et al. (2009).

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Chapter 2

Promoter Analysis of the *Catharanthus roseus Geraniol 10-Hydroxylase* Gene Involved in Terpenoid Indole Alkaloid Biosynthesis

2.1 Introduction

The terpenoid indole alkaloids of *Catharanthus roseus* is originated from the condensation of the indole trypatamine and secologanin, which is found to be limited in hairy root and cell culture (Morgan and Shanks,2000). Secologanin is the end product of the terpenoid pathway where the first committed step is the hydroxylation of geraniol to 10-hydroxyl geraniol by the cytochrome P450 enzyme geraniol 10-hydroxylase (G10H: Collu et al.,2001). The gene encoding a cytochrome P450 reductase (CPR), presumably acting as the electron donor for G10H, has been identified and characterized (Meijer et al.,1993). *G10H* has been suggested as a potential site for regulatory control in secologanin biosynthesis (Collu et al.,2001; Collu et al.,2002). Previous studies showed that increase activity of G10H has been associated with the increase accumulation of TIA products (Schiel et al.,1987; Wang et al.,2010). Responsiveness to hormonal or pathogenic signals, such as jasmonic acid (JA) and fungal elicitors is a hallmark of TIA pathway enzymes(Cardoso et al.,1997; Pasquali et al.,1999 ; Wei,2010). In *C. roseus* the mRNA levels of several key TIA pathway genes, including *TDC*, *STR*, *SGD* and *CPR*, increase dramatically upon exposure to jasmonic acid and fungal elicitors (van der Fits and Memelink,2000; Memelink et al.,2001). A similar response to JA induction by G10H has also been reported (Collu et al.,2001). However, the expression of *C. roseus G10H* in response to fungal elicitors has not been studied. A number of JA-responsive transcription factors, including transcriptional activators ORCA2 and ORCA3 (van der Fits and Memelink,2000) as well as repressors ZCT1, ZCT2 and ZCT3 (Pauw et al.,2004), are able to interact with the promoters of *TDC*, *STR* and *SGD*. These findings present a complex regulatory mechanism of the TIA pathway that involves multiple transcription factors responding to various developmental and environmental signals. The expression of *G10H* is not regulated by of ORCA3. Moreover, Overexpression of *ORCA3* did not result in TIA accumulation due to terpenoid deficiency, and loganin feeding result in the improvement of TIA accumulation (Morgan and Shanks,2000; van der Fits and Memelink,2000; Goklany et al.,2009; Wang et al.,2010). These observations suggest that the G10H is the bottle neck of TIA biosynthesis pathway and regulated by other JA- or elicitor-responsive transcription factors (Verpoorte and Memelink,2002). The availability and characterization of the *G10H* promoter will facilitate the identification of additional regulators capable of interacting with the promoter

and lead to further understanding of the mechanisms underlying TIA biosynthesis. Here we report the isolation and characterization of the *C. roseus G10H* promoter. Our results suggest that the promoter contains a number of *cis*-elements and transcriptional enhancers. The isolated promoter can control gene expression in transgenic *C. roseus* and tobacco cells and are responsive to induction by methyl jasmonate (MeJA) and fungal elicitors. The presence of several unique transcription factor binding sites in the promoter infers the possible roles of these regulators in TIA biosynthesis.

2.2 Materials and methods

2.2.1 Genome walking and isolation of *G10H* promoter

The *C. roseus G10H* promoter sequence was isolated by a PCR based genome walking procedure using the GenomeWalker Universal Kit (BD Biosciences, USA). Genomic DNA was isolated from *C. roseus* seedlings using DNeasy plant mini kit (Qiagen). Two non-overlapping gene specific primers (G10H1: 5′-GTGTGGTTGGTCGCCTAATAAATGGAG-3′ and G10H3: 5′-GGTAAGGTAATCCATGGAATGGAAGTG-3′) were designed based on the *G10H* cDNA sequence information available in the GenBank database. An approximately 0.5 kb fragment was isolated from *Eco*RV digested genomic DNA ligated to the GenomeWalker adapter sequence. The primary PCR was carried out using an adapter-specific primer and a gene-specific primer and followed by a second PCR amplification reaction using nested adapter-specific and gene-specific primers. All PCR reactions were amplified using Advantage 2 Polymerase mix (BD Biosciences, USA) as per manufacturer's instruction. The major product from the second PCR was gel purified and cloned into the pGEM-T Easy vector (Promega, USA). A plasmid containing the appropriate size insert was completely sequenced (Figure 2.1). Based on this sequence information a forward primer with an *Eco*RI restriction site (G10H-FW: 5′-GCG<u>GAATTC</u>TCGTTATAGAATTTCTCGCTTCA-3′) and a reverse primer with a *Hind*III restriction site (G10H-RV: 5′-AT<u>GAAGCTT</u>GGAATGGAAGTGTACAATTTG-3′) were designed. To verify the specificity of the sequence obtained by genome walking, the promoter fragment was re-amplified from the genomic DNA using these primers. The fragment was cloned and sequenced to check the integrity. The plasmid containing the *G10H* promoter was designated as pGEM-G10H.

2.2.2 Determination of the *G10H* promoter transcription start site by 5′-RACE

The transcription start site (TSS) of the *G10H* promoter was determined by 5′-rapid amplification of cDNA ends (5′-RACE). Total RNA was isolated from *C. roseus* seedlings using the RNeasy Plant mini kit (Qiagen, USA). RACE was carried out using the 5′-RACE System 2 (Invitrogen, USA) following protocols supplied by the manufacturer. A gene specific primer (G10RACE 1; Table 2.1) was used for the synthesis of first-strand cDNA. For amplification of the dC-tailed first-strand cDNA, the adapter primer from the kit and two nested gene specific antisense primers (G10RACE 2 and 3; Table 2.1) were used in subsequent PCR reactions. The 5′-RACE PCR products were cloned into pGEM-T Easy (Promega, USA) and 10 positive clones were sequenced to determine the transcription start site.

2.2.3 Construction of plasmids for protoplast transient expression assay

A series of G10H promoter fragments of various lengths (Figure. 2.2) were PCR-amplified from pGEM-G10H with appropriately designed primers (Table 2.1). PCR amplification was carried out in a 25 µl volume for 30 cycles under the following standard conditions: denaturation (94 °C for 30 sec), annealing (55 °C for 30 sec), and extension (72 °C for 30 sec) using Easy-A-high fidelity PCR cloning enzyme (Stratagene, USA). Each PCR-amplified fragment was gel-purified, digested with *Eco*RI and *Hind*III and cloned into the corresponding sites of the protoplast expression vector pUCPMAGUS (Day and Miti,1999). A total of eight plasmids were constructed, corresponding to coordinates −493 to +40 (pG10H1), −436 to +40 (pG10H2), −318 to +40 (pG10H3), −191 to +40 (pG10H4), −177 to +40 (pG10H5), −103 to +40 (pG10H6), −436 to −38 (pG10H7), and −436 to −188 (pG10H8).

2.2.4 Construction of plasmids for gain-of-function experiments

For gain-of-function experiments, five *cis*-regions (corresponding to positions −191 to −147, −266 to −188, −266 to −147, −318 to −188 and −318 to −147 relative to the TSS; Figure 2.3) were individually inserted upstream of the minimal promoter, pG10H6 (−103 to +40 relative to TSS). The *cis*-regions were individually PCR-amplified from the plasmid pGEMG10H to generate fragments of the following general structure: 5′-*Eco*RI-*cis*-region–*Hinc*II-3′. The G10H minimal promoter was PCR-amplified as a *Hinc*II–*Hind*III fragment. The minimal promoter with the respective *cis*-region was then cloned into the corresponding site of the pUCPMAGUS protoplast expression vector. The resulting constructs were designated pG6a, pG6b, pG6c, pG6d and pG6e (Figure 2.3). Each promoter fragment was verified by DNA-sequencing.

2.2.5 Protoplast isolation and electroporation

Isolation of protoplasts from tobacco cell suspension cultures (*Nicotiana tabacum* L. cv. *Xanthi* 'Brad') and electroporation of tobacco protoplasts with plasmid DNA containing the promoter fragment fused to a *β-glucuronidase* (*GUS*) gene were performed as described previously (Pattanaik et al.,2006). A Gene Pulser II apparatus (BioRad, USA) with the Capacitance Extender II (Model 165–2107) was used for electroporation. An aliquot of 750 µl containing 2×106 protoplasts in a 0.4 cm electroporation cuvette was electroporated with 10 µg of plasmid DNA (200 Vand 950 µF capacitance for 50–60 milli sec). A plasmid containing the fire-fly luciferase coding sequence under the control of *CaMV35S* promoter and *rbc*S terminator was co-electroporated with each promoter construct as an internal control. After 20–22 hr, protoplasts were harvested for GUS and luciferase activity assays. All constructs were tested in at least three independent experiments. The GUS activity was normalized against luciferase activity. A promoter-less GUS plasmid was used as a negative control in this transient expression assay.

2.2.6 β-Glucuronidase and Luciferase assays

Fluorometric assays to measure GUS activity in transfected protoplasts or plant tissue and histochemical staining to localize the distribution of GUS activity in plants were performed according to protocols described in Jefferson et al.,1987 . Total protein concentration in plant extracts was determined using the Bio-Rad protein assay (Bio-Rad, USA) with bovine serum albumins as standard. Luciferase activity in transfected protoplasts was measured using a Luciferase assay system (Promega, USA) and a luminometer (Model No. TD2020; Turner Designs, USA).

2.2.7 Construction of plant expression vectors for transformation of tobacco and *C. roseus*

The *G10H* promoter fragment isolated from pG10H1GUS (−493 to +40 relative to the TSS) was cloned into the plant expression vector pKYLX71-GUS (Schardl et al.,1987) at *EcoR*I and *Hind*III sites. The resulting plant expression vector was designated pKG10HGUS. The construct was introduced into *Agrobacterium tumefaciens* strain C58C1:pGV3850 by freeze–thaw. Tobacco plants (*Nicotiana tabacum* cv S*amsun* NN) were transformed with the *Agrobacterium* containing pKG10HGUS as described previously (Pattanaik et al.,2004). Twelve independent plant lines were generated for this construct. Regenerated, kanamycin-resistant tobacco plants were grown under greenhouse conditions. Transformation of *C. roseus* with *Agrobacterium rhizogenes* for production of transgenic hairy root cultures was conducted as previously described

(Choi et al.,2004). The plasmid pKG10HGUS was mobilized into *Agrobacterium rhizogenes* R1000 by freeze–thaw method. After removal of the roots, 1-week-old in vitro germinated *C. roseus* seedlings were immersed in the bacterial suspension for 30 min and then transferred onto solid MS basal medium (Murashige and Skoog,1962) for co-cultivation. Seedlings infected with *A. rhizogenes* R1000 without the binary vector were used as control. After 48 hr the explants were transferred to half-strength MS basal medium containing cefotaxime (400 mg/L). Hairy roots developed at the cut surface of explants in 3–4 weeks.

2.2.8 Analysis of transgenic tobacco plants: isolation of RNA and RT-PCR analysis

Total cellular RNA from leaves and roots of transgenic tobacco seedlings expressing the pKG10GUS construct was isolated using the RNeasy plant mini kit (Qiagen, Chatsworth, USA). Total RNA (4 μg) was treated with RNase-free DNase (Sigma, USA) and used for synthesis of first-strand cDNA in a total volume of 20 μl using Superscript II Reverse Transcriptase (Invitrogen, USA). For the no-reverse-transcriptase control, an individual reaction was performed in parallel without addition of reverse transcriptase. One μl of the RT products was used in the subsequent PCR reaction with appropriately designed forward and reverse primers (Table 2.1) to detect the full-length GUS transcript. The PCR reaction was performed in a total volume of 25 μl using GoTaq green master mix (Promega, USA) for 30 cycles (94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 2 min). As a negative control, the primer pair was tested against DNase-treated RNA to confirm cDNA dependence of amplification. PCR products were analyzed on an ethidium bromide-stained agarose gel.

2.2.9 Jasmonate induction, fungal elicitor treatment and GUS assay

The transgenic tobacco seedlings and hairy roots induced from hypocotyl segments of *C. roseus* were used for elicitor treatments. MeJA (Bedoukian Research, USA) was diluted with DMSO and added to liquid MS medium at a final concentration of 100 μM. Only DMSO was added to the control treatment. The seedlings or hairy roots were incubated for 8–20 hr in the medium and then either stained using GUS histochemical staining buffer containing 1 mg/ ml X-Gluc (5-bromo-4-chloro-3-indolyl-β-*D*-glucuronide; Gold Biotech, USA) or assayed for GUS activity using MUG (4-methylumbelliferyl-β-*D*-glucuronide; Sigma, USA). For fungal elicitor treatments, transgenic seedlings or transformed hairy roots were incubated for 12–24 hr in 0.5–1.5% yeast extract in water (Difco,USA). Control (water only) and treatment samples were then stained with X-Gluc or used for GUS activity assay. The pictures of the GUS-stained roots were

captured using an Olympus BX51 microscope with Olympus DP12 digital camera. The color intensities of the roots were quantified using the ImageJ software.

2.3 Results and discussion

2.3.1 Structure and sequence analysis of the *G10H* promoter

An approximately 0.5 kb *G10H* promoter fragment was isolated by PCR-based genome walking (Figure 2.1). Sequence analysis using PLACE (www.dna.affrc.go.jp/PLACE; Higo et al.,1999) and the NSITE-PL program (www.softberry.com) reveals that the G10H promoter contains several consensus eukaryotic regulatory domains. The TATA-box is present 31-bp upstream of the major transcription start site (TSS). The TATA proximal sequences up to −103 of the *G10H* promoter are relatively GC-rich while the distal regions are AT-rich (Figure 2. 1). A CAT-box-like sequence (CAAT) is present 19-bp upstream of the TATA-box sequence. In addition to the TATA-box and CAT-box, several other potential regulatory elements with roles in regulation of gene expression are present in *G10H* promoter. These include a prolamin box (P-box) like sequence (CTTTTACA) at position −331 to −324, a G-box (CACGTG) at position −185 to −180, a Myb-type transcription factor recognition sequence (TGGTTAG) at position −172 to −166, an AT-box (AATATTTTTAAA) at position −147 to −136 and a W-box (TGACCT) at position −191 to −186 relative to the TSS (Fig. 2.1). In addition, the *G10H* promoter contains six GT-1 transcription factor recognition sequences and six Dof (DNA binding with one finger) transcription factor recognition core sequences (AAAG and CTTT). The P-box, or '−300 elements', is found in the promoters of many cereal seed storage protein genes. This *cis*-element is identified on the basis of both its highly conserved nucleotide sequence (TGTAAAG) and position (−300 region) relative to translation start codon of the prolamin genes. The P-box has been shown to interact with endosperm-specific Dof transcription factors and may be involved in activation of storage protein gene expression during endosperm development (Vicente-Carbajosa et al.,1997). The G-box (CACGTG) is a highly conserved DNA sequence that has been identified in the promoter regions of many plant genes regulated by a variety of environmental and physiological factors. G-box or G-box-like sequences are reported to be present in the promoters of other TIA biosynthetic pathway genes. G-boxes have also been shown to effect gene expression in response to light, abscisic acid (ABA) and MeJA (Ishige et al.,1999). The W-boxes are present in the promoters of many plant defense-related genes. The WRKY transcription factors, which are involved in the regulation of various plant physiological processes including pathogen defense and trichome development, bind specifically to the W-box elements (Eulgem et

al.,2000). The AT-box has been found in the 5′ upstream region of many plant genes regulated by light, and the nuclear factor AT-1 binds specifically to this AT rich sequence (Datta and Cashmore,1989). GT elements were first identified in the promoter region of the photo-regulated pea *ribulose bisphosphate carboxylase/oxygenase* (*Rubisco*) small sub-unit as box II. The GT-elements have since been identified in the promoter regions of many plant genes including some that are not regulated by light (Zhou,1999). The GT-1 transcription factors recognize the consensus sequence (G/A)NNN(G/A)GT(A/T)AA(A/T)(A/T) (Ouwerkerk et al.,1999). The promoter regions of several genes involved in TIA biosynthesis in *C. roseus* contain the consensus GT-elements that bind the nuclear factor GT-1 (Cardoso et al.,1997; Pasquali et al.,1999). Mutagenesis of the GT elements in the *C. roseus TDC* promoter significantly lowers its response to UV light (Ouwerkerk et al.,1999). Dof proteins are plant specific transcription factors which bind to DNA with an AAAG core sequence or its reverse complement sequence CTTT. These transcription factors have been isolated from many monocot and dicot plants and play critical roles as transcriptional activators or repressors in plant growth and development (Yanagisawa,2004). The abundance of Dof binding sites in the *G10H* promoter is interesting. Several genes involved in *C. roseus* TIA biosynthesis are regulated by multiple zinc finger proteins. Three two-fingered transcription factors have been demonstrated to bind both *TDC* and *STR* promoters and act as transcriptional repressors (Pauw et al.,2004). These zinc finger repressors function in junctions with multiple activators to provide combinatorial control of environmental responses and timing of gene expression. To date no Dof protein has been directly implicated in *C. roseus* TIA biosynthesis. While *G10H* differs from *TDC*, *STR* and *SGD* in the response to ORCA transcription factors, it is not clear whether the two-fingered factors affect the *G10H* expression. Because *G10H* is likely to be regulated by different elicitor-responsive transcription factors than those controlling *TDC*, *STR* and *SGD*, it will be worthwhile to explore the possibility that Dof proteins play a significant role in regulation of *G10H* expression. Comparison of the known promoter sequences of *C. roseus* TIA biosynthetic pathway genes, such as *TDC* and *STR*, reveals that some of the *cis*-elements described above are unique to the *G10H* promoter. These elements include W-box, MYB-transcription factor recognition sequence, AT-box and P-box-like sequence (TGTAAAG/CTTTTACA). The presence of such novel *cis*-elements suggests that the *G10H* promoter may be regulated by a different transcriptional cascade.

2.3.2 Transcription start site of the *G10H* promoter

The TSS of the *G10H* promoter was determined by 5′-RACE using total RNA isolated from *C. roseus* seedlings followed by cloning and sequencing of amplified PCR products. More than ten 5′-RACE products were sequenced and the mRNA 5′ end points suggested two possible TSS (Figure 2.1). One starts at an adenine (A) base located 31-nucleotides downstream of the TATA box and the other one starts at an adenine 4 bp upstream. Joshi,1987 has analyzed over 75 published genomic DNA sequences from several higher plant species and found that an adenine flanked by pyrimidine bases is present at the TSS in the majority of cases. The adenine 31 nucleotides downstream of the TATA box conforms with this consensus and is therefore considered to be the major TSS and designated as position +1 (Figure 2.1).

2.3.3 5′and 3′-end deletion analysis

For functional characterization of the *G10H* promoter, the 533 bp promoter fragment was subjected to progressive 5′-and 3′-end deletion analysis. Eight promoter fragments were generated by PCR amplification and cloned upstream of the *GUS* reporter gene to create transcriptional fusions. A schematic map of deletion constructs and the corresponding GUS activities of each construct are shown in Figure 2. 2. Constructs containing the promoter fragments fused to the *GUS* gene were introduced into tobacco protoplasts for transient expression assays. The expression level of the full-length promoter (construct 1; Figure 2. 2) was 1.32 nmol MU/min/mg protein, and this value was considered to represent full (100%) promoter activity for comparison with the deletion constructs. Deletion of the *G10H* promoter from the 5′-end down to −318 had small effects on the promoter activity (constructs 2 and 3). However, further deletion down to −191 (construct 4) markedly reduced the promoter strength; less than 40% of the GUS activity remained in comparison to the full-length promoter. Further deletion of 14 bp (construct 5) resulted in an additional 10% loss in activity. Deletion to position −103 (construct 6) decreased the promoter activity to approximately 20% of that of the full-length promoter. This promoter region (−103 to +40) with minimal activity contains the TATA-box but no other known regulatory elements and is thus considered as the minimal promoter. Sequence analysis, discussed above, indicates that the promoter region between −318 bp and −147 bp contain a number of potential *cis*-elements. These *cis*-regulatory elements are well-characterized and have been shown to be involved in the regulation of promoter activities in many plants. Our deletion analysis indicates that these *cis*-elements are critical for maximal activity of the *G10H* promoter; deletions of these elements lead to significant reductions in promoter strength. The

deletion analysis also indicates that the TATA box is important for maximal promoter activity; the 3′-end deletion constructs 7 and 8 that lack the TATA box exhibit considerably lower GUS activities compared to that of the full-length promoter (Figure 2.2).

2.3.4 Gain-of-function experiments

While loss-of-function analyses such as deletion studies can be informative especially in determining the minimum requirement for promoter functions, gain-of-function experiments often further define the regulatory functions of promoter sub-regions. In order to further characterize the *cis*-elements that influence *G10H* promoter activity, the region containing the majority of *cis*-elements (−318 to −147) was divided into four sub-regions (−191 to −147, −266 to −188, −266 to −147, −318 to −188, and −318 to −147, respectively). These sub-regions were fused with the minimal promoter (construct 6; Figure 2.2) to generate constructs 6a–6e (Figure 2.3). These constructs were analyzed using a tobacco protoplast transient expression assay. As shown in Figure 2.3, GUS activity of construct 6a was 2.5-fold higher than the minimal promoter. Construct 6b (−266 to −188) has a comparable level of GUS activity as the full-length *G10H* promoter. Constructs 6c, 6d and 6e showed higher activities than the full-length *G10H* promoter. The highest GUS activity from these constructs (construct 6e) is approximately 20% above that of the full-length promoter. Taken together these data implicate the presence of three potential transcription-enhancing *cis* regulatory sequences. The first enhancing sequence is present in the 45-bp region between −191 and −147 as it significantly enhances transactivation of the minimal promoter (6a; Figure2.3). The second, and perhaps even stronger enhancing sequence is present in the 78-bp region between −266 and −188 (6b; Figure 2.3). Combining these two enhancing sequences increases GUS activity higher than that observed for the full-length promoter (6c; Figure 2.3). The third enhancing sequence is present in the 52-bp region between −318 and −266. Construct 6d, which contains the second and third enhancing sequences, has a similar enhancing effect to construct 6c which contains the first and second enhancing sequences (6d; Figure 2.3). The combination of all three enhancing sequences results in the highest improvement in transactivation (6e; Figure 2.3). Transcriptional activity of many plant and animal promoters is often the result of combinatorial or synergistic interactions of different *cis*-elements with cognate transacting nuclear factors (Benfey and Chua,1990). Because isolated enhancing sequences lead to higher transcriptional activity than that of the full-length promoter, our results also implicate the presence of negative regulatory elements within the G10H promoter. The region between −318 to −188 is AT rich and contains several putative GT-1 binding sites. The presence of

potential GT-1 binding sites in the enhancer region of the promoter suggests that this nuclear factor may play a role in the G10H promoter function.

2.3.5 *G10H* promoter-controlled *GUS* expression in transgenic tobacco plants and *C. roseus* hairy roots

Twelve independent tobacco transgenic lines were developed for the construct pKG10HGUS. Seedlings of transgenic lines (R1 progeny) showing a segregation ratio (KanR:KanS) of 3:1 for the *kanamycin* (*Kan*) marker gene were selected for further analysis. RT-PCR analysis was conducted using total RNA isolated from leaves and roots of 4-week-old seedlings to amplify the full-length GUS transcript. In all selected transgenic lines the expected 1.8 kb *GUS* product was detected in both leaves and roots (data not shown). Histochemical staining of whole seedlings indicated *GUS* expression was localized to leaves and roots (Figure 2.4A–E). No GUS staining was observed in hypocotyls. In roots, GUS expression was restricted to actively growing cells around the apical meristem and appeared to decrease at older developmental stages (Figure 2.4C–E). No GUS accumulation was observed in the root cap, root hair and older developing regions of the roots (Figure 2.4D). These results show that in addition to controlling transient gene expression in tobacco protoplasts, the *G10H* promoter is capable of conferring stable gene expression in transgenic tobacco plants. The biosynthetic pathway of terpenoid indole alkaloids (TIA) in *C. roseus* has been studied extensively in the past two decades. Experiments with germinating seedlings have demonstrated that alkaloid biosynthesis and accumulation are developmentally regulated and the genes involved in the TIA biosynthesis are preferentially expressed in actively growing tissues such as young leaves and root tips (St-Pierre et al.,1999). RNA blot analysis, in situ RNA hybridization and immunocytochemistry have been used to locate the tissue/cell-type involved in TIA biosynthesis (St-Pierre et al.,1999; Burlat et al.,2004). Northern blot analysis showed that the transcriptional expression of *G10H* is higher in roots and actively growing aerial organs (young leaf, internode) as compared to mature organs (mature/old leaf, fruit; Burlat et al.,2004). In situ RNA hybridization and immunocytochemistry experiments have revealed that mRNAs of genes, including *G10H*, known to be involved in biosynthesis of the terpenoid part of TIA accumulate in plant vascular cells. In contrast, genes involved in the earlier steps of the TIA pathway, such as *TDC* and *STR*, are expressed exclusively in epidermal cells of young leaves. In roots expression of *TDC* and *STR* is restricted to protoderm and cortical cells around the apical meristem. These mRNA do not appear to accumulate in root caps and steles (St-Pierre et al.,1999; Burlat et al.,2004). Recently more sophisticated techniques including laser-capture microdissection and carborundum abrasion have been applied to localize MIA

metabolites and expression of the biosynthetic pathway genes at the cellular level (Murata and Luca,2005). These experiments indicate that *G10H* transcripts are expressed in epidermal and laticifer cells as well as in vascular cells of leaves. Our results of *G10H* controlled *GUS* expression in transgenic tobacco corroborate with the organ/ tissue specific localization *G10H* transcripts in *Catharanthus* tissue described above. In transgenic tobacco roots, the GUS expression pattern is identical to the gene expression patterns observed for *TDC* and *STR* in *C. roseus* roots (St-Pierre et al.,1999). In this study *C. roseus* hairy roots were also developed from hypocotyls of young seedlings by *A. rhizogenes*-mediated transformation of the pKG10HGUS vector. Histochemical staining of *C. roseus* seedlings showed intense GUS staining only in hairy roots (Figure 2.5A–B). *GUS* expression was not observed in hairy roots developed from *C. roseus* seedlings inoculated with the wild-type *A. rhizogenes* control (data not shown).

2.3.6 Responses of *G10H* promoter to methyljasmonate and fungal elicitor induction in transgenic tobacco seedlings and *C. roseus* hairy roots

Expressions of several *C. roseus* TIA pathway genes are responsive to induction by JA or its volatile derivative MeJA (Collu et al.,2001). G10H mRNA is undetectable in Northern blot analysis of *C. roseus* suspension cells without JA treatment but accumulates to high levels following JA induction. A similar JA induction profile has been observed for a *cytochrome P450 reductase* (*CPR*) in the same cells. Other genes involved in the *C. roseus* TIA pathway, including *TDC*, *STR* and *SGD*, also respond to JA induction however with different kinetics (Collu et al.,2001). To investigate the effect of JA on *G10H* promoter activity, transgenic tobacco seedlings and *C. roseus* hairy roots expressing pKG10HGUS were treated with 100 μM MeJA for 8–20 hr. While untreated control roots (in the presence of 0.1% DMSO) showed basal GUS activity, JA treatment resulted in greater than 8-fold increase of GUS activity (Figure 2.6). GUS activity of MeJA treated tobacco seedlings was also higher than the untreated ones (Figure 2.7). These data agree well with the JA responsive expression of *C. roseus G10H* and suggests that the *G10H* promoter contains elements that are recognized by JA responsive factors. G-boxes and CAT-boxes in plant gene promoters have been implicated as JA responsive elements (Menkens et al.,1995; Guerineau et al.,2003). The GCC-box (AGCCGCC) may play a role in JA mediated gene activation (Brown et al.,2003). However, a GCC-box has not been identified in the *C. roseus G10H* promoter. It has also been suggested that palindrome-forming inverted repeats in the promoter are recognized by JA-responsive factors (Guerineau et al.,2003); however, no significant inverted repeat has been detected in the *G10H* promoter. Fungal elicitors induce responses from the *C. roseus* TIA pathway genes. In addition to *TDC* and *STR*, the cytochrome

P450 reductase gene, *CPR*, also responds to fungal elicitor induction (Collu et al.,2002). G10H is a cytochrome P450 enzyme and requires CPR for its function; therefore, we tested the effect of fungal elicitors on the *G10H* promoter activity to investigate the possibility of coordinated regulation of G10H and CPR by the same induction signals. After incubation with yeast extract, a significant increase in GUS activity was observed in both transformed *C. roseus* hairy roots (Figure 2.6) and transgenic tobacco seedlings (Figure 2.7) compared to the controls. Under our experimental conditions the increase of GUS activity correlated with increasing concentrations of yeast extract; GUS activities increased approximately 0.5-, 2- and 3-fold when 0.5%, 1% and 1.5% of yeast extract, respectively, were used to induce the *C. roseus* hairy root cultures (Figure 2.6). Presently it is difficult to pinpoint the promoter elements that are responsive to the induction. Although it has been hypothesized that the G-box is associated with the elicitor-responsive element, there is no evidence to suggest it is involved in the response of the *STR* promoter (Pasquali et al.,1999). Fungal elicitor activates the tobacco chitinase gene CHN48 via a cis-element containing W-boxes (Yamamoto et al.,2004). In the *C. roseus G10H* promoter the W-box is present adjacent to the G-box element (Figure 2.1). In this paper we report the isolation and characterization of the *C. roseus G10H* promoter. The major TSS maps to an adenine 40 bp upstream of the *G10H* start codon and 31 bp downstream of the TATA box. Many *cis*-elements are found between the -300 position and the TATA box. In other *C. roseus* TIA pathway promoters, the majority of *cis*-elements are located within the ~300-bp regions upstream of the TATA box (Pasquali et al.,1999). The *G10H* promoter responds to induction by both JA and fungal elicitors. A model to explain the effects of these elicitors on the *TDC* and *STR* promoters suggests that a putative membrane receptor responding to fungal elicitors signals the increase of JA levels which, in turn, activates the ORCA transcription factors that bind to and regulate the promoters (Pauw et al.,2004; Pauw et al.,2004). The fungal elicitor also induces the production of repressors such as ZCT proteins which repress the promoter activities. The simultaneous induction of both activators and repressors by JA and fungal elicitors provide a refined, combinatorial mechanism regulating the TIA pathway. However, ORCA proteins do not activate the expression of *G10H*, therefore it is unlikely that the same transcriptional cascade regulates the *G10H* promoter (van der Fits and Memelink,2000). The *G10H* promoter contains single WRKY and MYB binding site as well as multiple Dof and GT-1 binding sites (Figure 2.1). A number of JA-responsive transcription factors, including those from the AP2-, bHLH and two-fingered families, have been shown to be involved in regulation of the *C. roseus* TIA pathway (van der Fits and Memelink,2000; Chatel et al.,2003; Pauw et al.,2004). The *CPR* promoter is also responsive to the GT-1 transcription factor (Cardoso et al.,1997). At this time, however, the

participation of WRKY, MYB, Dof regulatory proteins is not well documented. The possible involvement of these transcription factors supports the notion that the *G10H* regulation utilizes a different transcriptional cascade than those of *TDC* and *STR*. This prospect hints at additional levels of complexity in the regulation of the TIA pathway and may serve as a guide in our continuing efforts to identify regulatory factors involved biosynthesis of valuable *C. roseus* secondary metabolites.

Table 2.1 Sequence of the oligonucleotides

Primer no	Primer sequence
G10 (− 493)F	5′ GCGAATTC TCGTTATAGAATTTCTCGCTTCA 3′
G10 (− 436)F	5′ GCGAATTC CTATCCAAATAAAAATTCAAATAATT 3′
G10 (− 318)F	5′ GCGAATTC GTCAAATTATAATTATCTATTCGTA 3′
G10 (− 266)F	5′ GCGAATTC CTAAAAACTTTTAATTATGAAAATAT 3′
G10 (− 191)F	5′ GCGAATTC TGACCTCACGTGGGTTGATTG 3′
G10 (− 177)F	5′ GCGAATTC TTGATTGGTTAGTAATATAAAAAGT 3′
G10 (− 103)F	5′ GCGAATTC GTAACTCGAGCTTCTCGAGTT 3′
G10 (+ 40)R	5′ ATGAAGCTT GGAATGGAAGTGTACAATTTG 3′
G10 (− 38)R	5′ GCTAAGCTT ACTACTGGAGAATGGA3′GATTGA 3′
G10 (− 147)R	5′ GCGGTCGAC TTATTTACTTTTTATATTACTAACC 3′
G10 (− 188)R	5′ GCTAAGCTT GTCATTAAATAGATGAATATTATTAT 3
G10 RACE1	5′ GAATTCAAAACTTTTCGAAGACT 3′
G10 RACE2	5′ TCCTGTTTTTGAAGAACTTCTTTC 3′
G10 RACE3	5′ CGTGTTTTTTGGAAAGTTTTGC 3′
GUS-FW	5′ ATGGGTTTACGTCCTGTAGAAACC 3′
GUS-RV	5′ TCATTGTTTGCCTCCCTGCTG 3′

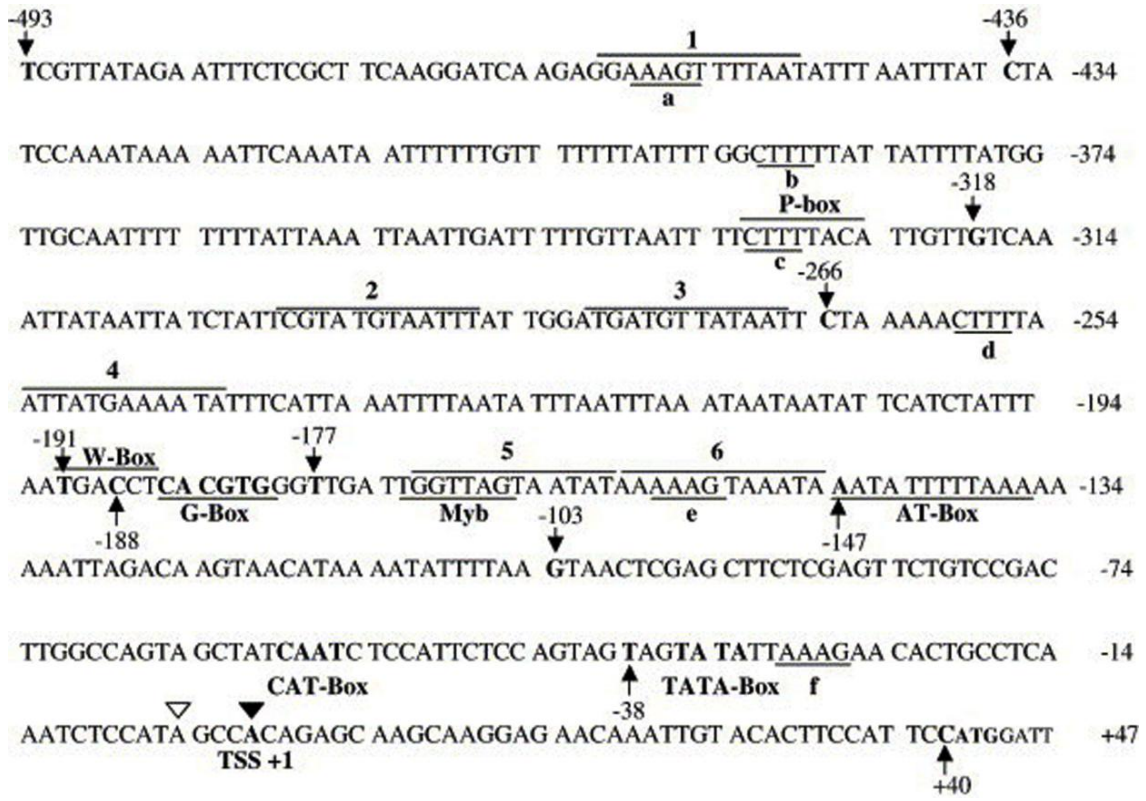

Figure 2.1 The DNA sequence of *Catharanthus roseus G10H* promoter. A 533-bp fragment (− 493 to + 47) with respect to the transcription start site (TSS) is presented. The two possible TSS are indicated with reversed triangles. The major TSS is indicated by the solid triangle and designated as + 1. An arrow above or below the sequence indicates the end point of 5′ or 3′ deletion fragments, respectively. The TATA and CAT boxes are shown in bold. Important *cis*-elements are indicated in bold. Potential GT-1 binding sites are indicated by number 1 to 6. Putative Dof transcription factor binding core sequences are underlined and labeled as **a** through **f**.

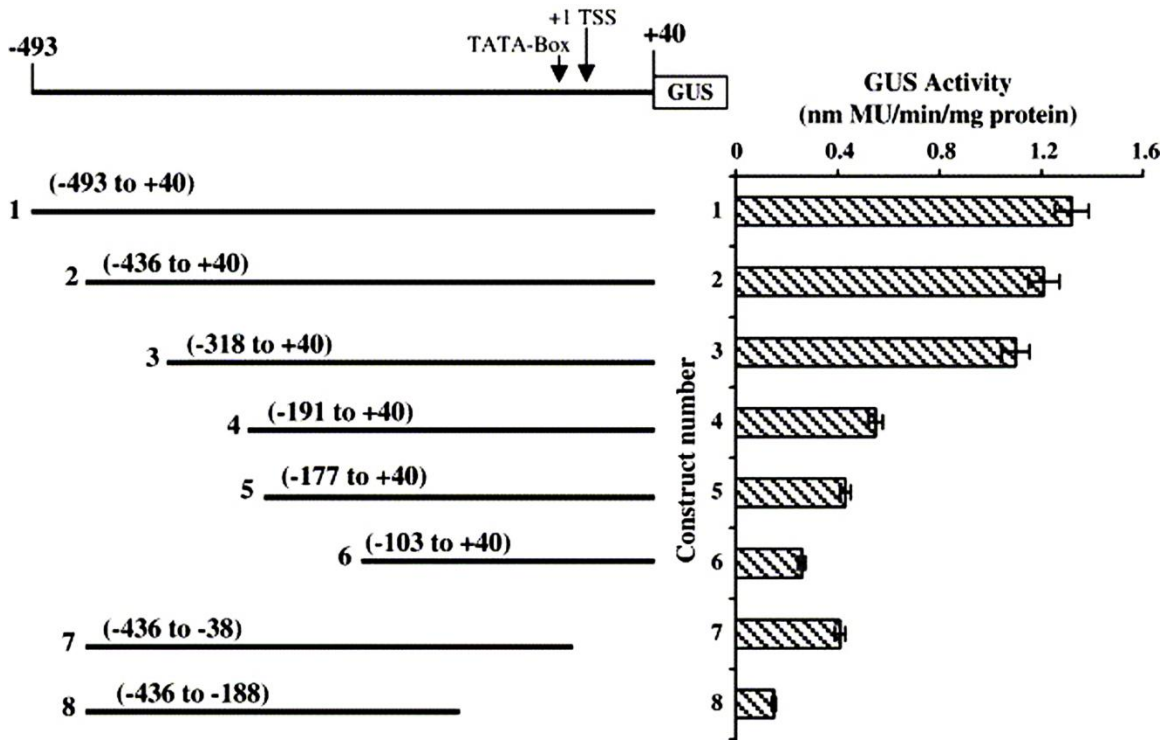

Figure 2.2 Deletion analysis of the *G10H* promoter-*GUS* constructs. The left panel shows a schematic map of the promoter deletion constructs. The 5′- and 3′-end coordinates of the deletion fragments are given in parentheses. At the top, the relative position of the TATA box, transcription start site (TSS, + 1) and the *G10H* promoter coordinates are indicated. The right panel shows the corresponding GUS activities of each construct. The GUS activities were determined in the protoplast transient expression assay. Each construct was assayed at least three times in three independent experiments. The relative GUS activity (nm MU/min/mg protein) with standard deviation is presented.

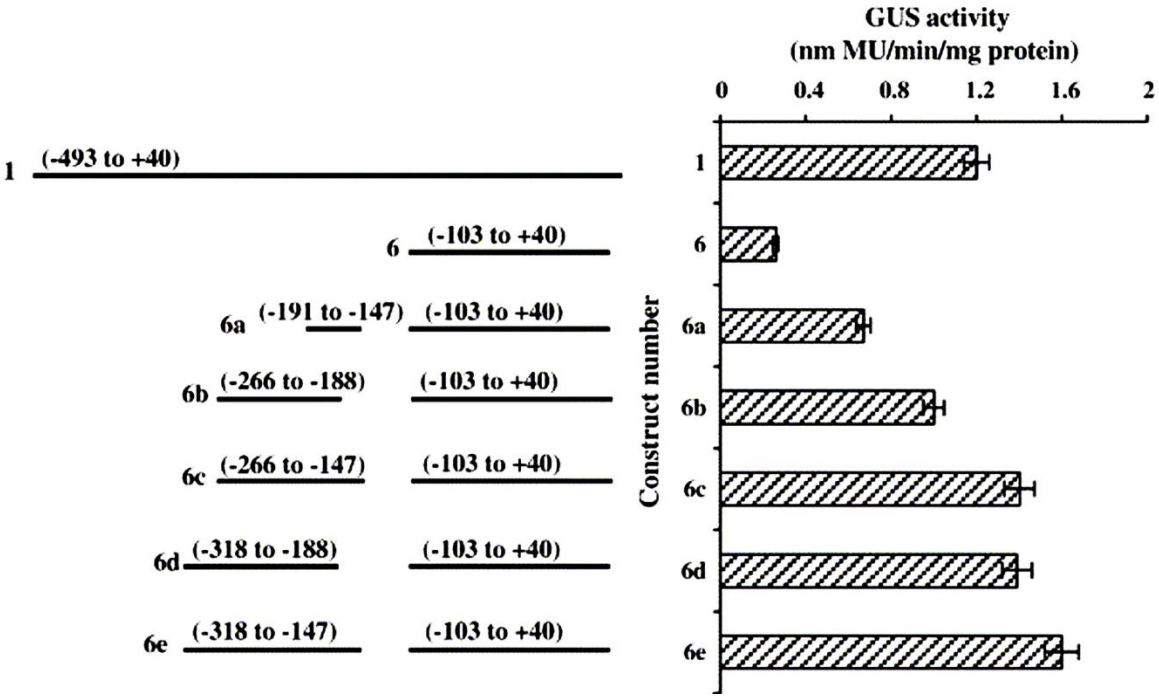

Figure 2.3 Gain-of-function analysis of the *G10H* promoter. The left panel shows a schematic map of the constructs. Construct 1 represents the full-length promoter and construct 6 represents the minimal promoter (equivalent to construct 6 in Figure 2.2). In constructs 6a to 6e, 5′-fragments of selected length are fused to the minimal promoter. The right panel indicates the corresponding GUS activities of each construct. The GUS activities were determined using the protoplast transient expression assay. Each construct was assayed at least three times in three independent experiments. The relative GUS activity (nm MU/min/mg protein) with standard deviation is presented.

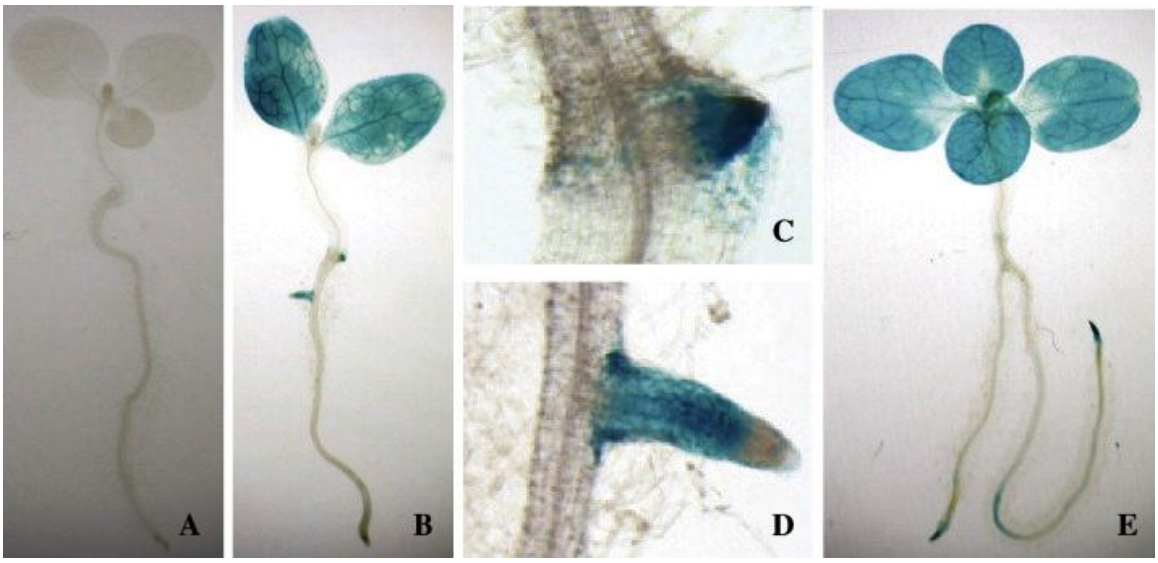

Figure 2.4 Histochemical localization of GUS activity in transgenic tobacco seedlings: 2 weeks old, control (A) and transgenic (B) seedlings were stained for GUS activity. (C–D) Close-up view of developing root shows GUS localization in root tip. (E) A 4-week-old seedling shows GUS staining in leaves and root tips.

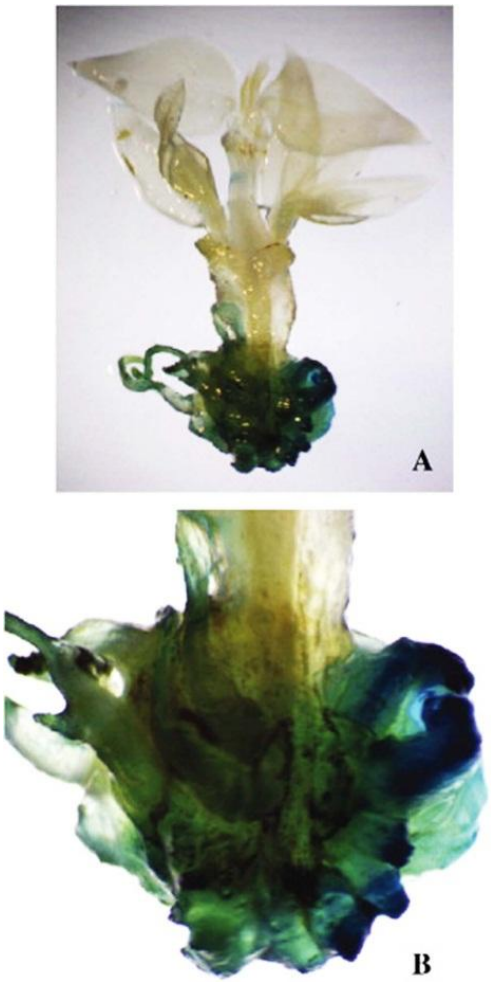

Figure 2.5 Histochemical localization of GUS activity in transgenic *C. roseus* hairy roots. (A) A 4-week-old *A. rhizogenes*-inoculated seedling shows GUS activity in transgenic hairy roots. (B) A close-up view of young developing root show GUS localization in root tip.

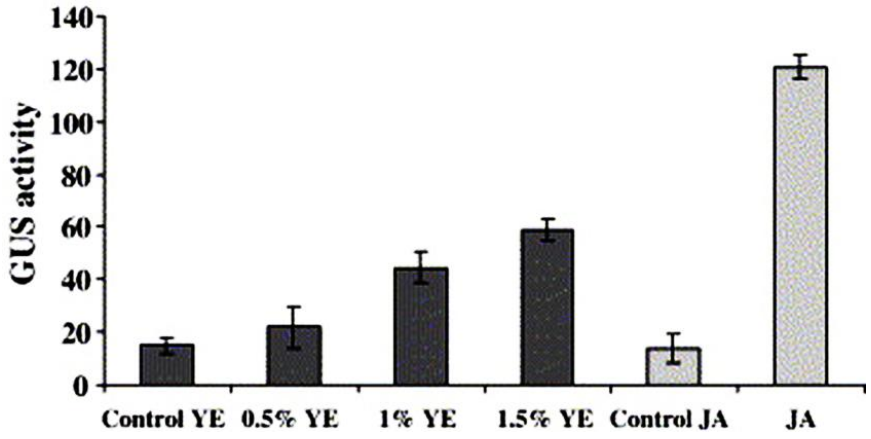

Figure 2.6 Fungal elicitor and methyl jasmonate (MeJA) induction of the *G10H* promoter activity in *C. roseus* hairy roots. For fungal elicitor induction, 0.5–1.5% of yeast extract (YE) was added to transgenic *C. roseus* hairy roots in water and incubated for 12 h at room temperature. Roots incubated in water served as control (Control YE). Following incubation, roots were stained for GUS activity. For JA induction, the transgenic *C. roseus* roots, with or without 100 μM MeJA treatment for 8 hr, were stained for GUS activity. GUS activity is presented as the average color densities of the stained root as quantified by the ImageJ software in an Olympus BX51 microscope with Olympus DP12 digital camera.

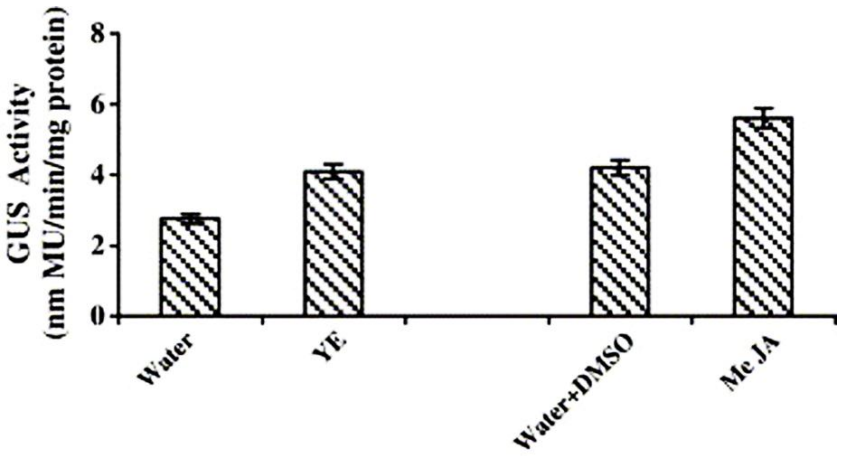

Figure 2.7 Effects of fungal elicitor (yeast extract) and methyljasmonate (MeJA) on *GUS* expression in transgenic tobacco seedlings. For fungal elicitor induction, transgenic tobacco seedlings were incubated in 1.5% yeast extract in water for 24 hr at room temperature. Seedlings incubated in water served as control (Control YE). Following incubation, seedlings were assayed for GUS activity. For JA induction, transgenic tobacco seedlings, were treated for 20 hr with or without 100 μM JA and assayed for GUS activity.

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Chapter 3

The Transcription Factor CrWRKY1 Positively Regulates the Terpenoid Indole Alkaloids Biosynthesis in *Catharanthus roseus*

3.1 Introduction

Catharanthus roseus (Madagascar periwinkle) produces a large array of terpenoid indole alkaloids (TIAs). A number of genes encoding TIA pathway enzymes and transcriptional regulators from *C. roseus* have been isolated and characterized (Liu et al.,2007; Costa et al.,2008). Attempts to increase TIA production by ectopic expression of genes encoding several rate-limiting enzymes have met with less than satisfactory results. For instance, despite an observation that overexpression of the *tryptophan decarboxylase* (*TDC*) gene has led to moderate increase of alkaloid accumulation (Hong et al.,2006), it is generally believed that TDC activity does not correlate positively with TIA production (Goddijn et al.,1995; El-Sayed and Verpoorte,2007). A more promising approach is ectopic expression of transcription factors (TFs) that regulate the TIA pathway in order to enhance alkaloid production (Memelink and Gantet,2007). Specific TFs are often capable of coordinating the transcriptions of multiple biosynthetic pathway genes, making them particularly effective in metabolic pathway engineering. Significant increase of ajmalicine production in *C. roseus* cells has been achieved by overexpression of an *Arabidopsis* TF, *Agamous-like 12* (Montiel et al.,2007). TIA biosynthesis responds strongly to a group of chemical or fungal elicitors that are known to regulate gene transcription, providing an explanation as to why the manipulation of TFs often results in substantial effects to the TIA pathway (El-Sayed and Verpoorte,2007). Promoter analyses of several structural and TF genes related to *C. roseus* TIA pathway have revealed the presence of chemical and elicitor responsive *cis* elements, leading to the cloning of jasmonate responsive TFs including the AP2-like *ORCA2* and *ORCA3* (Menke et al.,1999; Ouwerkerk and Memelink,1999; van der Fits and Memelink,2000), G-box-binding factors *CrGBF1* and *CrGBF2* (Siberil et al.,2001), P-box binding factor *CrBPF1* (van der Fits et al., 2000), zinc finger repressors *ZCT1*, *ZCT2* and *ZCT3* (Pauw et al.,2004) and the basic helix-loop-helix TF, CrMYC2 (Zhang et al.,2011). We have analyzed all available promoters of the TIA pathway genes and found the W-box (TTGACC/T) element in almost all of these promoters. The number of W-boxes in each promoter varies, ranging from one in *G10H* to two in *CPR* and four, in *TDC* and *deacetylvindoline-4-O-acetyltransferase* (*DAT*) (Ouwerkerk and Memelink,1999; Suttipanta et al.,2007; Wang et al.,2010). The W-box is a cognate binding site for WRKY TFs. The

characteristic of WRKY TFs is a conserved WRKY domain that consists of the peptide motif WRKYGQK and a zinc finger (Yamasaki et al.,2005). WRKY TFs form a large, plant-specific TF family and play dynamic roles in, among other biological processes, biotic and abiotic stress response (Rushton et al.,2010). WRKY TFs function alone or in combination with other regulators to activate, repress or de-repress transcription. WRKY TFs are known to be involved in alkaloid biosynthesis (Kato et al.,2007). In addition, when *Medicago truncatula* is exposed to fungal elicitors or methyl jasmonate (MeJA), a large number of *WRKY* genes are up-regulated, some of which are involved in production of defense compounds such as flavonoids and terpenoids (Naoumkina et al.,2007). Response to fungal elicitors and phytohormones such as methyl jasmonate (MeJA) is a hallmark of TIA pathway regulation. We therefore hypothesized that WRKY TFs are involved in the transcriptional regulation of the *C. roseus* TIA pathway. However, despite three reported sequences of putative *C. roseus WRKY* genes, obtained from EST sequencing (Murata et al.,2006), no WRKY TF from *C. roseus* has been functionally characterized to date. In this report, we describe the isolation and functional characterization of a WRKY TF, CrWRKY1, from *C. roseus*. *CrWRKY1* is preferentially expressed in roots and induced by phytohormones such as MeJA, gibberellic acid (GA) and ethylene. The overexpression of *CrWRKY1* in *C. roseus* hairy roots up-regulates several key pathway genes, especially *TDC*, and the ZCT TFs while it represses the transcriptional activators, *ORCA2*, *ORCA3* and *CrMYC2*. We show that CrWRKY1 activates the *TDC* gene by directly targeting its promoter. Moreover, transgenic CrWRKY1 hairy roots accumulate significantly higher levels of serpentine compared to control. The preferential expression of *CrWRKY1* in the root suggests that it is a key factor in determining the root-specific accumulation of serpentine in *C. roseus* plants. This work represents the first functional characterization of a *C. roseus* WRKY TF.

3.2 Materials and methods

3.2.1 Plant materials, isolation of *WRKY* cDNA and quantification of *CrWRKY1* gene

Catharanthus roseus (L.) G. Don cvs. Cooler Apricot (Swallowtail Garden, USA) seeds were surface sterilized and germinated on half-strength Murashige and Skoog (MS) basal medium. Ten-day old seedlings were treated with 100 μM MeJA and used for total RNA isolation as described previously (Suttipanta et al.,2007). Partially degenerate PCR primers (Table 3.1) were designed based on the highly conserved WRKY domain. A 180-bp product was PCR amplified by the degenerate primers using first strand cDNA synthesized from RNA isolated from MeJA-induced seedlings, and cloned into the pGEM Easy vector (Promega, USA) followed by

sequencing. To isolate the full-length cDNA of the *C. roseus* WRKY genes, 5'- and 3'-RACE were applied as described previously (Suttipanta et al., 2007). The predicted polypeptide sequences of the *C. roseus* WRKY and other available WRKY proteins were compared using the Basic Local Alignment Search Tool (BLAST) service (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment of nucleotide and deduced amino acid sequences were performed using the CLUSTAL W program (Larkin et al.,2007) with the default parameters through the service of European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). A phylogenetic tree was constructed and visualized using Neighbor-Joining (N-J) method through MEGAv4.1 software (Tamura et al.,2011). The statistical reliability of individual nodes of the newly constructed tree was assessed by bootstrap analyses with 1,000 replications. To study the effects of phytohormones on the expression of CrWRKY1, 10-day old seedlings (100 mg fresh weight) were exposed to 100 µM of MeJA, 1 ppm of ethylene or 10 µM of GA following reported procedures (El-Sayed and Verpoorte,2004). After the treatments, the seedlings were harvested at 0 min, 30 min, 1.0 hr, 2.0 hr, 4.0 hr and 8.0 hr and immediately frozen in liquid nitrogen. cDNA synthesis using total RNA from hypocotyl, young and mature leaves, root and fruit were performed as described earlier (Suttipanta et al.,2007). Reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR) were performed as described by Pattanaik et al.,2010 . The primers used in qPCR for several pathway genes are listed in Table 3.1. The comparative Ct method (Applied Biosystems bulletin) was used to measure the transcript levels. The *C. roseus* 40S ribosomal protein S9 (Rps9) gene was used as an internal control in qPCR to normalize the amount of total mRNA in all samples. All PCR reactions were performed in triplicate and repeated at least twice.

3.2.2 Plant expression vectors and hairy root transformation

The *CrWRKY1* gene was PCR-amplified and cloned into a modified pCAMBIA2301 vector, containing the *CaMV-35S* promoter and *rbc*S terminator, to generate pCrWRKY1 plasmid (Figure 3.6). *C. roseus* hairy roots were developed as described previously (Choi et al.,2004; Suttipanta et al.,2007). The pCrWRKY1 plasmid was mobilized into *Agrobacterium rhizogenes* R 1000 by freeze-thaw method. After removal of the roots, 10 day-old seedlings were immersed in the *Agrobacterium* suspension for 30 min and then transferred onto solid MS basal medium for co-cultivation. The pCAMBIA2301 vector was used as vector-only control. After 48 hr the explants were transferred to half-strength MS basal medium containing 400 mg/L cefotaxime. Hairy roots usually formed at the cut surface of the explants in 3 weeks. After 6-7 weeks, root tips of approximately 5 mm in length were excised and transferred to one-third strength Schenk and Hilderbrandt (SH) basal medium containing 100 mg/L kanamycin and 400 mg/L cefotaxime.

Three independent hairy root lines were chosen for detailed analysis. For the construction of the CrWRKY1-SRDX plasmid, sequence encoding the SRDX repressor domain (LDLELRLGFA) was fused to the C-terminal end of CrWRKY1. The *CrWRKY1-SRDX* was subsequently cloned into the modified pCAMBIA2300 vector under the control of the *CaMV35S* promoter and *rbc*S terminator (Figure 3.6B).

3.2.3 Genomic DNA isolation and PCR

Genomic DNA was isolated from independent CrWRKY1-overexpression, CrWRKY1-SRDX and empty-vector control hairy roots using the DNeasy Plant mini kit (Qiagen,USA). To verify the presence of both pCAMBIA and Ri T-DNAs in the transgenic lines, *CrWRKY1*, *kanamycin* (*Kn*), *ß-glucuronidase* (*GUS*), *rol*B and *rol*C genes were amplified from the genomic DNA using gene-specific primers (Table 3.1). All PCR reactions were performed using iProof High Fidelity DNA polymerase (Bio-Rad, USA) following the thermal cycling conditions recommended by the manufacturer. Recombinant protein production and electrophoretic mobility shift assay. The *CrWRKY1* gene was cloned into the *BamH*I/*EcoR*I sites of pGEX 4T-1 vector (Amersham, USA) to generate a glutathione-*S*-transferase (GST) fusion protein. The constructs were verified by DNA sequencing and transformed into *Escherichia coli* BL21 (DE3) cells. Cell cultures at OD600 of ~0.6 were induced by adding IPTG to a final concentration of 1 mM. After induction for 3 hr at 30°C, the cells were harvested by centrifugation and lysed using CelLytic B (Sigma, USA) according to the manufacturer's instructions. The GST fusion proteins were bound to Glutathione Sepharose 4B columns (Amersham, USA) and eluted using 50 mM Tris-HCl, pH 7.2, 150 mM NaCl and 30 mM glutathione.

3.2.4 Electrophoresis mobility shift assay (EMSA)

For EMSA experiments, three DNA probes, with or without biotin labeling, were synthesized based on the W-box elements of the *TDC* promoter (GenBank Accession no. X67662): Probe 1 (5' CAAAAA<u>TTTGACT</u>ATAC<u>TTGACT</u>ATTAGTG 3') is identical to the TDC promoter region (-1179 to –1150 relative to transcription start site, TSS) with two W-boxes (underlined), Probe 2 (5' GACTGCATG<u>TTGACC</u>TAAAATTATG 3') is a native TDC promoter sequence (-583 to -559 relative to TSS) containing a single W-box, and mProbe 2 (5'GACTGCATG<u>TTAATC</u>TAAAATTATG 3') is identical to Probe 2 except the W-box core sequence was destroyed by mutation. Complementary oligonucleotides, biotin-labeled at the 5'-end of each strand, were synthesized by Integrated DNA Technologies (Coralville, USA) and

annealed to produce double-stranded probes for EMSA. The DNA binding reactions were carried out in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 5% glycerol and 100 ng polydI:dC in a final volume of 20 μl. Purified proteins were incubated with 0.25- 0.5 nM DNA probe at room temperature for 90 min. The DNA-protein complexes were resolved by electrophoresis on 6% nondenaturing polyacrylamide gels and then transferred to Biodyne®B modified membrane (0.45 μm; Pierce, USA). The band-shifts were detected by a chemiluminescent nucleic acid detection module (Pierce, USA) and exposed to X-ray films.

3.2.5 Yeast one-hybrid assay

The pHIS2 vector (Clontech, USA), containing the *HIS3* nutritional reporter gene downstream of a multiple cloning site and minimal promoter of *HIS3* locus, was used for making the reporter plasmid. Three tandem copies of the W-box containing region of the *TDC* promoter (-583 to -559 relative to TSS) was synthesized and ligated into the *Eco*RI and *Sac*I sites of the pHIS2 vector, upstream of the *HIS3* minimal promoter, to generate the reporter plasmid (pHIS2-TDC). A reporter plasmid with the mutated W-box sequence (pHIS2-mTDC) was also made to serve as a control. The full-length *CrWRKY1* cDNA was cloned into the yeast expression plasmid, pAD-GAL4-2.1 (Stratagene, USA), to generate the effector plasmid. The reporter and the effector plasmids were transformed into yeast strain Y187 (Clontech, USA) and transformants were selected on synthetic drop-out (SD) medium lacking leucine and tryptophan. Transformed colonies were then streaked on SD-his-leu-trp medium with 100 mM 3-amino-1,2,4-triazole (3-AT) to check *TDC* promoter activation.

3.2.6 Plasmid construction for *TDC* promoter assay and CrWRKY1 nuclear localization

The two reporter plasmids used in the protoplast assay consist of the firefly luciferase coding sequence under the control of a minimal *CaMV 35S* (−46 to +8) promoter with three tandem copies of the W-box region of the *TDC* promoter (-583 to -559 relative to TSS) upstream and an *rbc*S terminator. The W-box core sequence was mutated in one reporter plasmid to serve as a control. The effector plasmids consist of the *CaMV35S* promoter and the *GAL4* activation domain (*GALAD*) fused to *CrWRKY1* coding sequences and an *rbc*S terminator. A plasmid containing the *β-glucuronidase* (*GUS*) gene under the control of the *CaMV35S* promoter and *rbc*S terminator was used as an internal control. For sub-cellular localization, two plasmids were constructed, one containing the enhanced green fluorescent protein (eGFP) and the other

containing a CrWRKY1-eGFP fusion. The expression of both genes was under the control of the *CaMV35S* promoter and *rbc*S terminator.

3.2.7 Protoplast isolation and electroporation

Protoplast isolation from *C. roseus cell* suspension cultures (DSMZ no. 510) and electroporation with supercoiled plasmid DNA were performed using protocols previously developed for tobacco protoplasts (Pattanaik et al.,2010). The reporter, effector, and internal control plasmids were electroporated into *C. roseus* protoplasts; luciferase and GUS activities in transfected protoplasts were measured as described previously (Suttipanta et al.,2007). The luciferase activity was normalized against GUS activity and expressed as fold activation relative to the reporter-only control. For sub-cellular localization, the plasmids, containing either eGFP or CrWRKY1-eGFP, were individually electroporated into *C. roseus* protoplasts and visualized under a fluorescent microscope after 20 hr incubation at room temperature.

3.2.8 Enzyme assay, alkaloid extraction, TLC, HPLC and LC-mass spectrometry

For TDC activity assay and alkaloid extraction we followed the protocols described by Miranda-Ham (Miranda-Ham et al.,2007). In routine assays, 20-µl aliquots of hairy root extracts or commercial alkaloid standards were applied onto thin layer chromatography (TLC) plates (silica G60; Merck, Germany). The TLC was run on solvent ethyl acetate: NH_4OH: chloroform (75:2:23), and alkaloids were identified under UV (λ=254 nm and 366 nm) and by color reaction with ceric ammonium sulfate (CAS). The alkaloid extracts were further analyzed using liquid chromatography-electrospray ionization mass/mass spectroscopy (LC-MS/MS) with a Varian 1200 L Triple Quadruple MS/MS (Varian, United Kingdom) equipped with an UV-DAD spectrophotometer. The LC was carried out on a 250 mm x 4.5 mm, 5 µm C-18 column (Zorbax Eclipse XDB; Agilent Technologies, USA) with a flow rate of 0.2 ml/min. The mobile phase was H_2O/0.1 % formic acid (A) and methanol/0.1 % formic acid (B). A linear gradient from 90 % A and 10% B to 10% A and 90%B was applied in 30 min. The MS/MS detector equipped with an electrospray ionization (ESI) system was operated with a capillary voltage of 30-60 kV and heated at 300°C. Nitrogen was used as flowing dry gas at a pressure of 20 psi and nebulizing gas at a pressure of 50 psi. Analysis was performed in scan mode ranging from m/z 100 to 900. Collision-induced dissociation experiments were performed in the ion trap using helium as collision gas. The collision energy varied from 6 to 27. The isolation width of the parent ion for following MS fragmentation events was set at +0.5. Alkaloids were verified by their retention

time (Rt), diode array profiles and MS/MS spectra compared with authentic standards. Serpentine is detected at Rt ~12 min with λ max of 249 nm/306 nm/367 nm and +MS: 349$[M+H]^+$, +MS2(349): 317 ; catharanthine at Rt ~17 min with λ max of 225 nm/283 nm and +MS: 337$[M+H]^+$, +MS2(337): 173, 144; ajmalicine at Rt ~22 min with λ max of 226 nm/244 nm/283 nm and +MS: 353 $[M+H]^+$, +MS2 (353): 321, 222, 210, 144; and tabersonine at Rt ~24 min with λ max of 227 nm/292 nm/267 nm and +MS: 337$[M+H]^+$, +MS2(377): 305. For quantitative determination of serpentine, catharanthine, ajmalicine and tabersonine, RP-HPLC was performed using a 250 mm x 4.5 mm, 5 μm C-18 column and a linear solvent gradient from 80% - 20% 5 mM phosphate buffer and 20% - 80% acetonitrile, at a flow rate of 1.0 ml/min, and monitored by a Waters 2998 photodiode array detector (Waters, USA). The authentic alkaloid standards were used to establish standard curves for quantification. Data were analyzed using the Masslynx software (Waters, USA). Chromatographic peaks were integrated and compared with the respective standard curves to calculate the total amount of alkaloids in each sample.

3.3 Results

3.3.1 Isolation and expression profiling of *CrWRKY1*

We aimed to isolate WRKY TFs that are induced by MeJA and have corresponding expression profiles as key TIA pathway genes. Degenerate PCR primers (Table 3.1), based on conserved sequences of WRKY domains, amplified bands of approximately 180-bp from mRNA isolated from MeJA-treated *C. roseus* tissues. DNA sequencing yielded a total of six unique open reading frames containing the WRKYGQK sequence. Functional characterization of one of the WRKY genes, designated as CrWRKY1 (GenBank accession no. HQ646368) was described here. *CrWRKY1* encodes a 259-amino acid protein, having a calculated molecular mass of 29.3 kD and a deduced pI of pH 9.4. Sequence alignment (Figure 3.1) revealed that CrWRKY1 shares high amino-acid sequence identity with the *Nicotiana tabacum* NtWRKY3 (42%), *Glycine max* GmWRKY58 (45%), and *Arabidopsis* AtWRKY 70 (40%), all of which are classified as group III WRKY TFs (Zhang and Wang,2005). Phylogenetic analysis of selected WRKY proteins placed CrWRKY1 close to NtWRKY3 (Figure 3.1B). CrWRKY1 possesses one WRKY domain together with a zinc-finger that is unique to Group III WRKY proteins (Eulgem et al.,2000). A putative bipartite nuclear localization signal (RKKSAGVKYRKGSYKK at amino acid 92-108) was also identified in CrWRKY1. To demonstrate that CrWRKY1 is indeed nucleus-localized, we fused *CrWRKY1* in-frame to the enhanced green florescent protein gene, *eGFP*, and expressed the fusion gene in *C. roseus* cell suspension-derived protoplasts. While control eGFP

accumulated throughout the cell (Figure 3.3 A), CrWRKY1-eGFP fusion protein was localized to the nucleus (Figure 3.3B). The expression of *CrWRKY1* in various *C. roseus* tissues was examined using qPCR (Figure 3.4). Significant expression of *CrWRKY1* could be detected in fruit tissue and mature leaf, whereas the expression is barely detectable in hypocotyl, young leaf and flower. However, the highest expression of *CrWRKY1* (approximately 2.5-fold of that in fruit) was found in the root.

3.3.2 Induction of *CrWRKY1* expression by jasmonate, ethylene and gibberellic acid

To investigate the effect of phytohormones on *CrWRKY1* expression, 10-day old *C. roseus* seedlings were treated with MeJA, ethylene and gibberellic acid (GA) in a time-course study. The expression of *CrWRKY1* was measured by qPCR using RNA isolated from treated plants (Figure 3.5). Compared to the uninduced control, *CrWRKY1* expression increased approximately 5-fold within 30 min after exposure to 100 μM MeJA and gradually reduced during the next 8-hr course. Effects of ethylene (1 ppm) on *CrWRKY1* expression reached its peak in 2 hr and returned to basal level in the next 2 hrs. By contrast, the effect of GA (10μM) on *CrWRKY1* expression is longer lasting, reaching a more than 4X increase within 30 min and maintaining the high expression level for at least 8 hr (Figure 3.5).

3.3.3 Activation of the *TDC* gene by *CrWRKY1* in transgenic hairy roots

We generated a number of independent hairy root lines expressing *CrWRKY1* under the control of the *CaMV-35S* promoter. The frequency of co-transformation of Ri T-DNA and transgene T-DNA was approximately 25%. The presence of both pCAMBIA and Ri plasmid T-DNAs in independent *CrWRKY1*-overexpression and empty vector control hairy-root lines was verified byPCR using gene specific primers (Figure 3.6 C). Two empty-vector controls and three overexpression lines were chosen for further analysis. In the CrWRKY1-overexpressing hairy roots, the *CrWRKY1* gene was expressed 17- to 22-fold higher than the empty-vector control (Figure 3.7 A). The expression of several other pathway genes were also up-regulated; among them the most striking was the *TDC* gene which increased ranging from 7- to 9-fold in the hairy roots compared to the control. Although to a lesser extent than *TDC*, expression of *anthranilate synthase* (*AS*), *1-deoxy-D-xylulose-5-phosphate synthase* (*DXS*), *secologanin synthase* (*SLS*) and *strictosidine* β-*D-glucosidase* (*SGD*) increased in *CrWRKY1*-overexpressing hairy roots by an average of 1.2-, 0.7-, 2.3- and 2.4-fold, respectively, compared to the control (Figure 3.7A). Overexpression of *CrWRKY1* did not significantly affect the expression of other pathway genes

including *G10H*, *cytochrome P450 reductase* (*CPR*) and *strictosidine synthase* (*STR*). We also measured the expression of several TF genes known to be involved in the TIA pathway in *CrWRKY1* hairy roots. The expression of *ORCA2, ORCA3* and *CrMYC2* were suppressed by an average of 90%, 80% and 60%, respectively, compared to that of vector only control (Figure 3.7 B). Overexpression of *CrWRKY1* also led to increases in expression of *ZCT1, ZCT2 and ZCT3* by an average of 1.4-, 0.3- and 3.2-fold, and had little effect on the expression of the two G-box binding factors, *GBF1* and *GBF2* (Figure 3.7 B). Because *TDC* is the gene most affected in the CrWRKY1 hairy roots, we applied gene silencing technology to further clarify the role of CrWRKY1 in regulation of *TDC*. We employed chimeric repressor silencing technology [CRES-T; (Hiratsu et al., 2003)] in which the SRDX repression domain was fused to the C-terminus of CrWRKY1, and obtained hairy roots overexpressing *CrWRKY1-SRDX*. Transgenic status of the CrWRKY1-SRDX lines was verified by PCR-amplifying the transgenes from genomic DNA using gene specific primers (Figure 3.6 C). Two empty-vector control and two CrWRKY1-SRDX hairy-root lines were chosen for further analysis. qPCR were performed to measure the expression of pathway and TF genes using RNA isolated from the CrWRKY1-SRDX hairy roots. In general, an opposite expression pattern was observed for each gene compared to the *CrWRKY1*-overexpression lines (Figure 3.7 A and B). Compared to the empty vector control, *TDC* expression in the CrWRKY1-SRDX lines decreased by an average of 83%, while expression of *AS* and *DXS* decreased by an average of 47% and 88%, respectively (Figure 3.7 A). In the same repression hairyroot lines, expression of the three transactivator genes, *ORCA2*, *ORCA3* and *CrMYC2*, are 1-, 4-and 3-fold of those of vector-only control, while expression of the three transrepressors, *ZCT1, ZCT2 and ZCT3,* decreased by an average of 71%, 33% and 72%, respectively (Figure 3.7 B).

3.3.4 Increase of TDC activity in CrWRKY1-overexpressing hairy roots

TDC can convert the phytotoxic tryptophan analogue 4-methyl tryptophan (4-MT) into the nontoxic 4-methyl tryptamine and is thus a useful selectable marker for plant cells (Goddijn et al.,1993). To test whether CrWRKY1 hairy roots, in which the *TDC* gene is highly expressed, confer 4-MT resistance, we screened the hairy roots for growth in the presence or absence of 4-MT (Figure 3.8 A-F). At 250 μM 4-MT, CrWRKY1 hairy roots sustained growth and formed new roots (Figure 3.8 B), whereas empty vector control roots were severely stunted (Figure 3.8 E). Even at 500 μM 4-MT, CrWRKY1 hairy roots, although somewhat stunted, continued their growth (Figure 3.8 C) while the control roots did not survive (Figure 3.8 F). These results indicate that elevated TDC activity is associated with the CrWRKY1 hairy roots.

Soluble proteins were extracted from hairy roots grown on medium without 4-MT and assayed for TDC enzyme activity using tryptophan as substrate. The TDC activities in empty vector control, CrWRKY1 and CrWRKY1-SRDX hairy roots were 21.6 + 3.8 and 37.7 + 2.2 and 17.3 +5.3 pM tryptamine/s/mg total soluble protein, respectively, representing a 73% increase of TDC activity in CrWRKY1 hairy roots and 20% decrease of the enzyme activity in CrWRKY1-SRDX hairy roots. Correspondingly, tryptamine content in control, CrWRKY1 and CrWRKY1-SRDX hairy roots were 30.1 + 2.3, 76.0 + 9.9 and 23.3 + 4.6 μg/g dry weight, respectively, representing a 150% increase in CrWRKY1 hairy roots and 23% decrease in CrWRKY1-SRDX hairy roots compared to the control.

3.3.5 Binding of CrWRKY1 to the W-boxes in the *TDC* promoter

Electrophoretic mobility shift assay (EMSA) was used to determine the binding affinity of CrWRKY1 to the *TDC* promoter. We first purified the recombinant, GST-tagged CrWRKY1 protein (GST-CrWRKY1) from *E. coli* using GST affinity chromatography which yielded approximately 160 μg of purified protein per liter of cell culture. For EMSA experiments, three DNA probes, with or without biotin labeling, were synthesized based on the W-box elements of the *TDC* promoter. Probe 1 (5' CAAAAATTTGACTATACTTGACTATTAGTG 3') is identical to the *TDC* promoter region (-1179 to –1150 relative to transcription start site, TSS) where two W-boxes (underlined) are adjacent to one another. Probe 2 (5' GACTGCATGTTGACCTAAAATTATG 3') is a native *TDC* promoter sequence (-583 to -559 relative to TSS) containing a single W-box. The third probe, mProbe 2 (5' GACTGCATGTTAATCCTAAAATTATG 3'), is identical to Probe 2 except the W-box sequence was destroyed by mutation in the W-box core sequence. As shown in Figure 3.9 A, the recombinant CrWRKY1 bound to Probe 1 and Probe 2 and resulted in mobility shifts (lane 2 in both Figure 3.7A Gel 1 and 2); however the protein did not bind mProbe 2 (lane 2 in Figure 3.9 A Gel 3). The W-box binding specificity of GST-CrWRKY1 is further confirmed by a competition experiment showing that binding of the labeled probes by CrWRKY1 could be eliminated by increasing concentrations (250X and 500X in excess) of unlabeled Probe 1 and Probe 2 (lanes 2 and 3 in Figure. 3.9 A Gel 1 and 2). Binding of CrWRKY1 to the *TDC* promoter was also verified *in vivo* using yeast one-hybrid. The reporter plasmid used in the yeast one-hybrid assay contains three tandem repeats of the W-box containing region of the *TDC* promoter (-559 to-583 relative to TSS) driving expression of the *HIS3* selection gene. The W-box core sequence was mutated in one reporter plasmid to serve as a control. The chimeric CrWRKY1 fused with the yeast GAL4-activation domain (ADCrWRKY1) was co-transformed into yeast cells with the

individual reporter plasmid. Yeast colonies expressing the *TDC* W-box reporter grew on triple (-His-Leu-Trp) selection medium with 3-AT (Figure 3.9 B), indicating transactivation of the *TDC* promoter by AD-CrWRKY1. The yeast cells transformed by AD-CrWRKY1 and reporter with the mutated W-box failed to grow on the triple selection medium, indicating that the W-box elements are essential for binding and activation by AD-CrWRKY1. Binding of CrWRKY1 to the *TDC* promoter was further confirmed by a transient protoplast assay (Figure 3.9 C). Luciferase reporter plasmids containing tandem repeats of the *TDC* W-box or the mutated W-box sequence (mTDC) were electroporated into *C. roseus* protoplasts alone or with AD-CrWRKY1. The luciferase activity in protoplasts electroporated with the *TDC* reporter+AD-CrWRKY1 was approximately 1.7-fold of that in the protoplasts electroporated with reporter alone. Furthermore, AD-CrWRKY1 failed to activate the mTDC luciferase reporter containing the mutated W-box sequence (Figure 3.9 C).

3.3.6 Alkaloid accumulation in hairy roots overexpressing or repressing CrWRKY1

Quantification of alkaloids in vector-only control, CrWRKY1 and CrWRKY1-SRDX hairy roots were measured on equal dry weight basis. Based on the color reaction with ceric ammonium sulfate (CAS) and UV absorbance characteristics, the major alkaloids were identified by TLC as serpentine, ajmalicine, catharanthine and tabersonine. These alkaloids were subsequently analyzed by reverse phase (RP)-HPLC with a photodiode array detector in combination with liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) (Figure. 3.10, 3.11 and 3.12). On average, the two independent lines of control hairy roots produced 131.5+23.7, 87.5+6.5, 12.8+1.4 and 11.2+3.0 μg/g of dry roots of serpentine, catharanthine, ajmalicine and tabersonine, respectively (Figure 3.10). In contrast, the three independent CrWRKY1 lines showed variable but significant increases of serpentine (291.5+73.2 μg/g of dry roots) and ajmalicine (15.4+1.6 μg/g), as well as significant decrease of catharanthine (45.5+5.1 μg/g) and tabersonine (9.0+3.0 μg/g). The three independent lines of CrWRKY1-SRDX produced comparable levels of serpentine and ajmalicine to those of the control lines; however, they produced significantly higher levels of catharanthine (100.2+15.1 μg/g) and tabersonine (19.3+1.6 μg/g).

3.4 Discussion

3.4.1 The phytohormone-inducible CrWRKY1 belongs to the group III WRKY superfamily and preferentially expresses in roots

JA induction of a number of genes encoding *C. roseus* TIA pathway enzymes and TFs is well established. Phytohormones, including JA and GA, have been shown to induce the expression of certain *WRKY* genes in other species (Naoumkina et al.,2008; Ramamoorthy et al.,2008). The presence of W-boxes in the majority of characterized promoters of TIA-associated genes suggests that *WRKY* genes are involved in regulating these genes. In this study we functionally and biochemically characterize the *C. roseus WRKY* gene, *CrWRKY1*. CrWRKY1 possesses the characteristics of the Group III WRKY, featuring a single WRKY domain in the center of the polypeptide and the unique zinc finger motif C-X6-C-X23-H-XC (Figure 3.1). The lack of both activation or repression activities, in protoplast and yeast one-hybrid assays, suggests that it mainly functions by recruiting a transcriptional activator or repressor to the targeted promoters. Although WRKY TFs can bind to W-boxes as a monomer, they are also known to form homodimers, as well as, heterodimers with other TFs. In *Arabidopsis*, different WRKY18, WRKY40 and WRKY60 complexes play distinct roles in response to pathogens (Xu et al.,2006). Similar to these *Arabidopsis* WRKY factors, CrWRKY1-GFP is also localized in the nucleus (Figure 3.3). Expression analysis of *CrWRKY1* in various tissues reveals that it preferentially expresses in roots of *C. roseus* (Figure 3.3). The biosynthesis of TIAs is highly compartmentalized in *C. roseus*, presumably due to spatial and temporal transcriptional regulation. *In situ* RNA hybridization and immunocytochemical analyses show that *TDC* and *STR* mRNA are heavily present in the epidermis of stems, leaves and flower buds while also associating with cells around the apical meristem of root tips (St-Pierre et al.,1999). Hence, TFs associated with the TIA pathway are expected to display tissue- or cell-specific expression patterns. In addition to response to JA induction, TIA accumulation in *C. roseus* is also induced by other phytohormones such as ET and GA. The productions of ajmalicine in cell suspensions, hairy roots and shoot cultures of *C. roseus* are greatly enhanced by JA and ethylene, but not salicylic acid. The hormone-induced increases of the TIAs are, in general, accompanied by the increase of TDC activity (Vázquez-Flota et al.,2009). CrWRKY1 is highly up-regulated by MeJA, ET and GA; however, the different induction patterns of the three chemicals suggest separate induction pathways are involved in governing *CrWRKY1* expression.

3.4.2 CrWRKY1 activates the *TDC* gene by targeting its promoter

TDC converts tryptophan to tryptamine, a starting substrate for biosynthesis of TIAs. In a recent elegant endeavor to re-direct the *C. roseus* TIA pathway to produce non-natural TIAs, all natural TIAs were eliminated as a result of RNAi gene silencing of *TDC*, creating a precursor-free system that can be explored for production of novel TIAs by feeding unnatural starting substrates (Runguphan et al.,2009; Runguphan and O'Connor,2009). TDC is clearly a key enzyme in the TIA pathway, and thus, understanding the transcriptional regulation of *TDC* is important. Here we present multiple lines of evidence to show that CrWRKY1 regulates *TDC* expression. First, *TDC* is highly upregulated in CrWRKY1 hairy roots (Figure 3.7). Second, consistent with increased *TDC* expression, TDC enzyme activity is elevated greater than 73% in CrWRKY1 hairy roots compared to control roots. In addition, CrWRKY1 hairy roots are significantly more resistant to 4-MT inhibition than control roots (Figure 3.8). Relying on detoxification of 4-MT as selection has led to the isolation of ORCA3, an AP2-like TF that up-regulates *TDC* (van der Fits and Memelink,2000). Overexpression of *ORCA3* increases TDC activity and accumulation of tryptamine in *C. roseus* suspension cells (van der Fits et al.,2000), but not in hairy roots (Peebles et al.,2009). In contrast, CrWRKY1 hairy roots not only have increased TDC activity, but also accumulate nearly 2-fold more tryptamine than control roots. In addition, *CrWRKY1-SRDX* expression results in an approximately 20% decrease of cellular TDC activity and a 23% reduction in tryptamine accumulation in hairy roots. EMSA, yeast one hybrid and *C. roseus* protoplasts transactivation assays were used to demonstrate the physical interaction of CrWRKY1 to W-box elements in the *TDC* promoter (Figure 3.9 A-C). Supporting evidence for binding of CrWRKY1 to the *TDC* promoter in *C. roseus* cells also comes from CrWRKY1-SRDX hairy roots. *TDC* expression is reduced significantly in CrWRKY1-SRDX hairy roots (Figure 3.7 A), suggesting that the CrWRKY1-SRDX repressor binds to the *TDC* promoter though the possibility that CrWRKY1 activates *TDC* by controlling the expression of an unknown TDC-activator cannot be excluded. However, this seems unlikely as it would require the existence of the same mechanism in yeast cells. Overexpression of *CrWRKY1* does not affect the expression of *STR*, which lacks the W-box consensus sequence in its promoter. Taken together, these results support that CrWRKY1 activates the *TDC* gene by targeting its promoter.

3.4.3 Overexpression or repression of *CrWRKY1* affects accumulation of serpentine in hairy roots

In roots of naturally grown *C. roseus*, serpentine and ajmalicine are the major alkaloids. The TIA pathway is highly branched and diversified from strictosidine onward. Enzymes and genes involved in the catharanthine pathway have yet to be isolated. The better characterized vindoline pathway starts with hydroxylation of tabersonine. Although no gene has been cloned for the serpentine pathway, it is believed that a cathenamine reductase converts cathenamine to ajmalicine, which is then metabolized to serpentine, putatively by a peroxidase. Overexpression of *CrWRKY1* appears to preferentially activate the serpentine pathway at the expense of the catharanthine and tabersonine pathways, as indicated by the significant increase of serpentine and reduced production of catharanthine and tabersonine in CrWRKY1 hairy roots. Consistent with this conclusion is the observation that repression of *CrWRKY1* by CrWRKY1-SRDX results in increases of catharanthine by approximately 15% and tabersonine by approximately 72% (Figure 3.9). Feeding a synthetic tryptamine analogue to the *TDC*-silenced *C. roseus* hairy roots results in a significant increase of unnatural ajmalicine, but not serpentine, indicating that the putative ajmalicine peroxidase is not sufficient to convert the increased ajmalicine to serpentine (Runguphan et al.,2009). This can either be a consequence of the inability of the peroxidase to catalyze the modified ajmalicine or its persistent low activity in the *TDC*-silenced lines. CrWRKY1 hairy roots accumulate considerably more serpentine than ajmalicine, indicating that the peroxidase is highly active in conversion of ajmalicine to serpentine.

3.4.4 The roles of CrWRKY1 in TIA pathway regulation

JA induces TIA-associated transcriptional activators such as CrMYC2 and ORCA3 that stimulate pathway genes, including *TDC* and *STR* (Zhang et al.,2011). However, JA also induces the repressors ZCT and GBF, proteins that are known to inhibit the expression of *TDC* and *STR* (Memelink and Gantet,2007). The emerging picture of transcriptional control of TIA biosynthesis suggests simultaneous induction of activators and repressors by elicitors and developmental signals. These regulators form a dynamic network that fine-tunes the amplitude, timing and tissue specificity of gene expression. The previously identified TIA-associated TFs include the ORCA proteins, ZCTs, GBFs, CrMYC2 and several AT-Hook DNA-binding proteins (Vom Endt et al.,2007). Here we show that the newly characterized CrWRKY1 is a member of the TIA regulatory network. Our current understanding of CrWRKY1 illustrates complex interaction and co-regulation of the TIA pathway by multiple TFs (Figure 3.14). Phytohormones

(JA, GA and Ethylene) induce *CrWRKY1* expression. Overexpression of *CrWRKY1* up-regulates the *DXS* and *SLS* genes in the terpenoid pathway, as well as the *AS* and *TDC* genes in the indole pathway. Based on the observation that CrWRKY1 by itself lacks transactivational activity, we anticipate that CrWRKY1 functionally interacts with other TFs. Overexpression of *CrWRKY1* moderately up-regulates the *ZCT* genes, but strongly represses *ORCA3* and *ORCA2*. Expression of *CrWRKY1-SRDX* significantly up-regulates *ORCA3* but not *ORCA2* (Figur 3.7 B). We envision CrWRKY1 represses *ORCA3* by activating a yet to be identified *ORCA3*-repressing factor (ORF; Figure 3.14). Memelink and colleagues propose that *ORCA3* is dually controlled by sets of qualitative (on/off switch) and quantitative regulators (Vom Endt et al.,2007). In this scenario, quantitative regulators, such as the AT-Hook DNA-binding proteins, control the strength and timing of*ORCA3* expression, while a potential repressor (ORF) acts as an on/off switch by occupying the *ORCA3* promoter and preventing transactivation. CrWRKY1 may be an activator of *ORF* that is up-regulated when *CrWRKY1* is overexpressed, resulting in the severe repression of *ORCA3*. Repression of *ORF* by CrWRKY1-SRDX de-represses the *ORCA3* promoter, leading to up-regulation of *ORCA3*. Alternatively, CrWRKY1 regulates *ORCA3* by repressing *CrMYC2* which controls the JA-responsive expression of *ORCA3* (Zhang et al.,2011). Our results are in agreement with recent observations showing that JA induction increases TIA accumulation in *C. roseus* hairy roots, yet, overexpression of *ORCA3* does not enhance TIA production (Peebles et al.,2009). Correspondingly, JA elicitation, but not *ORCA3* overexpression, up-regulates *TDC* expression. In general, the transcript levels of *TDC*, *DXS*, *G10H*, *SGD* and *ORCA2* are lower in the *ORCA3*-overexpressing lines than in the JA-induced lines. The authors have thus concluded that (1) a negative regulatory mechanism overrides the activation of these genes by ORCA3, and (2) another strong positive regulator of the TIA pathway exists in *C. roseus* (Peebles et al.,2009). Here we demonstrate that CrWRKY1 is a negative regulator of ORCA3 and a positive regulator of the TIA pathway. The genetic interaction of *CrWRKY1* with *ORCA3* is compelling. Overexpression of *CrWRKY1* results in up to 3-fold increase of serpentine in *C. roseus* hairy roots (Figure 3.10); however, overexpression of *TDC* does not result in similar levels of serpentine production (Goddijn et al.,1995; Hughes et al.,2004). Therefore, up-regulation of *TDC* alone is evidently not sufficient to explain the large increase of serpentine induced by CrWRKY1. In addition, feeding the precursor, tryptamine, to *C. roseus* hairy roots does not lead to significant increases in serpentine or ajmalicine production (Goklany et al.,2009) simply increasing the concentration of tryptamine cannot explain how CrWRKY1 preferentially activates the serpentine branch of the pathway. We speculate that CrWRKY1 genetically interacts with other genes to regulate the TIA pathway (Figure 3.14). The

activator, ORCA3, apparently pushes the flux towards the vindoline and catharanthine pathways. Previous work has demonstrated that ORCA3 activates the vindoline pathway enzyme, tabersonine 16-hydroxylase (T16H) (Memelink and Gantet,2007) and when sufficient precursors from the terpenoid pathway become available, as the result of *G10H* overexpression, ORCA3 preferentially activates the catharanthine pathway (Wang et al.,2010). Conversely, CrWRKY1 induces the serpentine pathway by repressing *ORCA3* and activating the serpentine branch genes, including the putative peroxidase gene converting ajmalicine to serpentine. The reduction of catharanthine in CrWRKY1 hairy roots (Figure 3.10) is also consistent with the repression of *ORCA3* by CrWRKY1. In roots of naturally grown *C. roseus*, serpentine and its isomers account for approximately 65% of the alkaloids (Ferreres et al.,2010). This is consistent with our results showing high *CrWRKY1* expression and relatively low *ORCA3* expression in roots. Therefore, we believe that CrWRKY1 plays a key role in determining the preferential accumulation of ajmalicine and serpentine in *C. roseus* roots. As a global regulator of the TIA pathway, CrWRKY1 possesses overlapping functions with the ORCA TFs. Similar to ORCA3, CrWRKY1 activates pathway enzymes including AS, DXS, SLS, SGD and TDC, as well as ZCT transrepressors (Figure 3.7). These results point to the presence of crosstalk within the TIA regulatory network. The picture of this network will no doubt become more complex as additional pathway enzymes and regulators are discovered and characterized. Nevertheless, the discovery and functional characterization of CrWRKY1 not only provides a biological explanation for root-specific production of serpentine, but also opens (Naoumkina et al.,2008) a new door to elucidating the molecular mechanisms underlying TIA biosynthesis. To the best of our knowledge, *CrWRKY1* is the first *C. roseus* WRKY gene to be functionally characterized. Other *C. roseus* WRKY genes may also participate in the regulation of the TIA pathway. CrWRKY1 is capable of activating the serpentine branch to completion. This unique characteristic is biotechnologically relevant due to the pharmaceutical values of ajmalicine and serpentine. The genetic interaction between CrWRKY1 and ORCA3, as well as other regulators, can be explored for engineering of selective TIAs. For example, silencing CrWRKY1 in *C. roseus* may help guide the pathway towards catharanthine and vindoline, the precursors of vinblastine and vincristine. Furthermore, CrWRKY1 hairy roots are an excellent system for gene discovery of the currently unknown enzymes that catalyze the serpentine pathway.

Table 3.1 Oligonucleotide used in PCR and qPCR

Gene	Description (accession no)	Primer pairs
AS	Anthranilate synthase (AF441857)	5'-GCGAACATTTGCAGATCCAT-3' 5'-GGCCGATTTGTTATTGTTCC-3'
TDC	Tryptophan decarboxylase (X67662)	5'-ATCCGATCAAACCCATACCA-3' 5'-CGTCATCCTCGACCATTTTT-3'
DXS	1-Deoxy-*D*-xylulose 5-phosphate synthase (AJ011840)	5'-TCGCTGCAGAACTTAGAGCA-3' 5'-GCCAACATCCCAAATGATTC-3'
G10H	Geraniol 10-hydroxylase (AJ251269)	5'-TTATTCGGATTCTGCCAAGG-3' 5'-TCCCCAAAGTGAATCGTCAT-3'
CPR	Cytochrome P450 reductase (X69791)	5'-TGGCAGAAAAGGCTTCTGAT-3' 5'-CTCAGCCTGTGTGCTATCCA-3
SLS	Secologanin synthase (L10081)	5'-GTTCCTTCTCACCGGAGTTG-3' 5'-CCCATTTGGTCAACATGTCA-3'
STR	Strictosidine synthase (X61932)	5'-ACCATTGTGTGGGAGGACAT-3' 5'-ATTTGAATGGCACTCCTTGC-3'
SGD	Strictosidine-β-D-glucosidase (AF112888)	5'-GGAGGCTTCTTGAGTGATCG-3' 5'-GCAAATTCACCAGTGGCATA-3
ORCA2	AP2-domain DNA-binding protein (AJ238740)	5'-GAAGATGCGGCATTAGCTTT-3' 5'-TTGAGGACGAAGATGACACG-3'
ORCA3	AP2-domain DNA-binding protein (AJ251249)	5'-CGGGATCCGAAATACAGAAA-3' 5'-GCCCTTATACCGGTTCCAAT-3'
ZCT1	Zinc finger DNA-binding protein (AJ632082)	5'-AGCCGAAAACTCATGCTTGT-3' 5'-CGCCTTTGCAACAGGTTTAT-3'
ORCA2	AP2-domain DNA-binding protein (AJ238740)	5'-GAAGATGCGGCATTAGCTTT-3' 5'-TTGAGGACGAAGATGACACG-3'
ZCT2	Zinc finger DNA-binding protein (AJ632083)	5'-CGTCAATTTCCATCGTTTCA-3' 5'-CCGATAGCGAATTCAAGTCC-3'
ZCT3	Zinc finger DNA-binding protein (AJ632084)	5-GACAAGCTTTGGGAGGACAC-3' 5'-GGCAAGGCAGGTAAGTTCAA-3'

Table 3.1 Oligonucleotide used in PCR and qPCR (continue)

Gene	Description (accession no)	Primer pairs
GBF1	G-box-binding protein (AF084971)	5'-AACAGGCTGAGACGGAAGAA-3' 5'-GACCCGTGCATTTTTCAACT-3'
GBF2	G-box-binding protein (AF084972)	5'-GGAAGGTGCCATCTACTCCA-3' 5'-CTAGATCGCCGAGCAGATTC-3'
CrMYC2	bHLH DNA binding protein(AF283507)	5'-TTTGGCAGTCGTCTGTTGTC-3' 5'-CAAAAGAACTCGCGGAAGAC-3'
rps9	40S ribosomal protein S9 (AJ749993)	5'-GAGGGCCAAAACAAACTTGA-3' 5'-CCCTTATGTGCCTTTGCCTA-3'
WRKY Degenerate	degenerate primers designed according to the sequences encoding conserved WRKY domain	5'-TGGMGIAARTAYGGNCARAAR-3' 5'-GAYGGITAYAAYTGGMGIAARTAY-3' 5'-RTGYTTICCYTCRTAIGTDGT-3'
CrWRKY1	WRKY DNA binding protein (HQ646368)	5'- GTACTTGGTCCCGACGATATTC-3' 5'-CGAAACATTCCTTCGTTTGTAAG-3'
GBF2	G-box-binding protein (AF084972)	5'-GGAAGGTGCCATCTACTCCA-3' 5'-CTAGATCGCCGAGCAGATTC-3'
CrMYC2	bHLH DNA binding protein(AF283507)	5'-TTTGGCAGTCGTCTGTTGTC-3' 5'-CAAAAGAACTCGCGGAAGAC-3'
rps9	40S ribosomal protein S9 (AJ749993)	5'-GAGGGCCAAAACAAACTTGA-3' 5'-CCCTTATGTGCCTTTGCCTA-3'
rol B	*role B* gene of *Agrobacterium rhizogenes* R1000	5'-CTTATGACAAACTCATAGATAAAAGGTT-3' 5'- TCGTAACTATCCAACTCACATCAC-3'
rol C	*rol C* gene *Agrobacterium rhizogenes* R1000	5'-CAACCTGTTTCCTACTTTGTTAAC-3' 5'- AAACAAGTGACACACTCAGCTTC-3'
GUS	ß-glucuronidase gene	5'-ATGGTAGATCTGAGGGTAAATTTC-3' 5'- GCTAGCTTGTTTGCCTCCCTG-3'
NptII	Neomycin phosphotransferase gene	5'-ATGGGGATTGAACAAGATGGA-3' 5'-TCAGAAGAACTCGTCAAGAAG -3'

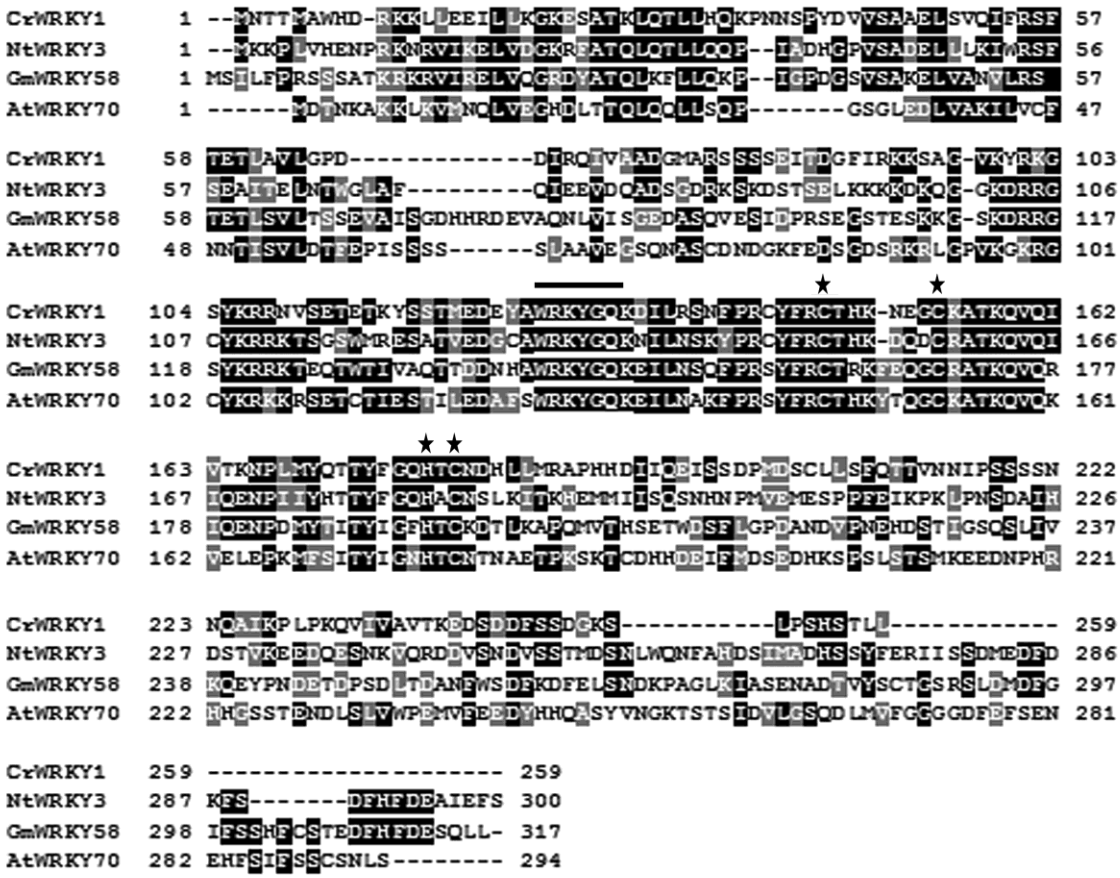


Figure 3.1 Multiple alignment and phylogenetic analysis of selected WRKY proteins. Deduced amino acid sequence of CrWRKY1 is aligned with homologues from *Nicotiana tabacum* (NtWRKY3; Accession no. AF193770), *Glycine max* (GmWRKY54; Accession no. EU375354) and *Arabidopsis thaliana* (AtWRKY70; Accession no. AF421157). The WRKY signature motif (WRKYGQK) is indicated by a straight line and the characteristic residues for the zinc-finger motif are marked by asterisks.

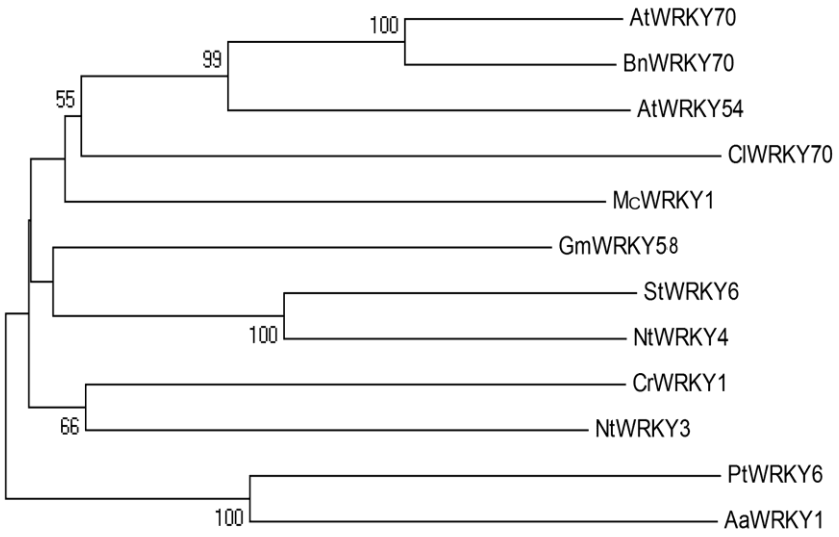


Figure 3.2 A neighbor-joining phylogenetic tree of CrWRKY1 and selected WRKY domain proteins from other plant species is constructed using the MEGA5 software. The statistical reliability of individual nodes of the newly constructed tree is assessed by bootstrap analyses with 1,000 replications. The WRKY proteins, their respective plant species, and GenBank accession numbers are as follows: AtWRKY70, *A. thaliana* (AF421157; At3G56400); BnWRKY70, *Brassica napus* (FJ384113); AtWRKY54, *A. thaliana* (NM_129637; At2G40750); ClWRKY70, *Citrullus lanatus* (GQ453670); McWRKY1, *Matricaria chamomilla* (AB035271); GmWRKY58, *Glycine max* (EU375354); StWRKY6, *Solanum tuberosum* (EU056918); NtWRKY3, *N. tabacum* (AF193770); CrWRKY1, *Catharanthus roseus* (HQ646368); NtWRKY4, *N. tabacum* (AF193771); PtWRKY6, *Populus tomentosa* x *P. bolleana* (GQ377426); AaWRKY1, *Artemisia annua* (FJ390842).

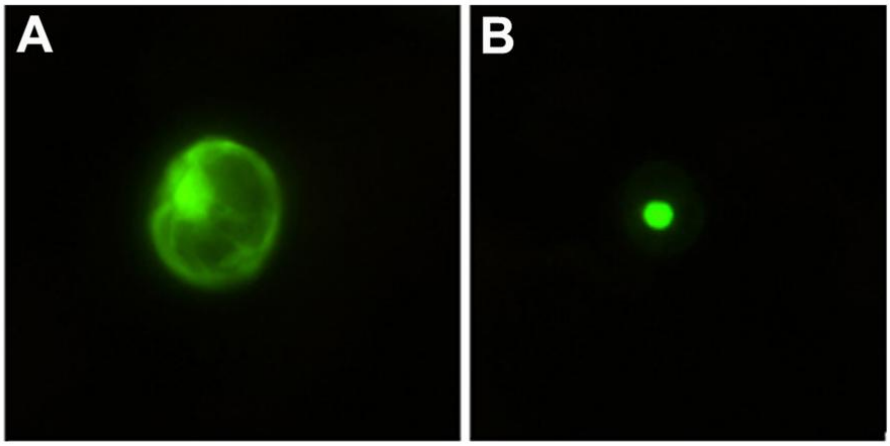


Figure 3.3 Sub-cellular localization of CrWRKY1 in *C. roseus* protoplasts. eGFP is accumulated throughout the cell (A) whereas CrWRKY1-eGFP is localized to the nucleus in *C. roseus* cells (B). The experiment was repeated two times and a representative result is shown here.

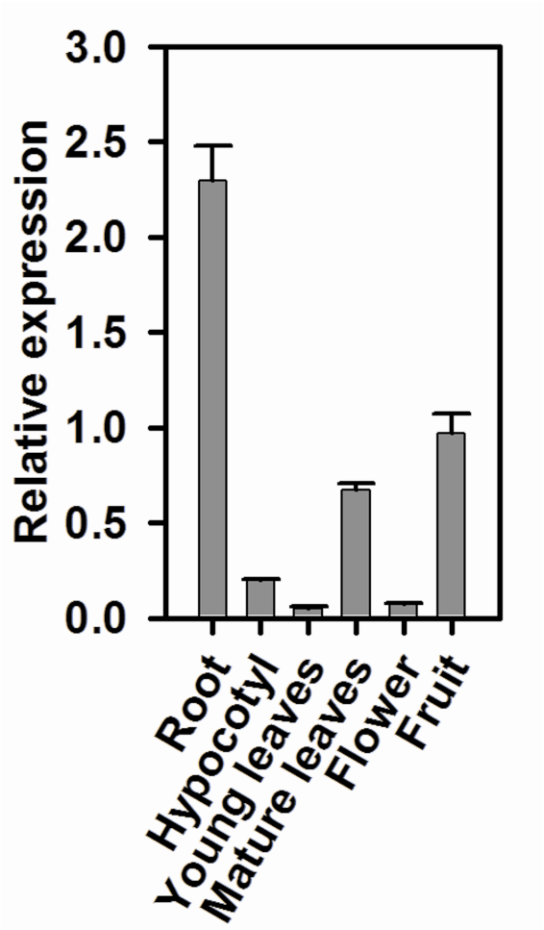


Figure 3.4 qRT-PCR analysis of CrWRKY1 expression levels in different tissues of *C. roseus*. The values expressed are relative to the expression level in fruit. Data are means and standard deviation from three biological replicates.

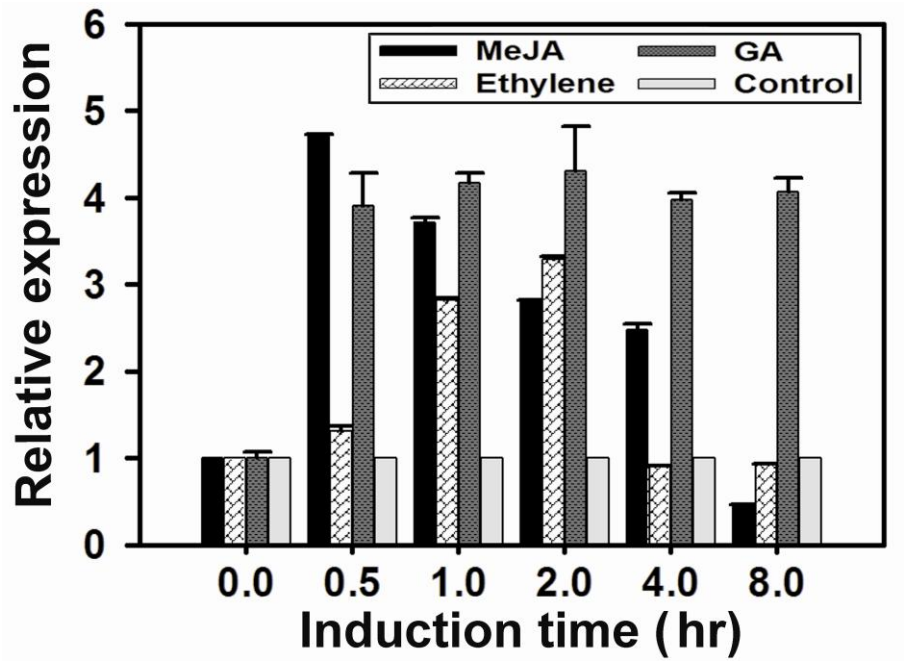


Figure 3.5 qRT-PCR analysis of *CrWRKY1* expression levels in *C. roseus* seedlings upon induction by methyl jasmonate (MeJA), gibberellic acid (GA) and ethylene. Seedlings without phytohormone induction served as control. All experiments were performed in triplicate and error bars represent standard deviation.

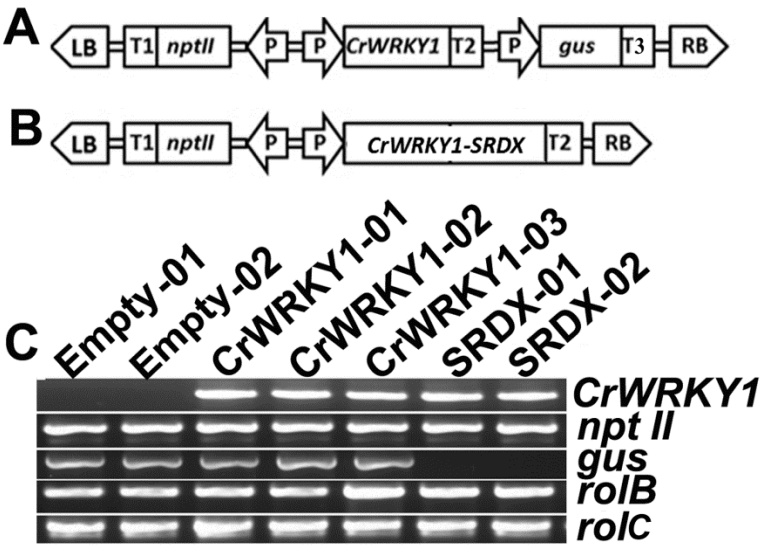
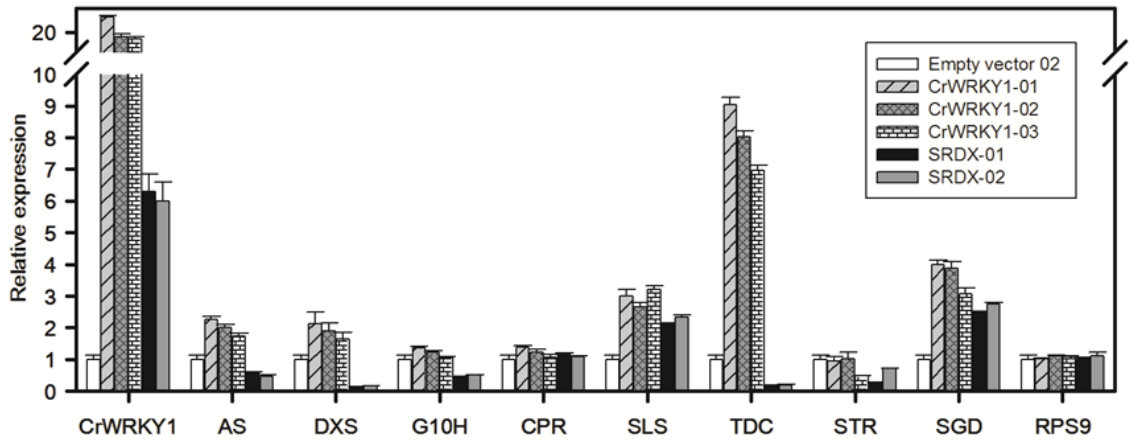


Figure 3.6 Plant expression vectors used for generating transgenic hairy roots and PCR confirmation of the presence of transgenes in transgenic hairy roots. (A) modified pCAMBIA2301 vector containing the *CrWRKY*1 cDNA under the control of the *CaMV35*S promoter and *rbc*S terminator. (B) modified pCAMBIA2300 vector containing the *CrWRKY1-SRDX* under the control of the *CaMV35S* promoter and *rbc*S terminator. (C) *CrWRKY1*, *npt*II, *GUS*, *rolB* and *rolC* genes were PCR amplified from genomic DNA isolated from transgenic empty vector control, CrWRKY1 and CrWRKY1-SRDX hairy root lines using gene specific primers (Table 3.1). *GUS*; *ß-glucuronidase*; LB; left T-DNA border, *nptII*; *neomycin phosphotransferase*, P; *CaMV35S promoter*, RB; right T-DNA border, T1; *CaMV35S polyA*, T2; *rbc*S polyA, T3; *nos* polyA.

A



B

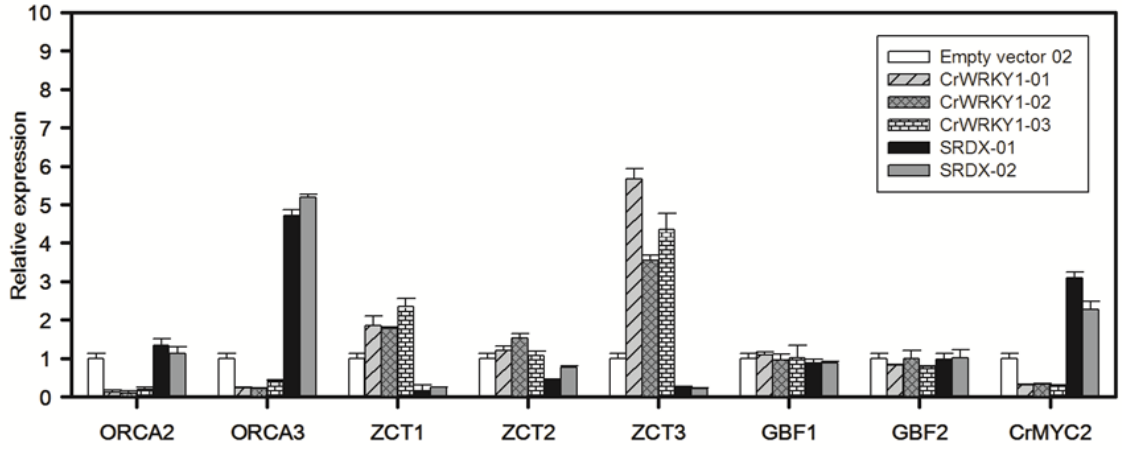


Figure 3.7 Relative expression levels of selected TIA pathway genes in CrWRKY1 and CrWRKY1-SRDX hairy roots. Expression levels of these genes are normalized to the empty vector control line. The Rps9 gene was used as an internal control. Relative expression levels of structural genes (A) and transcription factor (TF) genes (B) in CrWRKY1 and CrWRKY1-SRDX hairy roots were measured by qPCR. AS: *anthranilate snthase*; DXS: 1-*deoxy-D-xylulose-5-phosphate* synthase; G10H: geraniol 10-hydroxylase; CPR: cytochrome P450 reductase; SLS: secologanin s*ynthase*; TDC: *tryptophan decarboxylase*; STR: *strictosidine synthase*; SGD: *strictosidine-β-glucosidase*; ZCT: *zinc-finger C. roseus TF*; GBF: *G-box binding factor*; CrMYC2: *C. roseus bHLH TF*. Each relative gene expression represents the average of three measurements, with error bars representing standard deviation.

Figure 3.8 Inhibition of CrWRKY1-overexpressing and empty vector control hairy roots by 4-methyl tryptophan (4-MT). Hairy roots were grown for 90 days on one-third SH medium containing 0 μM (A: CrWRKY1; D: control), 250 μM (B: CRWRKY1; E: control) and 500 μM (C:CrWRKY1; F: control) 4-MT.

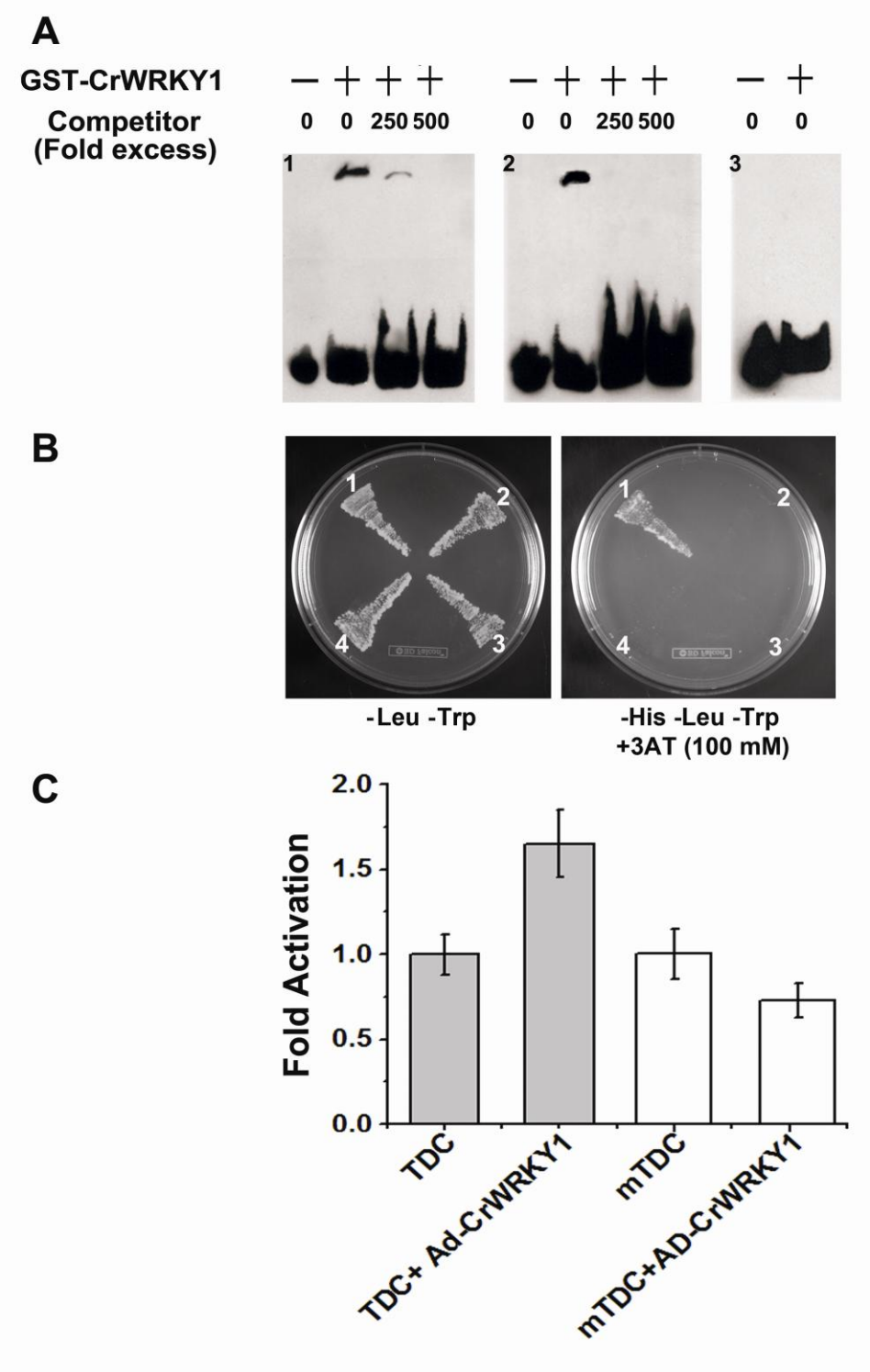


Figure 3.9 Binding of CrWRKY1 to W-box elements in the TDC promoter. (A)Autoradiographs show up-shifted bands of the EMSA assay using purified GST-CrWRKY1 protein and Probe 1 (containing two W-boxes), Probe 2 (containing one W-box) or mProbe 2 (containing a mutated

W-box). Gel 1 shows the products of DNA-binding reactions in the absence and presence of GST-CrWRKY1 or labeled and unlabeled Probe 1. Gel 2 is the same as Gel 1 except that labeled and unlabeled Probe 2 were used. Gel 3 shows the reaction products of mProbe 2 in the absence and presence of GST-CrWRKY1 proteins. (B) Yeast one-hybrid assays demonstrate CrWRKY1 activation of the *C. roseus TDC* promoter. The plasmid harboring a GAL4 activation domain/CrWRKY1 fusion (pAD-CrWRKY1) was co-transformed with the p*TDC*-HIS2 or p*mTDC*-HIS2 reporter plasmid. The transformants were grown in either double selection medium (SD-Leu-Trp) or triple selection medium (SD-His-Leu-Trp) with 100 mM 3-amino-1,2,4-triazole (3-AT). 1. pHIS2-*TDC*+pAD-CrWRKY1; 2. pHIS2-m*TDC*+pADCrWRKY1; 3. pHIS2-TDC+pAD; 4. pHIS2-m*TDC*+pAD. (C) CrWRKY1 binds to W-box elements of the TDC promoter in *C. roseus* protoplasts. The reporter plasmid (TDC), consisting of the luciferase gene under the control of tandem repeats of W-box elements upstream of a minimal 35S promoter, was either transformed alone or co-transformed with AD-CrWRKY1 into *C. roseus* protoplasts. Another reporter plasmid (mTDC), that is identical to the TDC reporter except the W-box sequence is mutated, was also transformed alone or together with ADWRKY1 into the protoplasts. The relative levels of activation of the luciferase gene are presented. Experiments were performed in triplicate.

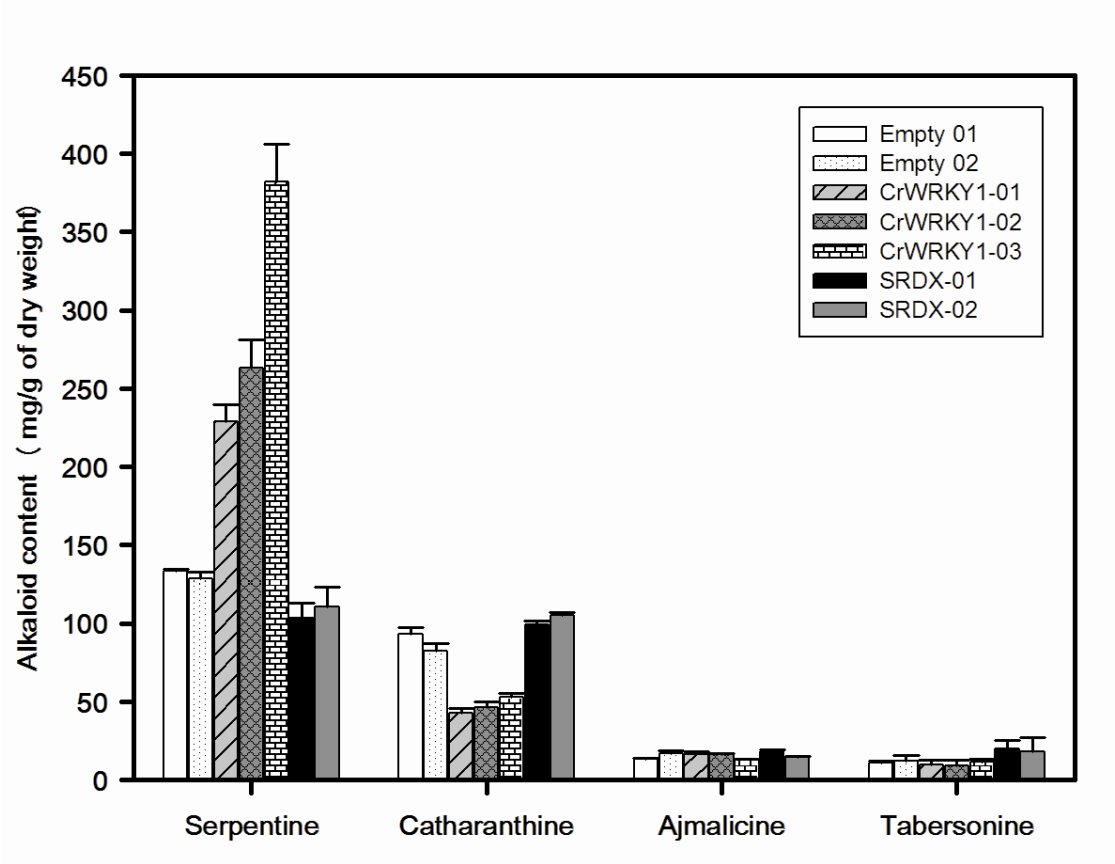


Figure 3.10 Accumulation of serpentine, catharanthine, ajmalicine and tabersonine in two empty vector control, three independent CrWRKY1, and three independent CrWRKY1-SRDX hairy root lines. On an equal dry weight basis, alkaloid extracts from control, CrWRKY1 and CrWRKY1-SRDX lines were analyzed by HPLC-DAD and LC-MS/MS, and the levels of serpentine, ajmalicine and catharanthine were estimated based on peak areas. All experiments were performed in triplicate. Error bars represent standard deviation.

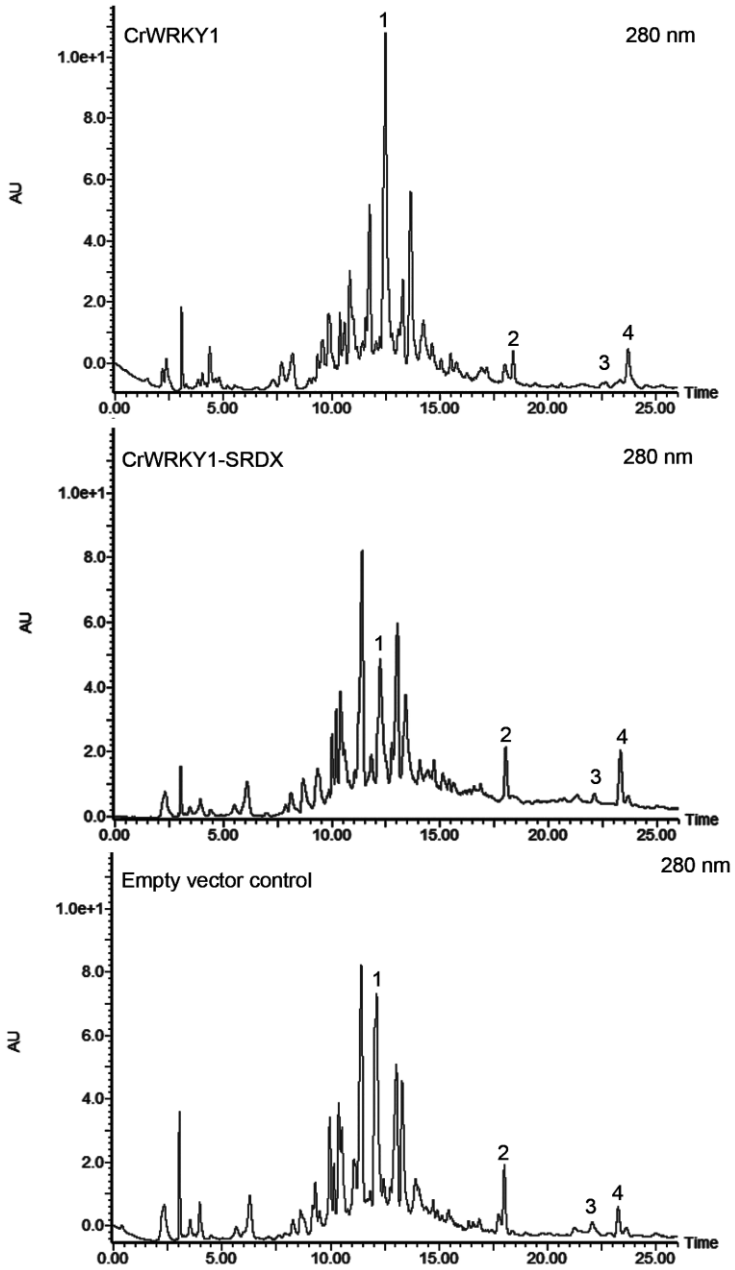


Figure 3.11 Alkaloid productions in CrWRKY1, CrWRKY1-SRDX and empty vector control hairy roots measured by reverse-phase HPLC-DAD. Peaks 1 to 4 were identified by their retention time, UV-spectra (Figure 3.11) and MS/MS spectra (Figure 3.12) as serpentine, catharanthine, ajmalicine and tabersonine, respectively. AU: absorbance unit.

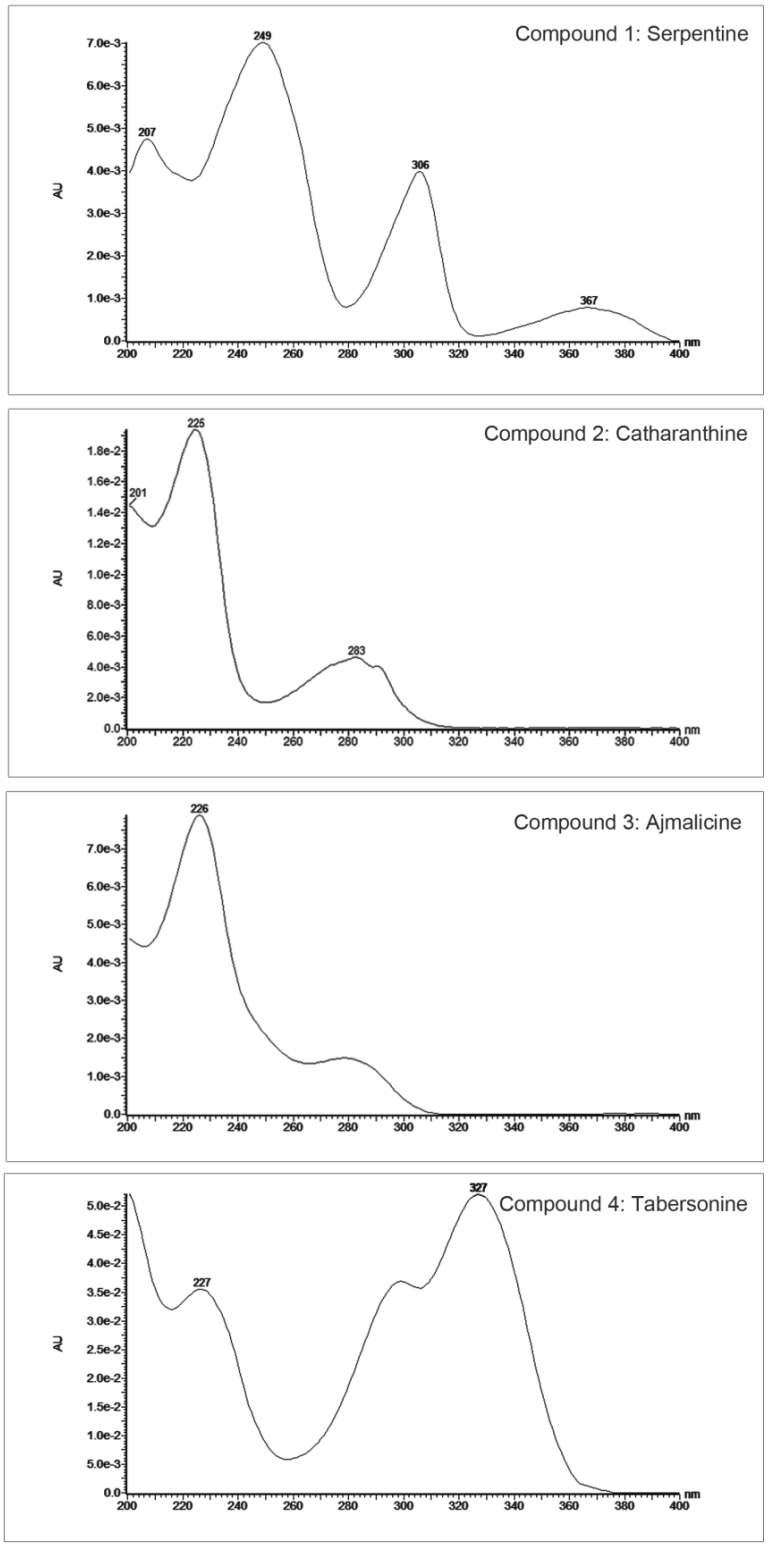
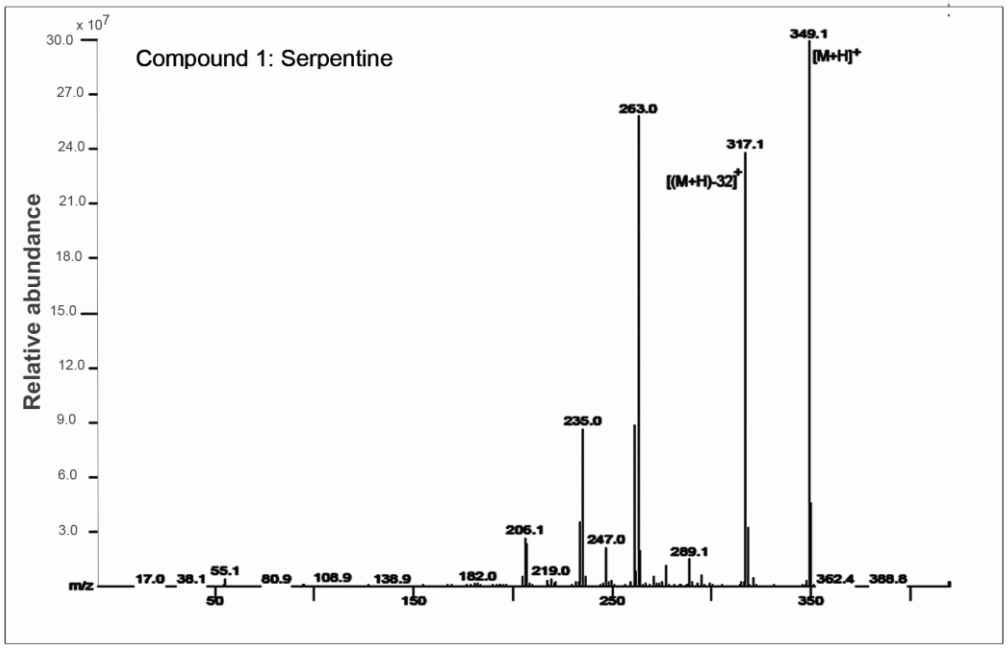
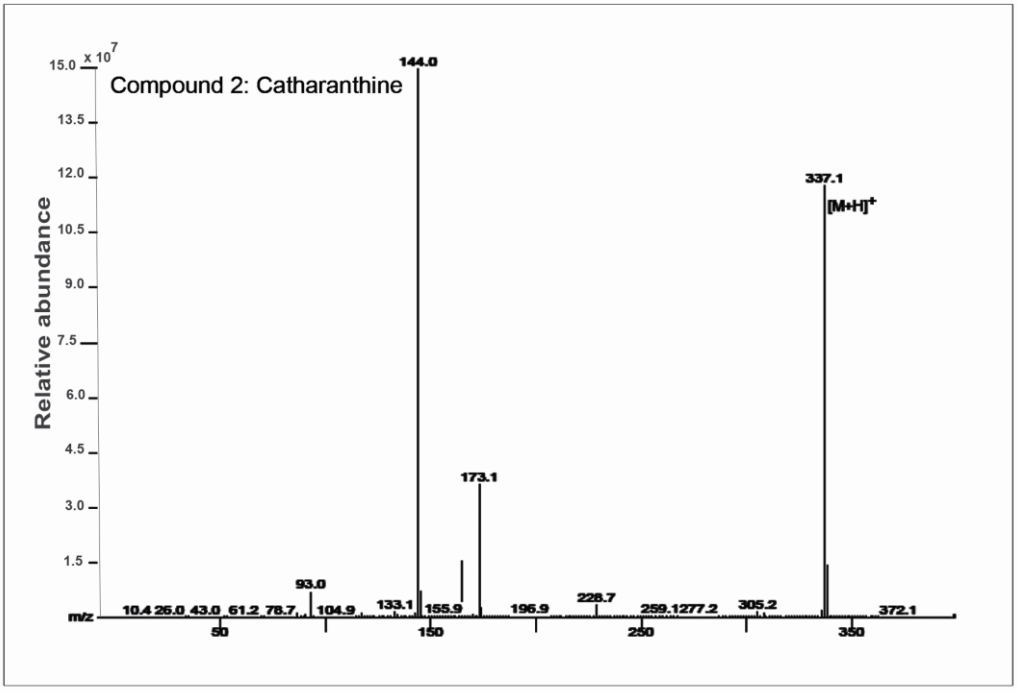


Figure 3.12 UV-spectra of sepentine, catharanthine, ajmalicine and tabersonine. AU: absorbance unit.


Compound 1: Serpentine
x 10^7
Relative abundance
m/z
349.1
[M+H]+
263.0
317.1
[(M+H)-32]+
235.0
206.1
247.0
289.1
219.0
55.1
17.0
38.1
80.9
108.9
138.9
182.0
362.4
388.8
50
150
250
350


Compound 2: Catharanthine
x 10^7
Relative abundance
m/z
144.0
337.1
[M+H]+
173.1
93.0
10.4
26.0
43.0
61.2
78.7
104.9
133.1
155.9
196.9
228.7
259.1
277.2
305.2
372.1
50
150
250
350

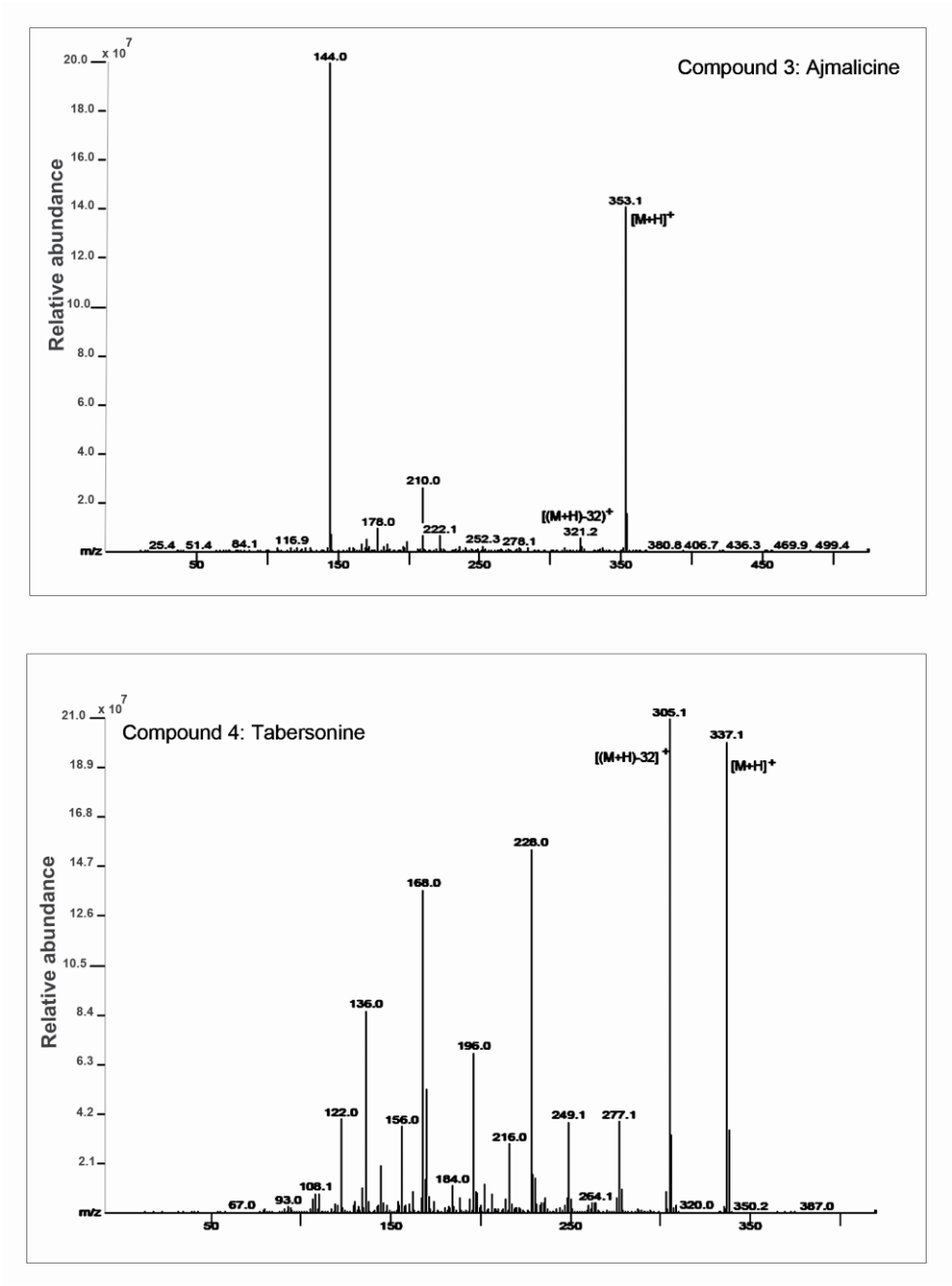


Figure 3.12 MS/MS spectra for serpentine, identified as +MS: 349[M+H]+, +MS2(349): 317; catharanthine, identified as +MS: 337$[M+H]^+$, +MS2(337): 173, 144; ajmalicine, identified as +MS: 353 $[M+H]^+$, +MS2(353): 321, 222, 210, 144, and tabersonine, identified as +MS:337$[M+H]^+$, +MS2(377): 305.

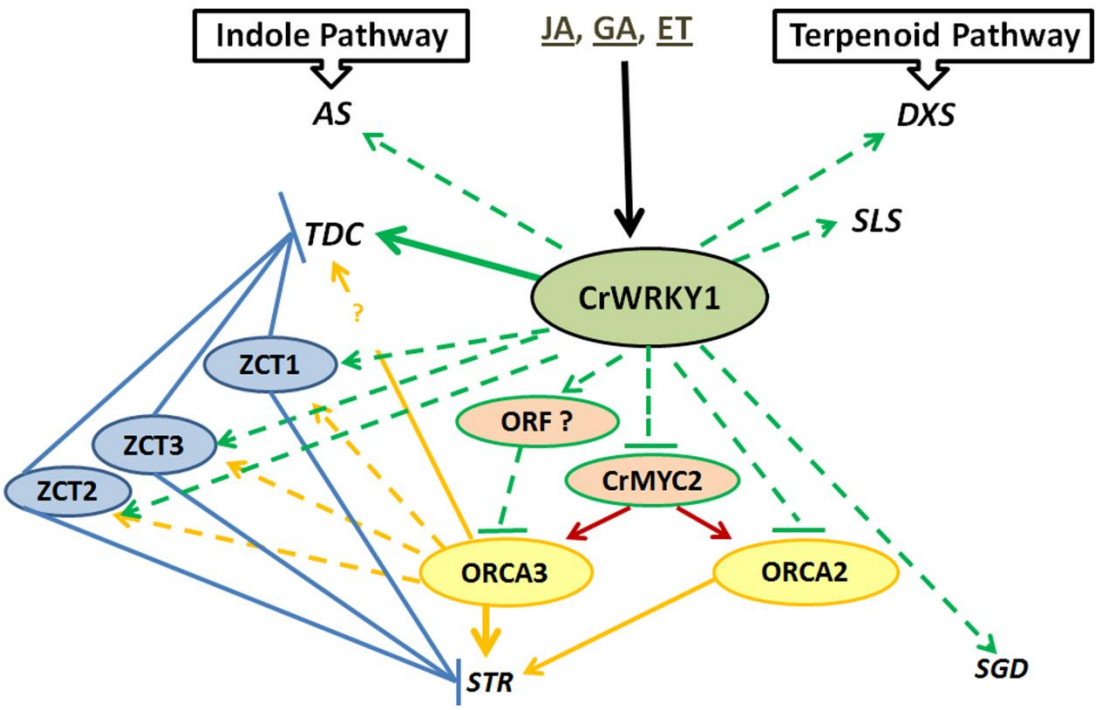


Figure 3.14 Working model summarizing the interaction of CrWRKY1 with TIA pathway genes. AS and TDC, terpenoid pathway genes, DXS and SLS, and downstream genes, STR and SGD, as well as the ORCA, CrMYC2 and ZCT regulators. ET: ethylene; GA: gibberellic acid; JA: jasmonic acid; ORF: ORCA3 repressing factor. Dashed lines represent interactions that may be direct or indirect. Solid lines indicate potentially direct interactions. Lines with arrows represent transactivation and lines with bars represent transrepression.

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Chapter 4

Isolation of CrWRKY2, a *Cattharanthus roseus* Transcription Factor that Regulates the Terpenoid Indole Alkaloid Biosynthesis

4.1 Introduction

Plants are exposed to many forms of stress, including pathogen and herbivore attack, or adverse light, water, temperature, nutrient or salt conditions. Survival depends on the ability to perceive and launch rapid response mechanisms to diversified external signals. The response of stress signals often results in the biosynthesis of one or more of the major secondary signaling molecules such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA). The production of these hormones, which act synergistically or antagonistically together, generates a signal transduction network leading to a cascade of events responsible for the physiological adaptation of the plant to the external stress, regulation of plant growth and development and defense response (Bari and Jones,2009).

The WRKY transcription factors represent one of the best-characterized classes of plant defense transcription factors and are at the forefront of research on plant defense responses. They belong to the WRKY-CGM1 superfamily of zinc finger proteins that have been classified as large plant-specific transcription factors (Eulgem and Somssich,2007). However, WRKY proteins have also been reported in non-plant species, e.g. a primitive protozoan (Pan et al.,2009), *Dictyostelium discoideum*, a slime mold and *Chamydomonas reinhardtii*, unicellular green algae (Rushton et al.,2010). Recently, more than 80 members were also found in gymnosperm *Pinus monticola* (Liu and Ekramoddoullah,2009). The name of WRKY is derived from the WRKY domain, a 60 amino acid stretch containing the conserved amino acid sequence of WRKYGKQ together with a zinc finger-like motif. The classification of WRKY TFs is based on both the number of WRKY domains present and the pattern of zinc finger motifs. Group I WRKY are defined by the presence of two WRKY domains, whereas, group II and III contain only a single domain. Both group I and II have a C_2H_2-type zinc finger motif ($CX_{4-5}CX_{22-23}HXH$) with a C-terminal active DNA binding domain. Group III has a C_2HC zinc finger motif comprised of $CX_7CX_{23}HXC$ amino acid sequences. Most WRKY proteins contain a basic nuclear localization signal (Eulgem et al.,2000). All WRKY proteins studied to date show preference of binding to their cognate *cis*-acting element, the W-box (TTGAACC/T), a motif frequently found in the promoter of defense-regulated genes (Yu et al.,2001).

The majority of WRKY TF genes characterized in *Arabidopsis thaliana* and other plant species appear to mediate cross talk between JA and SA signaling pathways (Li et al.,2004; Li et al.,2006) and aid plants in defense against phytopathogens and abiotic stress (Robatzek and Somssich,2002; Marè et al.,2004; Ulker and Somssich,2004; Park et al.,2005; Knoth et al.,2007; Zou et al.,2007; Zhou et al.,2008; Zhou and Shao,2008). Increasing evidence show that WRKY TFs also play diverse roles in regulating plant development, trichrome initiation, senescence and carbohydrate metabolism (Miao et al.,2007). In recent years, many WRKY genes have been isolated from medicinal plants. For instance, CjWRKY1 from *Coptis japonica* represents the first discovery of a transcriptional regulator of benzyl isoquinoline alkaloid (BIA) biosynthesis. (Kato et al.,2007). A full-length cDNA for AaWRKY1 was isolated from a cDNA library of the glandular secretory trichomes of *Atemesia annua* where artemisinin is synthesized and sequestered. AaWRKY1 is involved in artemisinin biosynthesis by regulating amorpha-4, 11-diene synthase (ADS) gene expression (Ma et al.,2009). GaWRKY1, the othologue of AtWRKY 18 isolated from cotton (*Gossypium arboreum*), has been proposed to regulate the sesquiterpene biosynthesis pathway by interacting with the W-box palindrome with two reversely oriented TGAC repeats in the (+)-δ-Cadinine synthase (CAD1) promoter. Moreover, the coordinated expression of GaWRKY1 and CAD1-A was observed in floral organs and in response to fungal elicitor treatment (Xu et al.,2004).

The *Catharanthus roseus* plant produces over 130 different terpenoid indole alkaloids (TIAs) (Verpoorte et al.,2000). Important TIAs from *C. roseus* include vincristine (anti-cancer), vinblastine (anti-cancer), ajmalicine (anti-hypertensive), and serpentine (sedative). The precise functions of TIAs in plant are still poorly understood, however, the accumulated evidence support a role for these molecules in response against insect attacks (Guirimand et al.,2010; Bond et al.,2011).

Due to their economic importance, many studies have focused on the characterization of TIA biosynthesis in *C. roseus* and strategies to increase the production of valuable alkaloids. Current yields from whole plant are low; production of these compounds is subject to unpredictable weather conditions. Thus, cell and organ cultures are being pursued as important sources of TIA production (Guillon et al.,2006). One strategy for enhancing TIA production from cell culture is the use of elicitors, such as MeJA, ET and fungal elicitors, to induce plant stress responses leading to the up-regulation of TIA genes and alkaloid biosynthesis (van Dam et al.,1993; Vázquez-Flota and Luca,1998; van der Heijden et al.,2004; Memelink,2009; Vázquez-Flota et al.,2009). In *C. roseus*, elicitors appear to regulate production of TIAs at the transcriptional level

by inducing the expression of enzymes involved in the TIA biosynthesis pathway. By using T-DNA activation tagging Van der Fits and Memelink isolated the jasmonate-responsive AP2 domain transcription factor, ORCA3 (octadecanoid-responsive *Catharanthus* AP2/ERF domain), from *C. roseus* cell culture. Over-expression of *ORCA3* led to increased expression of *Asα*, *TDC*, *DXS*, *CPR*, *STR*, *SGD* and *D4H*. However *G10H* and *DAT* transcripts were not affected by *ORCA3* over-expression (van der Fits and Memelink,2000). Recently, the *G10H* gene was transformed independently, or co-transformed with *ORCA3*, into *C. roseus* hairy roots. Analysis of alkaloid accumulation showed higher levels of catharanthine in all transgenic clones. The highest accumulation was observed in transgenic lines containing both *G10H* and *ORCA3* (Wang et al.,2010) suggesting ORCA3 do not regulate the expression of *G10H*. Analysis of the *G10H* promoter revealed it contains unique binding sites of several transcriptional factors, suggesting regulation by a different transcriptional cascade (Suttipanta et al.,2007). Sequence analysis of the *STR* and *TDC* promoters revealed that both contain G-box and G-box–like elements. By using the G-box element as bait for yeast one hybrid (Y1H), two G-box binding elements, *CrGBF1* and *CrGBF2*, were isolated (Siberil et al, 2001). However, CrGBFs do not appear to be involved in the stress induction process. In transgenic tobacco, deletion of the G-box could not reduce CrGBFs elicitor responsiveness to elicitors (Pasquali et al.,1992). Another G-box binding factor, the CrMYC1 (basic helix-loop-helix; bHLH) transcription factor, was induced by fungal elicitor and MeJA, suggesting that CrMYC1 may be involved in the regulation of gene expression in response to these signals (Chatel et al.,2003). The identification of a MYB-type transcription factor, CrBF1 (*C. roseus* box P-binding factor-1), revealed its role in elicitor response, activating a signal transduction pathway downstream of protein phosphorylation and calcium influx. The zinc finger-binding proteins ZCT1, ZCT2 and ZCT3 (members of the transcription factor IIIA-type zinc finger family), were found to bind to *STR* and *TDC* promoters. The repression activity of ZCTs on STR and TDC has been suggested to counteract the transactivation of these promoters by ORCA2 or ORCA3 (Pauw et al.,2004). Recently, a novel jasmonic–responsive transcription factor, CrMYC2 (basic-Helix-Loop-Helix), has been characterized. CrMYC2 binds to the quantitative JERE (jasmonic acid responsive region) sequence in ORCA3 promoters and is involved in the timing and regulation control of *ORCA3* expression (Zhang et al.,2011). Although the isolation of transcription factors regulating the TIA biosynthesis pathway is progressing, many transcription factors involved in TIA biosynthesis remain uncharacterized.

Due to the potential importance of WRKY transcription factors in regulating biosynthesis of secondary metabolites, the *CrWRKY2* cDNA, which encodes a group I WRKY protein, was

isolated from *C. roseus* seedlings. *CrWRKY2* is expressed predominantly in the aerial tissue of *C. roseus*. CrWRKY2 is involved in SA/JA–mediated plant responses, as well as plant development and senescence. The CrWRKY2 gene was transformed into *C. roseus* hairy root cultures to investigate the transgenic effect of *CrWRKY2* overexpression on TIA biosynthesis. Overexpression of *CrWRKY2* in hairy roots increased the transcript levels of 13 TIA biosynthetic and regulatory genes demonstrating CrWRKY2 participates in TIA pathway regulation. The accumulation of specific alkaloids in CrWRKY2 hairy roots was analyzed by HPLC. The increased amounts of caharanthine, tabersonine, and vindoline detected in CrWRKY2 hairy roots coincide with the up-regulation of TIA pathway and regulator genes.

4.2 Materials and methods

4.2.1 Plant materials

Catharanthus roseus (L.) G. Don cvs. Cooler Apricot seeds (Swallowtail Garden, CA, USA) were surface-sterilized by treatment with a solution of 5% (w/v) NaOCl (commercial bleach, 4% active chlorine) containing 1% triton X 100 (Fisher Scientific, PA) and shaken for 20 min. Seeds were rinsed with sterile distilled water. Sterilized seeds were germinated on solidified MS (Murashige and Skooge) basal medium consisting of MS inorganic salts, 100 mg/l myo-inositol, 0.4 mg/l thiamine HCl, 3% sucrose, and 10 g/l agar. The pH of medium was adjusted to 5.8 before autoclaving. 25 ml of medium was dispensed into each plastic Petri dish. Approximately 25 seeds were used per plate and care was taken to avoid any contact between seeds. The plates were sealed with laboratory film and grown using a 16 hr photoperiod at 25 ^{0}C. Individual 10-day-old seedlings were harvested for biotic/abiotic inductions and 15-day-old seedlings were subjected to *Agrobacterium rhizogenes* transformation.

4.2.2 Isolation of *CrWRKY2* cDNA

Partially degenerate PCR primers were designed according to the conserved amino acid sequence of the WRKY domain. The internal fragments of *C. roseus* WRKYs were amplified by the degenerate primers using first strand cDNA synthesized from total RNA isolated from MeJA-induced seedlings. PCR products longer than 500 bp were cloned into the pGEM Easy vector (Promega, USA) and followed by sequencing. Full-length cDNA of *C. roseus WRKYs* was obtained using the 5′ and 3’-RACE System 2 (Invitrogen, USA) following protocols supplied by the manufacturer. The gene specific primers, CrWRKY2-FW and CrWRKY2-RV, were used to amplify the full-length cDNA of *CrWRKY2*.

The predicted polypeptide sequences of the *C. roseus* WRKY and other available WRKY proteins were compared using the Basic Local Alignment Search Tool (BLAST) service software 5.2.2 and BLAST software online (http://www.ncbi.nlm.nih.gov/BLAST). Alignment of CrWRKY2 with WRKY proteins from other plant species was performed by ClustalW (Thompson et al.,1994) with default parameters through EMB net (http://www.ch.embne.org/software/clustalW.html). The conserved amino acid and sequence homology of CrWRKY2 to other proteins were highlighted and shaded using BOXSHADE 3.2.2 (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis of CrWRKY2 and other available WRKY proteins was carried out by MEGA 5 software (Tamura et al.,2011). The online tool PSORT program (http://psort.ims.u-tokyo.ac.jp) was used to predict the localization of CrWRKY2 protein (Nakai and Kanehisa,1991).

4.2.3 Tissue–specific expression of *Cr2WRKY* and effects of biotic and abiotic stress on *Cr2WRKY* transcript

For tissue-specific expression analysis, roots and stems were harvested from 15-day-old seedlings. Young leaves, mature leaves, flower buds, opened flowers and fruit were obtained from the same plant at the flowering stage. All plant tissues were frozen in liquid nitrogen and stored at -80^{0}C.

The seedlings at the cotyledon stage were subjected to different treatments. Seedlings were sprayed with 100 µM MeJA and 10 µM SA. ET gas was diluted with air to 10 ppm and exposed with seedlings in an airtight container. Wounding was induced by needle flicking under the cotyledon. Seedlings treated with sterile water were used as control. After treatments, the seedlings were harvested at 0 min, 30 min, 1.0 hr, 2.0 hr, 4.0 hr and 8.0 hr, immediately frozen in liquid nitrogen and kept in -80^{0}C for RNA extraction. All experiments were conducted at least in triplicate. Chemicals were purchased from Sigma (USA).

4.2.4 Generation of transformed *C. roseus* hairy roots

The *CrWRKY2* gene was PCR-amplified and cloned into a modified pCAMBIA2301 vector, containing the CaMV-35S promoter and rbc*S* terminator, to generate CrWRKY2/pCAMBIA 2301 plasmid. Plasmids CrWRKY2/pCAMBIA 2301 and pCAMBIA 2301 (empty vector control), were transformed into *Agrobacterium rhizogenes* strain R1000 by freeze-thaw method. A single transformed colony from each combination growing on solid LB kanamycin (50 mg/l) agar medium was harvested and used to start a 20 ml culture grown at 28 ^{0}C and 200 rpm for 16

hr in liquid LB/kanamycin medium. 15 day-old seedlings were cut at stem tips to remove their roots. Bacterial suspension cultures of each respective construct were diluted with liquid MS basal medium to half strength. The explants were incubated with the bacterial solution for 30 min, and then explants were transferred onto solid MS basal medium. Freshly co-cultivated explants were kept in darkness for 24 hr to prevent the bacteria from drying under illumination during the window of competence for transformation of wounded tissue (Bhadra et al.,1993). After 48 hr of co-cultivation, the explants were rinsed three times with half-strength liquid MS basal medium supplemented with 800 mg/l cefotaxime, then transferred onto half-strength solid MS basal medium with 400 mg/L cefotaxime. After 6-7 weeks, hairy roots formed on explants were cut into 1-cm in length and cultured on selective medium containing one-third-strength SH (Schenk and Hildebrandt) basal medium, 30 g/l sucrose, 6 g/l agar, 100 mg/l kanamycin and 400 mg/l cefotaxime. Hairy-root cultures were grown in dark at 27 ^{0}C and subcultured at 4-week intervals. On kanamycin selective medium, non-transformed roots died in 45 days. The hairy roots were cleared of bacterial growth after three or four subcultures, and then subcultured once to the same medium without antibiotic. For the first transfer from solid to liquid culture, approximately 100 mg of actively growing bacteria-free root clones were used to inoculate 50 ml of antibiotic-free liquid one–third SH medium in 150 ml Erlenmeyer flasks. The hairy roots were grown at 27°C in darkness on an orbital shaker at 125 rpm. Subsequent clones were maintained in liquid culture under the same conditions for a subculture cycle of 3 to 4 weeks.

4.2.5 Identification of Cr2WRKY insert in hairy root

Genomic DNA was extracted from independent lines of CrWRKY2 overexpression and empty-vector control hairy roots using the DNAeasy plant mini kit (Qiagen, USA). The presence of *Neomycin Phosphotransferase (npt II)*, *β-glucuronidase (GUS*), *rolB* and *rolC* in hairy roots was confirmed by PCR amplification of genomic DNA and used to verify the transgenic lines. All specific primers used for PCR amplification are listed in table 4.1.

4.2.6 GUS histochemical assay

Histochemical β-glucuronidase (GUS) activity of the hairy roots was assayed by histochemical staining as described by (Jefferson et al.,1987). Both putative CrWRKY2 and empty vector control transgenic hairy root segments in lengths of 1-2 cm were incubated at 37 ^{0}C in the dark in 5-bromo-4chloro-indolyl-β-*D*-glucuronide (X-Gluc) solution. The X-Gluc solution contained 50 mM Na_3PO_4 (pH 7.0), 10 mM, Na_2EDTA 0.1% (v/v), Triton X-100, 0.1 M $K_3[Fe(CN)]_6$, 0.1M

$K_4[Fe(CN)_6]$, 0.25 mM X-Gluc, and 20% methanol. The roots were subsequently washed in an ethanol gradient at room temperature (30 min in 70% ethanol, 30 min in 40% ethanol and 30 min in 20% ethanol). After rehydration, the roots were kept in water.

4.2.7 Isolation of alkaloid from hairy roots and HPLC analysis

The fresh weight of the harvested hairy root culture was measured after the cultures were rinsed 3-times with sterile water and blotted to remove excess media. The cultures were then freeze-dried. The dry weight was measured after lyophillization. Approximately 1 g dry weight of the ground tissues was extracted with 10 ml of MeOH in a sonicating bath for 1 hr at room temperature. The supernatant was removed and the cell debris was re-extracted in the same manner. The supernatant were combined and concentrated in a centrifuge-vacuum at room temperature. The dried extract was dissolved with 2 ml of 1M HCl and extracted with 5 ml of hexane two times. The hexane portion was discarded. The aqueous portion was basicified to pH 9.0 with NH_4OH, and subsequently extracted with 5 ml of ethyl acetate two times. The combined ethyl acetate was washed with sterile water, evaporated until dryness, and re-dissolved with 100 µl methanol.

HPLC analysis was performed using a 250 mm x 4.5 mm, 5 µm, A Zorbax Eclipse XDB column (Agilent Technologies, USA), at 27 0C. The injection volume was 10 µl. The HPLC-solvent system used to quantify serpentine, vindoline, catharanthine, and tabersonine was adapted from (Tikhomiroff and Jolicoeur,2002). The mobile phase consisted of a mixture of 5mM Na2HPO4 (pH adjusted to 6 with H3PO4) (Solvent A) and acetronitrile (solvent B). Flow-rate was 1.5 ml/min. The eluent profile (volume of A: B) was 0-20 min, linear gradient from 80:20 to 20:80. The column was then washed with isocratic elution with 20:80 mixtures of the same solvents for 30 min and then re-equilibrated for 10 min. The separation of each sample was monitored by a Waters 2998 photodiode array detector (Waters Corporation, USA). In this elution system, serpentine is detected at Rt ~10.3 min with λ max of 249 nm/306 nm/367 nm, vindoline at Rt ~ 17.0 min with λ max of 249 nm/306 nm/367 nm, catharanthine at Rt ~17 min with λ max of 225 nm/283 nm, catharathine at Rt~19 min with λ max of 225 nm/283 nm, and tabersonine at Rt ~17 min with λ max of 227nm/ 288 nm/327 nm. Chromatographic peaks were detected at 254 and 280 nm. The amount of alkaloid in each sample was quantified by comparison to authentic standard curves. Solutions of standards were prepared in methanol (0.25 mg/ml) and used at different concentrations for the preparation of the calibration graphs, which were linear in the range of 0.25–25 mg. Quantification was repeated three times for each root line.

4.2.8 cDNA synthesis and qPCR quantification

Total RNA was isolated from *C. roseus* tissue using the RNeasy plant mini kit (Qiagen, USA), and then treated with RNase-free DNase (Sigma, USA) to remove potential genomic DNA contamination. The first-strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's protocol.

The qRT-PCR was performed on an Applied Biosystem Step OneTM Real-Time PCR system. In each run, 2 µl of diluted cDNA was used as template. The samples were added to 18 µl of reaction mixture containing 6.2 µl of sterile MQ-water, 10 µl of Power SYBR green PCR master mix (Applied Biosystems, USA), and 0.9 µl of each forward and reverse 20 pico mole (pm) primers. The list of primers used is mentioned in Table 1. Real-time PCR amplification was carried out for 40 cycles at 94 ^{0}C for 10 sec, 76 ^{0}C for 15 sec, and 72 ^{0}C for 55 sec. The temperature range for analysis of the melting curve was 60 ^{0}C to 90 ^{0}C over 30 sec. The final PCR products were checked for homogeneity by melting curve analysis according to manufacturer's instructions (Agilent Technologies, USA). All reactions were performed in triplicate. The relative gene expression was quantified using the comparative CT (threshold cycle) method as previously described (Shalel-Levanon et al.,2005). *CrRPS*9 was used as an endogenous control gene.

PCR primers (Table 1) were designed for putative regulatory molecules, TIA biosynthetic genes, and an internal control using the Primer3 program (www.genome.ei.mit.edu/cgi-bin/primer/primer3_www.cgi). The set of criteria used to facilitate real-time PCR measurement followed a predicted melting temperature of 60 ^{0}C, primer length of 20-24 nucleotides, 45-55% guanine-cytosine (GC) content and a PCR amplicon length of 150-200 bp.

4.2.9 Statistical analysis

Data were analyzed using the Student's *t*-test. The expression profile of TIA pathway genes and regulators were clustered by hierarchical cluster analysis method using cluster program MeV v4.7.4 (Mar et al.,2011).

4.2.10 Primers

Primers used in this study are listed in Table 4.1.

4.3 Results

4.3.1 Cloning and sequence analysis of CrWRKY2

Primers based on the highly conserved amino acid sequence of WRKY proteins, were designed to isolate the internal fragment of *C. roseus* WRKYs. An expected 708 bp fragment was isolated from *C. roseus* cDNA. The full-length *CrWRKY2* cDNA was identified using RACE-PCR with primers designed according to the nucleotide sequence obtained. *CrWRKY2* contains 1002 bp and encodes 334 amino acids with the predicted molecular weight (MW) of 36981.24 Da and pI 9.0. The deduced protein sequence (Figure 4.2) possesses two WRKY domains followed with zinc finger motifs (C-X_4-C-X_{22-23}-H-X-H) categorizing it into group I of the WRKY superfamily (Eulgem et al.,2000). Sequence analysis of CrWRKY2 showed monopartite nuclear localization signal located outside the WRKY domains at amino acid positions 181-187 (PSAKRRS). CrWRKY2 exhibits high sequence identities (51%-59%) to group I WRKY proteins including, NtWRKY2 (*Nicotiana tabacum*: BAA77383.1), AtWRKY 58 (*Arabidopsis thaliana*: NP_186757.2), GhWRKY3 (*Gossypium hirsutum*: ADI52618.1), and CaWRKY2 (*Capsicum annuum*: ABA56495.2) (Figure 4.3). Alignment of the protein sequences revealed that the WRKY domains, putative nuclear localization site, and basic amino acids adjacent to the C-terminal WRKY domain are conserved; however little amino acid sequence conservation exists outside these regions.

4.3.2 Tissue-specific expression of *CrWRKY2* and effects of signaling molecules on *CrWRKY2* transcription

The expression pattern of the *CrWRKY2* gene in *C. roseus* tissues was investigated using semi-quantitative RT-PCR. As shown in Figure 4.4, *CrWRKY2* is expressed differently in all tissues analyzed. The expression of *CrWRKY2* was highly detected in stem (9.5-fold) and young leaves (6.2-fold) compared to roots (2.2-fold). However, a decrease of *CrWRKY2* transcripts was observed in mature leaves (1.0-fold). In flower organs, the accumulation of *CrWRKY2* mRNA increased towards flower maturation. *CrWRKY2* expression was up-regulated in the open flower stage (4.2-fold) and maintained at similar levels during fruit development (4.1-fold). These results indicate a constitutive basal expression of C*rWRKY2* in *C. roseus* that is controlled in a tissue- and developmental-dependent manner.

To investigate the hormonal control mechanism underlying *CrWRKY2* gene expression, the *C. roseus* seedlings were treated with MeJA, SA and ET under time course, and the *CrWRKY2*

transcript was examined by qPCR (Figure 4.5). The expression of *CrWRKY2* was weakly affected by water, indicating that this method of experiment did not affect gene expression. Strong expression of *CrWRKY2* was detected 24 hr after treatment with 100 µM MeJA and trended to maintain the higher level after 48 hr of induction (Figure 4.5 A). Exposure to 10 µM SA significantly decreased *CrWRKY2* to 10% of basal transcript levels within 4 h and this level persisted 48 hr post treatment (Figure 4.5 C). ET did not appear to affect *CrWRKY2* expression as no significant change of *CrWRKY2* transcript levels was observed during ET treatment (Figure 4.5 B). These results indicate that *CrWRKY*2 expression is positively affected by JA but negatively affected by SA. Since many WRKY proteins play roles in abiotic stress responses, *C. roseus* seedlings were wounded by needle flicking and the *CrWRKY2* transcript level was measured. The transcription level of *CrWRKY2* expression was rapidly reduced to 50% of basal level within 2 h after wounding and declined slowly thereafter (Figure 4.5 D). Interestingly, the expression pattern of *CrWRKY2* under wound induction is similar to SA induction. These results suggest CrWRKY2 might be involved in SA/JA signaling pathways and regulate plant response to wounding.

4.3.3 Transcription analysis of TIA pathway genes and regulators in CrWRKY2-overexpressing hairy roots

To generate overexpression lines, *C. roseus* seedlings were infected with *A. rhizogenes* strain R1000 harboring Ri plasmid and the modified pCAMBIA 2310-CrWRKY2. The plasmid pCAMBIA 2310-CrWRKY2 contains the coding region of the *CrWRKY2* gene under the control of the CaMV 35S promoter (Figure 4.6 A). While generating transgenic hairy root lines, nine completely sterilized clones were selected for transgenic analysis. The presence of *CrWRKY2* was successfully detected in four hairy root lines by PCR analysis of genomic DNA with specific primers to *Neomycin phosphotransferase* (*npt II*), *β-glucuronidase* (*GUS*), *rolB* and *rolC*. Control lines were generated with *A. rhizogenes* R1000 harboring Ri plasmid and empty vector-pCAMBIA 2310. PCR results indicated *rolB* and *rolC* genes from the Ri plasmid of *A. rhizogenes* R1000 and the *npt II* from the plant expression vector modified pCAMBIA 2301-CrWRKY2, were all integrated into the genome of transgenic hairy root cultures (Figure 4.6 B).

The phenotype of transgenic and control lines were white, slim and fast growing (Figure 4.7 A-D). In comparison to growth on solid medium, hairy roots cultured in liquid medium grew more rapidly and exhibited higher lateral branching (Figure 4.7 C-D). However, the hairy root cultures changed gradually from white to yellow-brown during subculture, and secretion of a yellow-

brown substance from the hairy roots into the culture medium was observed after 3 to 4 weeks. Total RNA was extracted from transgenic roots harvested after 3 weeks of subculturing.

To detect expression of transgenes in hairy root cultures, histochemical GUS activity was assayed on four putative CrWRKY2 hairy root and three putative empty vector hairy root lines. All hairy root cultures stained blue; different samples showed diverse GUS activities. These results indicate integration of the GUS gene in the T-DNA into the genome of transformed *C. roseus* hairy root cultures

To investigate the transcriptional regulation of *CrWRKY2* in overexpression lines, its expression level was measured using qRT-PCR. Transgenic root lines were harvested after 3 weeks of subculturing for RNA extraction. Expression analysis revealed that all transgenic lines exhibited increased *CrWRKY2* levels. The three lines with the highest *CrWRKY2* transcript levels were selected for further analysis.

Since WRKY transcription factors have been implicated in the regulation of alkaloid biosynthesis, a variety of TIA biosynthesis and regulatory genes were analyzed by qRT-PCR. A multivariate analysis was performed with $2^{-\Delta\Delta CT}$ value from real time PCR. Ribosomal protein subunit 9 (*Rsp9*) was used as an internal control to evaluate all hairy root lines. qRT-PCR analysis in CrWRKY2 hairy root lines showed differential transcript profiles for TIA genes and regulators in comparison to empty vector control (EV) hairy roots (Figure 4.8 A-C). In response to *CrWRKY2* overexpression, the mRNA transcripts of the TIA genes, *TDC* (3.3-6.7-fold), *STR* (4.6-12.2-fold), *SGD* (2.25-3.81- fold), *D4H* (3.21-4.28-fold), *T16H* (5.6-8.5-fold), *NMT* (1.66-1.77-fold), *DAT* (3.21-8.40-fold) and *MAT* (4.18-6.40-fold) increased in all three lines. However, variation of gene expression between the three lines was observed. Transcripts of *AS* (0.31-0.67-fold), *DXS* (0.17-0.33-fold), *CPR* (0.11-0.28-fold) and PRX (0.18-0.62-fold) were detected at low levels. On the other hand, overexpression of *CrWRKY2* did not affect the expression of *SLS* (1.02-1.20-fold) and *G10H* (0.87-0.94-fold).

Up-regulation of transcription factors of the TIA biosynthetic pathway was observed in *CrWRKY2*-overexpressing lines (Figure 4.8 C). *ORCA2* and *ORCA3* gene expression were examined in these transgenic lines. Interestingly, *ORCA2* and *ORCA3* expression were significantly enhanced by 4.6-16.2 fold and 14.0-17.3-fold, respectively. *CrWRKY*1 (2.5-7.4-fold) was moderately up-regulated in *CrWRKY2* overexpression lines. The transcription profiles of TIA pathway repressors, *ZCT1*, *ZCT2*, *ZCT3*, *GBF1* and *GBF2*, were also investigated.

Increased *ZCT1* (4.75-7.92-fold) and *ZCT3* (2.47-5.15-fold) transcripts were observed in *CrWRKY2* transgenic lines, whereas transcript levels of *ZCT2* (0.87-0.92-fold), *GBF1* (0.67-0.96-fold) and *GBF2* (0.74-0.82-fold) were only slightly altered.

Based on the $2^{-\Delta\Delta CT}$ value, a transcription matrix (genes versus transgenic hairy root lines) was constructed using hierarchical cluster analysis. Genes were grouped in two clusters (A and B) with distinct expression patterns in different transgenic lines (Figure 4.9). Cluster A was further subdivided into three subclusters, A1, A2 and A3. Subcluster A1 showed the grouping of *ORCA2*, *ORCA3* and *STR*, which are highly up-regulated in *CrWRKY2* overexpression hairy roots. Subcluster A2 included *TDC*, *T16H*, *DAT*, *CrWRKY1* and *ZCT1*, genes highly expressed in CrWRKY2 hairy root. *SGD*, *D4H*, *MAT* and *ZCT3* are grouped to subcluster which are moderately moderately up-regulated by*CrWRKY2* transgenic lines. Cluster B was also subdivided into two groups, B1 and B2. In *CrWRKY2* overexpression hairy roots, genes belonging to subcluster B1, such as, *AS*, *CPR*, *DXS*, *OMT*, and *PRX1*, were down-regulated, whereas expression of *SLS*, *G10H*, *NMT*, *ZCT2*, *GBF1*, *GBF2*, *MYC2*, grouped into subcluster B2, were considered to be only slightly changed.

4.3.4 Alkaloid production in transformed hairy roots

In order to determine any differences in alkaloid accumulation between the CrWRKY2 and empty vector control hairy root lines, serpentine, tabersonine, catharanthine and vindoline were quantified during late-exponential stages of growth rate. Three transgenic lines and two empty vector lines were harvested after 4 weeks of subculturing. Alkaloid extracts were partially purified by acid-basic extraction method. The quantitative analyses of specific alkaloids were carried out with HPLC. Identification of alkaloids from hairy root extracts was established by comparison of retention time and UV spectra with those of authentic standards.

In CrWRKY2 transgenic lines, increased accumulation of specific alkaloids was observed with a wide range of variation (Figure 4.10). Vinblastine and ajmalicine could not be detected by HPLC (Figure 4.11). The average content of serpentine in CrWRKY2 hairy root and empty vector control hairy root lines were 280 and 180 µg/g of dry root, respectively. This might be due to the increase of *TDC*, *STR* and *STR* transcripts which catalyze biosynthesis of upstream TIAs. The overall increase in tabersonine accumulation was 5.1-6.0-fold in transgenic lines compared to control lines. The average vindoline content in CrWRKY2 hairy roots was 24.2-30.4 µg/g dry weight higher than the empty vector control hairy root (3.1-4.8 µg/g dry root). Interestingly,

accumulation of vindoline was observed in *CrWRKY2* transgenic roots, and corresponding to the up-regulation of *T16H*, *OMT*, *D4H*, *DAT* and *MAT* transcripts. Although the accumulation of catharanthine and vindoline were enhanced in transgenic lines, the accumulation of vincristine and vinblastine were not detected, possibly as a consequence of CrPRX1 repression.

4.4 Discussion

In *C. roseus*, three partial sequences, from the EST database, showing significant homologies with WRKY genes have been identified from libraries constructed after MeJA treatment (Murata et al.,2006). However, no functional analyses of genes encoding these sequences have been published to date**.**

In the present study, a cDNA, designated *CrWRKY2*, encoding a polypeptide of 334 amino acids, was isolated and characterized. The deduced CrWRKY2 protein contains two typical WRKY domains followed by a zinc finger –like motif (C-X_4-C-X_{22-23}-H-X-H), and is classified as a member of group I WRKY transcription factors. The two WRKY domains of group I members play distinct functions: the C-terminal WRKY domain is responsible for DNA-binding, while the N-terminal WRKY domain is associated with the binding process and enhances the binding activity to the target gene (Ishiguro and Nakamura,1994). Consistent with its putative role as a transcription factor, the CrWRKY2 protein contains a nuclear targeting sequence at amino acid positions 181-187 (PSAKRRS).

In defense responses, plants activate a series of signal transduction events that lead to the expression of defense-related genes. SA, JA and ET are three key signaling molecules involved in plant disease and defense responses. Generally, SA-mediated signaling is relevant to defense responses against biotrophic pathogens and systemic acquired resistance (SAR) (Halim et al.,2006). Pathways involving JA and ET are mainly effective against necrotrophic pathogens, insects and wounding (Dong,1998). Through mutually antagonistic or synergistic interactions, two signaling pathways affect the outcome of the defense response (Kunkel and Brooks,2002). The expression pattern of *CrWRKY2* was examined under phytohormone treatments and wound induction. The expression of *CrWRKY2* could be induced by MeJA, but was repressed by SA and wounding. ET did not affect *CrWRKY2* expression. Previous studies reported that WRKY genes regulate crosstalk between SA- and JA-mediated signaling pathways (Loake and Grant,2007). However, there are few reports analyzing the effects of other signal molecules e.g. ET or ABA. (Dellagi et al.,2000). The effect of SA, JA and wounding on *CrWRKY2* gene expression suggests

CrWRKY2 may be a key regulatory gene involved in plant defense responses positioned downstream of SA and JA signaling pathways.

The spatial expression pattern of *CrWRKY2* in *C. roseus* was analyzed. *CrWRKY2* expression is higher in aboveground organs, mainly stem and young leaves. The developmentally-mediated expression of CrWRKY2 in leaves and floral organs strongly suggests its function is not likely limited to regulation of defense responses. The accumulation of *CrWRKY2* transcripts decreased in mature leaves. In contrast, *CrWRKY2* transcripts increased in open flowers and were maintained at higher levels during fruit development, which may attribute to its effect in plant developmental process.

We have demonstrated the regulatory role of CrWRKY2 in the TIA pathway by overexpression in hairy roots. Modulating CrWRKY2 levels has significant impact on TIA pathway genes and regulators. The analysis of separated CrWRKY2 transgenic lines showed a differential expression pattern of CrWRKY2 in each line. This variability is common in *Agrobacterium*-transformed plants and is attributed to gene copy number and position effects (Christey,2001).

In *C. roseus*, the biosynthesis of TIAs originates from the stereospecific condensation reaction of tryptamine and secologanin to strictosidine. This reaction is catalyzed by STR. Tryptamine is converted from tryptophan by tryptamine decarboxylase (TDC). The secologanin is from the C-methyl-*D*-erythritol 4-phosphate (MEP) pathway (Contin et al.,1999). Several genes in the MEP pathway have been cloned, including *1-deoxy-D-xylulose-5-phosphate synthase* (*DXS*); *1-deoxy-*D-xylulose-5-phosphate reductoisomerase (*DXR*); and *2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase* (*MECS*) (Chahed et al.,2000; Veau et al.,2000). Geraniol 10-hydroxylase (G10H) and secologanin synthase (SLS), two membrane-associated cytochrome P450-dependent mono-oxygenases involved in late stages of secologanin biosynthesis, have also been characterized (Irmler et al.,2000; Yamamoto et al.,2000; Collu et al.,2001). The structural diversity of TIAs comes first from the deglycosylation of strictosidine by strictosidine β-glucosidase (SGD), which may be associated with the endoplasmic reticulum (Geerlings et al.,2000). Unstable and versatile strictosidine aglycone is biochemically converted, by mostly uncharacterized enzyme reactions, to the corynanthe, iboga and aspidosperma classes of alkaloids such as ajmalicine, catharanthine and tabersonine, respectively. The pathway leading to the production of vindoline is even more complex since different cell types appear to participate in regulation, in addition to developmental, environmental and tissue-specific cues (St-Pierre et al.,1998; De Luca and Laflamme,2001; Murata and Luca,2005). The transformation of

tabersonine to vindoline requires six strictly ordered enzymatic reactions, involving hydroxylation of tabersonine by a cytochrome P450-dependent monooxygenase, tabersonine 16-hydroxylase (T16H); *O*-methylation by 16-hydroxytabersonine *O*-methyltransferase (16OMT); an uncharacterized hydration of the 2,3-double bond; a thylakoid-associated 2,3-dihydro 16-hydroxytabersonine-*N*-methyltransferase (NMT); a cytosolic 2-oxoglutarate dependent dioxygenase, desacetoxyvindoline 4-hydroxlase (D4H); and a cytosolic deactylvindoline-4-*O*-acetyltransferase (DAT) (De Luca and Laflamme,2001; Vázquez-Flota et al.,2002; van der Heijden et al.,2004) (Figure 4.1).

In CrWRKY2 overexpression lines, increased accumulation of *TDC*, *STR* and *SGD* transcripts were detected by qPCR. The expression of *G10H* and *SLS* were not affected by CrWRKY2. However, *AS*, *DXS* and *CPR* were down-regulated in CrWRKY2 hairy roots. The conversion of tabersonine into 16-hydroxytabersonine is considered to be a rate-limiting step in vindoline biosynthesis. T16H is present in the leaf epidermis and redirects the flux of tabersonine precursor to the vindoline branch (Levac et al.,2008). Moreover, enzymatic studies with extracts from young leaves of a low vindoline *C. roseus* cultivar showed lower T16H activity than those of *C. roseus* cv. Little Delicata (Magnotta et al.,2006). Here, the overexpression of *CrWRKY2* in transgenic hairy roots resulted in high up-regulation of *T16H* but decreased expression of *16OMT*.

In many *C. roseus* cell cultures, the lack of vindoline accumulation has been correlated with the lack of expression of *NMT*, *D4H* and *DAT*, the enzymes catalyzing the last three steps of vindoline biosynthesis. Studies with germinating seedlings have suggested that these three enzymes are expressed at later developmental stages than the rest of the pathway and only in aerial tissue (St-Pierre et al. 1998; Vazquez-Flota et al. 1997; Laflamme et al. 2001). In CrWRKY2 hairy root lines, we detected slight increase of *NMT*, whereas accumulation of *D4H* and *DAT* transcripts ranged between 3.1-4.2-fold, 3.3-8.4-fold, respectively. Moreover, the expression of *CrWRKY2* enhances the accumulation of minovincinine-*O*-acetyltransferase (MAT), a homolog of DAT. In hairy roots, MAT, whose function is to acetylate minovincinine and/or horhammericine, also appears to be involved in vindoline biosynthesis (Laflamme et al.,2001).

In *C. roseus*, five peroxidase genes have been reported but only two have been studied in detail. CrPRX1, which is reported to be involved in dimerization of vindoline and catharanthine to form α-3',4' anhydrovinblastine, does not express in root tissues (Costa et al.,2008). However, in this

study, we used qPCR to investigate the expression of *CrPRX1* in CrWRKY2 hairy root lines. Down-regulation of *CrPRX1* was observed in CrWRKY2 hairy roots indicating a negative effect of CrWRKY2 on *CrPRX1* expression.

Transcription factors are regulatory proteins that modulate the expression of specific groups of genes through sequence-specific DNA-binding and protein-protein interactions. They can act as activators or repressors of gene expression, mediating either an increase or a decrease in the accumulation of messenger RNA (Pierre,2004). More than ten transcription factor genes have been cloned and characterized in *C. roseus*. Upon overexpression, *ORCA3*, an AP2 domain transcription factor in *C. roseus* involved in JA-induced regulation of TIA biosynthesis, is known to increase expression of multiple genes of the MIA biosynthetic pathway resulting in increased accumulation of TIAs (van der Fits and Memelink,2000). The transcript accumulation of *ORCA3* was previously reported to be relatively low in hairy root clones in comparison with roots of plants grown in vivo (Taneja et al.,2010). In the *CrWRKY2* overexpression hairy roots, the expression of *ORCA3* was found to be 14.1-16.4- fold, in comparison to the empty vector. Similarly, *ORCA2* expression was highly up-regulated in CrWRKY2 hairy roots. ORCA2 activates the *STR* promoter and its expression is rapidly inducible with jasmonate (JA) and elicitors (Menke et al.,1999). However, the function of ORCA2 during TIA biosynthesis has not been reported until now. The transcript profiles of three MIA pathway repressors of zinc finger proteins (ZCT1, ZCT2 and ZCT3) and G-box binding factors (GBF1 and GBF2) were also observed (Figure4.8). ZCT proteins have been shown to repress the activity of two genes, *STR* and *TDC*, which are involved in biosynthesis of strictosidine, the central precursor for all MIAs (Pauw et al.,2004). GBF1 and GBF2, which play functional roles in the regulation of STR, have been shown to have high affinity for all G-boxes (Siberil et al.,2001). Increased *ZCT1* and *ZCT2* expression were observed in CrWRKY2 hairy roots, compared with empty vector control. Interestingly, Peebles et al (2009) reported a similar observation for *ORCA3* –overexpressing hairy roots, which also results in the increase of *ZCT* transcripts, suggesting that a negative regulatory scheme is overriding the positive up-regulation. The simultaneous induction of expression of activators and repressors upon elicitation and jasmonate treatment has also been demonstrated previously, clearly indicating the complex regulation of the MIA biosynthetic pathway (Gantet and Memelink,2002). In contrast to *ORCA2*, *ORCA3*, *ZCT1* and *ZCT3*, the accumulation of *ZCT2*, *GBF1*, *GBF2* and *CrMYC2* transcripts were not affected by *CrWRKY2* overexpression.

In general, the production of secondary metabolites is a highly ordered process with respect to plant development, and involves a wide array of gene expression which, in turn, controls the expression of pathways within organs, specific cell types and specific organelles. Tissue differentiation plays a significant role in the type of alkaloid produced (De Luca and St Pierre,2000). For instance, the biosynthesis of tabersonine and vindoline are restricted to leaves and stems of plants. Catharanthine is distributed equally throughout aboveground and underground tissues, while serpentine is the major alkaloid found in roots (De Luca et al.,1988; Shanks et al.,1998). Certain cell lines and select hairy root lines were shown to produce and accumulate catharanthine and tabersonine, but not vindoline (Shanks et al.,1998; Morgan and Shanks,2000). In this study, the effect of CrWRKY2 on TIA production was evaluated by measuring the concentration of four major root alkaloids: serpentine, tabersonine, vindoline and catharanthine.

In CrWRKY2 hairy roots, the high accumulation of tabersonine and catharanthine, in comparison to control roots, correlate with the increased accumulation of *TDC*, *STR* and *SGD* transcripts in the upper stream pathway. However, the accumulation of serpentine did not differ significantly between empty vector control roots and CrWRKY2 hairy roots (p value< 0.005). These results reflect the complexity of TIA biosynthesis and the regulatory function of CrWRKY2 to lead the TIA pathway towards tabersonine and catharanthine. Vindoline biosynthesis is essential in the formation of bis-indole alkaloids, vinblastine and vincristine. The enzymes catalyzing the complete pathway from tabersonine to vindoline are expressed only in aerial parts (Weatekemper et al.,1980; St-Pierre et al.,1999) and are present in sterile shoot cultures, a proposed source of vindoline (Endo et al.,1987; Campos-Tamayo et al.,2008). Vindoline is not synthesized in cell culture (Eilert et al.,1987; Vázquez-Flota et al.,2002) or in root cultures (Endo et al.,1987). Moreover, in standard hairy root cultures, generated from transformation with *A. rhizogenes*, biosynthesis of vindoline was not observed (Toivonen et al.,1989). The presence of tabersonine in hairy roots has raised speculation that this intermediate, together with catharanthine, is transported from its potential site of biosynthesis through the vasculature to the stem and leaves where tabersonine is further catalyzed to vindoline within laticifer and/or idioblast cell. In this study, however, CrWRKY2 hairy root extracts accumulated vindoline 6.0-8.1- fold more than the control hairy root cultures. The increased vindoline accumulation in CrWRKY2 hairy roots coincides with the up-regulation of *T16H*, *NMT*, *D4H*, *MAT* and *DAT*. Previously, many studies reported similar results indicating serpentine, vindoline and catharanthine were prominent components in *C. roseus* hairy root cultures during all stages of the growth cycle (Bhadra et

al.,1993; O'Keefe et al.,1997). In selected hairy root lines, vinblastine was also detected by a combination of HPLC and radioimmunoassay (Parr et al.,1988). Due to their differentiated characteristic, these hairy roots were found to produce vindoline suggesting the enzyme activities leading from tabersonine to vindoline must be present. In addition, three critical enzymes in vindoline biosynthesis, NMT, D4H and DAT, were found to be absent in cell culture (De Luca and Cutler,1987; Vázquez-Flota et al.,2002; Shukla et al.,2006; Shukla et al.,2010). In particular, the last enzyme, DAT, appears to be a major bottleneck. Laflamme et al., 2001 offer the explanation that the production of vindoline in non-aerial tissues is the consequence of the expression of another acetyltransferase, MAT, which catalyzes the 19-*O*-acetylation of minovicine and horhammericine and also acetylate deacetylvindoline. However, the poor efficiency of MAT could be responsible for the low amounts of vindoline found in hairy root cultures. Northern-blot analysis revealed that *DAT* was only expressed in specialized idioblast and laticifer cells within light-exposed tissue like leaves and stems, whereas, *MAT* was expressed mainly in the cortical of root tips (Laflamme et al.,2001). In *DAT*-overexpressing hairy roots, unexpected interference from MAT activity resulted in the accumulation of root-specific TIAs in cells (Magnotta et al.,2007). The dimeric alkaloids, vinblastine and vincristine, were not detected in both CrWRKY2 and empty vector control root cultures. This result is consistent with previous literature reports where vinblastine and vincristine were not detected in hairy root cultures of *C. roseus* (Moreno-Valenzuela et al.,1998; Tikhomiroff and Jolicoeur,2002). The absence of dimeric alkaloids in CrWRKY2 hairy roots can be a consequence of limited CrPRX1.

Table 4.1 Oligonucleotide used in PCR and qPCR

Gene	Description (accession no)	Primer pairs
AS	Anthranilate synthase (AF441857)	5'-GCGAACATTTGCAGATCCAT-3' 5'-GGCCGATTTGTTATTGTTCC-3'
TDC	Tryptophan decarboxylase (X67662)	5'-ATCCGATCAAACCCATACCA-3' 5'-CGTCATCCTCGACCATTTTT-3'
DXS	1-Deoxy-*D*-xylulose 5-phosphate synthase (AJ011840)	5'-TCGCTGCAGAACTTAGAGCA-3' 5'-GCCAACATCCCAAATGATTC-3'
G10H	Geraniol 10-hydroxylase (AJ251269)	5'-TTATTCGGATTCTGCCAAGG-3' 5'-TCCCCAAAGTGAATCGTCAT-3'
CPR	Cytochrome P450 reductase (X69791)	5'-TGGCAGAAAAGGCTTCTGAT-3' 5'-CTCAGCCTGTGTGCTATCCA-3
SLS	Secologanin synthase (L10081)	5'-GTTCCTTCTCACCGGAGTTG-3' 5'-CCCATTTGGTCAACATGTCA-3'
STR	Strictosidine synthase (X61932)	5'-ACCATTGTGTGGGAGGACAT-3' 5'-ATTTGAATGGCACTCCTTGC-3'
SGD	Strictosidine β-*D*-glucosidase (AF112888)	5'-GGAGGCTTCTTGAGTGATCG-3' 5'-GCAAATTCACCAGTGGCATA-3
T16H	Tabersonine 16-hydroxylase (P98183)	CATTAAGGCTTCATCCACCAG TCCGGGTTAAATTTCTCAGGT
NMT	16-methoxy-2,3-dihydrotabersonine-*N*-methyltransferase (ADP00410)	TTTGGCTTCATTATTGATGTTACC CTGGTGTCTTTAATAGGTTGTTCG
OMT	16-hydroxytabersonine -*O*-methyltransferase (ABR20103)	TCTCACGCCGTATTTCTGAAT GGCTGGTGTAAAAGGGATTGT
D4H	Desacetoxyvindoline-4-hydroxylase (AAB97311)	GGCTGGTGTAAAAGGGATTGT TCTCACGCCGTATTTCTGAAT
DAT	Deacetylvindoline – acetyltransferase (Q9ZTK5)	ATCGGTTGAGACAGAGACACTTC GATACGCACGTTTGGTATATGTTT
MAT	10-hydorxy-*O*-acetyl transferase (HQ901597)	ATGGAAAACGTTGACATCGA AATAATACAATTTTTAAAATGGGGTT GAA

Table 4.1 Oligonucleotide used in PCR and qPCR (continue)

Gene	Description (accession no)	Primer pairs
PRX1	peroxidase 1(CAJ84723)	TGTGGGAGAATTGTTTCTTGC CACTTGTGTTGCTTGTTGGTG
ORCA2	AP2-domain DNA-binding protein (AJ238740)	5’-GAAGATGCGGCATTAGCTTT-3’ 5’-TTGAGGACGAAGATGACACG-3’
ORCA3	AP2-domain DNA-binding protein (AJ251249)	5’-CGGGATCCGAAATACAGAAA-3’ 5’-GCCCTTATACCGGTTCCAAT-3’
ZCT1	Zinc finger DNA-binding protein (AJ632082)	5’-AGCCGAAAACTCATGCTTGT-3’ 5’-CGCCTTTGCAACAGGTTTAT-3’
ZCT2	Zinc finger DNA-binding protein (AJ632083)	5’-CGTCAATTTCCATCGTTTCA-3’ 5’-CCGATAGCGAATTCAAGTCC-3’
ZCT3	Zinc finger DNA-binding protein (AJ632084)	5-GACAAGCTTTGGGAGGACAC-3’ 5’-GGCAAGGCAGGTAAGTTCAA-3’
GBF1	G-box-binding protein (AF084971)	5’-AACAGGCTGAGACGGAAGAA-3’ 5’-GACCCGTGCATTTTTCAACT-3’
GBF2	G-box-binding protein (AF084972)	5’-GGAAGGTGCCATCTACTCCA-3’ 5’-CTAGATCGCCGAGCAGATTC-3’
CrMYC2	bHLH DNA binding protein(AF283507)	5’-TTTGGCAGTCGTCTGTTGTC-3’ 5’-CAAAAGAACTCGCGGAAGAC-3’
rps9	40S ribosomal protein S9 (AJ749993)	5’-GAGGGCCAAAACAAACTTGA-3’ 5’-CCCTTATGTGCCTTTGCCTA-3’
WRKY Degenerate	degenerate primers designed according to the sequences encoding conserved WRKY domain	5’-TGGMGIAARTAYGGNCARAAR-3’ 5’-GAYGGITAYAAYTGGMGIAARTAY-3’ 5’-RTGYTTICCYTCRTAIGTDGT-3’
CrWRKY1	WRKY DNA binding protein (HQ646368)	5’- GTACTTGGTCCCGACGATATTC-3’ 5’-CGAAACATTCCTTCGTTTGTAAG-3’
CrWRKY2	WRKY DNA binding protein	TGCTAGAAATAACGGCCAGAAT CCCTCTTTCAACTGTAGAAAAC

Table 4.1 Oligonucleotide used in PCR and qPCR (continue)

Gene	Description (accession no)	Primer pairs
rol B	*role B* gene of *Agrobacterium rhizogenes* R1000	5'-CTTATGACAAACTCATAGATAAAAGGTT-3' 5'- TCGTAACTATCCAACTCACATCAC-3'
rol C	*rol C* gene *Agrobacterium rhizogenes* R1000	5'-CAACCTGTTTCCTACTTTGTTAAC-3' 5'- AAACAAGTGACACACTCAGCTTC-3'
GUS	ß-glucuronidase gene	5'-ATGGTAGATCTGAGGGTAAATTTC-3' 5'- GCTAGCTTGTTTGCCTCCCTG-3'
NptII	Neomycin phosphotransferase gene	5'-ATGGGGATTGAACAAGATGGA-3' 5'-TCAGAAGAACTCGTCAAGAAG -3'

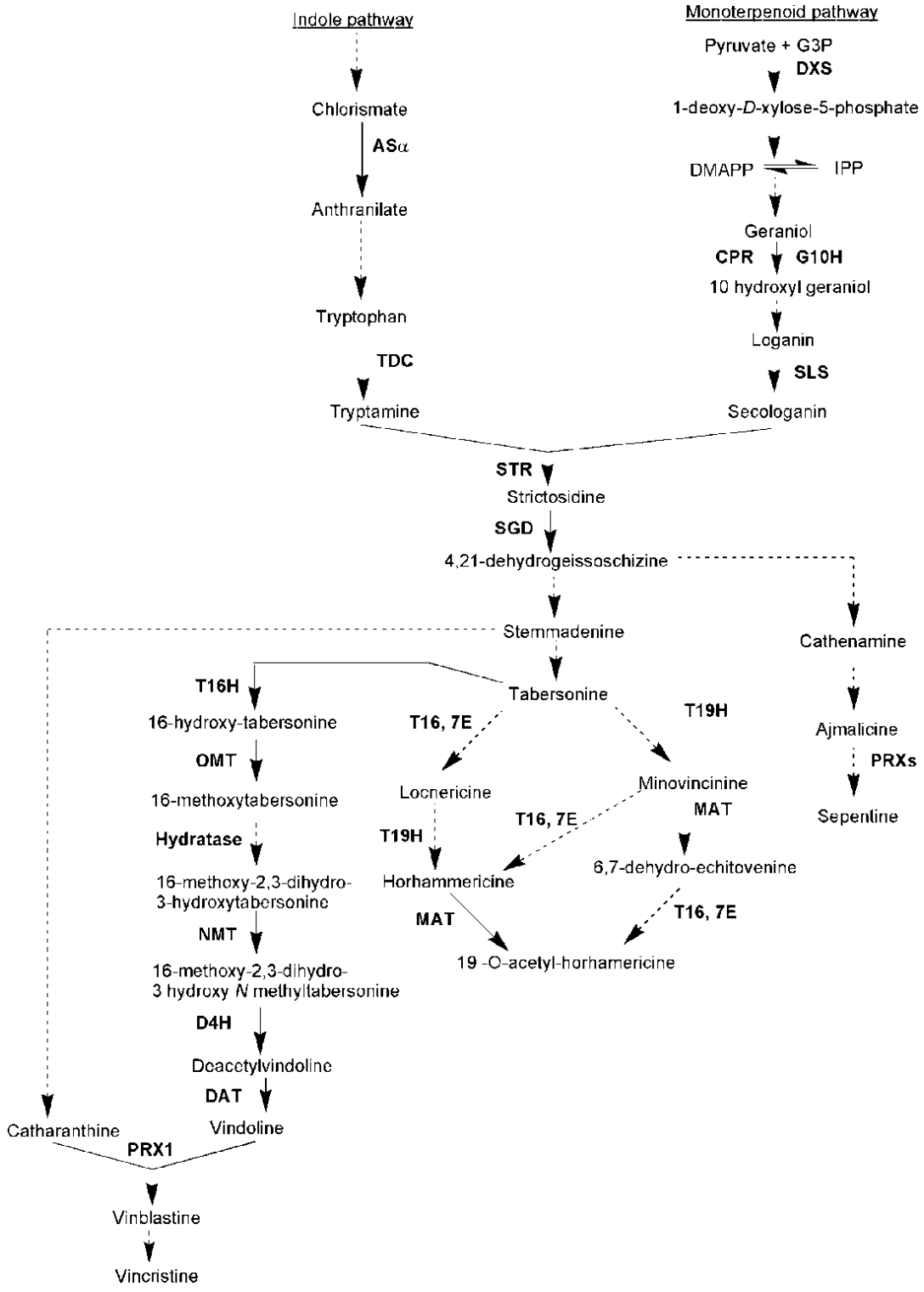


Figure 4.1 Terpenoid indole alkaloid (TIA) pathway. DXS, 1 –deoxy-D-xylulose 5-phosphate synthase; G10H, geraiol 10-hydroxylase; CPR, cytochrome P450 reductase; SLS, secologanin

synthase; Asα, anthanilate synthase α subunit; TDC, tryptophan decarboxylase; STR, strictosidine synthase, SGD, strictosidine β-*D*-glucosidase; T16H, tabersonine 16-hydroxylase; OMT, 16-hydroxytabersonine *O*-methyltransferase; D4H, deacetoxyvindoline 4- hydroxylase; NMT, 16-methoxy-2,3-dihydrotabersonine *N*-methyltransferase; DAT, deacetylvindoline acetyltransferase; T6,7E tabersonine 6,7-epoxidase; MAT, 10-hydorxy-*O*-acetyl transferase.

```
CrWRKY2      1 ------------------------------------------------------------
NtWRKY2      1 MAEN------EASSKRAPMRPTITLPPRA----SVENLFMGGPGG-LSPGPMTLVSSFFS
CaWRKY2      1 MAENEGLSSSSATSRGQLVRPTITLPPRN----SMDSLFSGG----LSPGPMTLVSSFFS
GhWRKY3      1 MAAEQG----KRSKLSAPTYPTITLPPRP----PIDGLFQSGSG--LSPGPMTLVSAFFS
AtWRKY58     1 MAVEDDVSLIRTTTLVAPTRPTITVPHRPPAIETAAYFFGGGDGLSLSPGPLSFVSSLFV

CrWRKY2      1 ------------------------------------------------------------
NtWRKY2     50 --DPASEYPSFSQLLAGAMASPAA---FSGQRQGFPPPPEMTVSISKGKESGSGDMDFGF
CaWRKY2     53 DNDPDSECRSFSQLLAGAMTSPAG---ISGVRPGFSPP----------------------
GhWRKY3     51 --DPDSTNRSFSQLLAGAMASPGAKLPYNPMDDSFMEVG----------FENGGEKNSGF
AtWRKY58    61 --------DNFPDVLT-----PDN-----------------------------------

CrWRKY2      1 ------------------------------------------------MSQQHMDNAPAEP
NtWRKY2    105 KQNRPSGLVITQSPMFTTPAGLSPARLLGSPLLFSPG-QGPFGMSHQQALAQVTAQAAHP
CaWRKY2     88 -----STAAPAMTPTFSVPPGLNPTNLFDG--FFSPG-QGPFGMSHQQVLAQLTAQASQP
GhWRKY3     99 KQNRPLNLGVGNSPWFTVPPGLSPSGLLNSPGLFCLSPQSPFGISHQQALAQVTAQAALV
AtWRKY58    72 ----------QRTTSFTH--------LLTSPMFFPPQ--SSAHTGFIQPRQQSQPQPQRP

CrWRKY2     14 QCLKMGSSE--------------------------------------------ASQSDTK
NtWRKY2    164 QSQMHIQPDYPSSSAAPAPSFSQFQSLTSNATANKQIPPPASDPN-VMKEASEVSLSDQR
CaWRKY2    140 QSQMHIQPDYSSSSAATALSMSPFQSLTSNTAANQQIPP-ALDPN-TIKESSDVSLSDQR
GhWRKY3    159 QSHVHAQPEYQTLSAAGSLEPSIPPSSGNPEETSQQMLS--SDPQSSAMEYLEASQFDKK
AtWRKY58   112 DTFPHHMP--PSISVAVHGRQSLDVSQVDQRARNHYNNP--------------GNNNNNR
                                WRKY Domain          *    *
CrWRKY2     30 VAA--VALDKPADDGYNWRKYGQKLVKAKEHPRSYYKCTHLNCPVKKKVERATDGHVAEI
NtWRKY2    223 SEPASSAVDKPADDGYNWRKYGQKHVKGSEYPRSYYKCTHPNCPVKKKVERSLDGQVTEI
CaWRKY2    198 SEPASFVVDKPADDGYNWRKYGQKQVKGSEYPRSYYKCTQPNCPVKKKVERSLDGQVTEI
GhWRKY3    217 SQP-CVAVDKPAEDGYNWRKYGQKQIKGCEYPRSYYKCTHPSCPVKKIVERSAEGLITEI
AtWRKY58   156 SYN-VVNVDKPADDGYNWRKYGQKPIKGCEYPRSYYKCTHVNCPVKKKVERSSDGQITQI
                    * *
CrWRKY2     88 TYKGQHNHEMPQPTNFQAKDVSNAGVINAGGKTELCSRSQTEN-DTLNVVKTSDSGARKY
NtWRKY2    283 IYKGQHNHQPPQSSKRSKESGNPNGNYNLQGPSELSSEGVAGTLNNSKDSMPSYSLRMTD
CaWRKY2    258 IYKGQHNHQPPQASKRSKESGNPNGNYNLQGTYEP------------KEGEPSYSLRMKD
GhWRKY3    276 IYKSTHNHEKPPPNKQPK--GGSDGNTNSQGNPELG--------SLAVAGNSNNLSEGKN
AtWRKY58   215 IYKGQHDHERPQ----NRRGGGGRDSTEVGG-------------------------AGQM
                                                Putative NSL
CrWRKY2    147 KAPRQLPSDQFPVNDEHDEMEDSAIVIDGENDDEPSAKRRS--TEVGARLPAPSTKTITE
NtWRKY2    343 QESSQATHDQVSGTSEGEEVGDTENLADGN--DERESKRRAIEVQTSEAASSASHRAVAE
CaWRKY2    306 QESSLAN-DQISGSSDSEEVGNAETRVDGRDIDERESKRRAVEVQTSEAVC--SHRTAPG
GhWRKY3    326 HESTQAV--ELPGFSDCEEGCDEESREERDD-DEPNPKRRN---STGEAAVVLSHKAVAD
AtWRKY58   246 MESSDDS-----GYRKDHDDDDDD---DEDDEDLPASKIRR------IDGVSTIHRTVTE
```

```
                                [WRKY Domain]          *    *    *
CrWRKY2    205 PKIVVQTRSEVDLLDDGYKWRKYGQKVVKGNPHPRSYYRCTYAGCNVRKHVERASIDAKA
NtWRKY2    401 PRIIVQTTSEVDLLDDGYRWRKYGQKVVKGNPYPRSYYKCTSQGCNVRKHVERAPSDPKA
CaWRKY2    363 PRIIGRTTREFDLLDDGYRWRKYGQKVVKGNPYPRSYYKCTSQGCNVRKHVERAASDPKA
GhWRKY3    380 AKIIVQTRSEVDLLDDGYRWRKYGQKVVKGNPHPRSYYKCTSAGCNVRKHVERASSDPKA
AtWRKY58   292 PKIIVQTKSEVDLLDDGYRWRKYGQKVVKGNPHPRSYYKCTTPNCTVRKHVERASIDAKA
                     * *
CrWRKY2    265 VVTTYEGKHNHDIPIAARNNGQNIPNSSSMQLK-PKRVAGRKSSAQRGMGFENKD---QL
NtWRKY2    461 VITTYEGEHNHDVP-AARNSSHNTTNNSVSQMR-PHNPVVDKQDATRRIGFSNNE--QQP
CaWRKY2    423 VITTYEGKHNHDVP-AARNSSHNTANNSTSQLR-PHNPVFDKPTAMRRSDFPSNE--QQP
GhWRKY3    440 VITTYEGKHNHDVP-AARNSSHNTVNNSLPQPKQQHDAVAEKHSLLQEIDFRN----VQG
AtWRKY58   352 VITTYEGKHNHDVP-AARNGTAAATAAAVGPSDHHRMRSMSGNNMQQHMSFGNNNNTGQS

CrWRKY2    321 QVFSQLKEGEIAA
NtWRKY2    517 IALLRLKEEQVT-
CaWRKY2    479 IALLRFKEEQIT-
GhWRKY3    495 PAVLRLKEEQIRV
AtWRKY58   411 PVLLRLKEEKITI
```

Figure 4.2 Alignment of CrWRKY2 with related WRKY proteins from other species. The deduced amino acid sequence of CrWRKY2 was aligned with NtWRKY2 (BAA77383.1), AtWRKY 58 (NP_186757.2) GhWRKY3 (ADI52618.1) and CaWRKY2 (ABA56495.2). Black and gray shading indicate conserve amino acid sequence. The WRKY signature motif (WRKYGQK) is indicted by a straight line, the characteristic residues for the zinc-finger motif are mark in asterisks and the nuclear localization sequences are high-lighted in red.

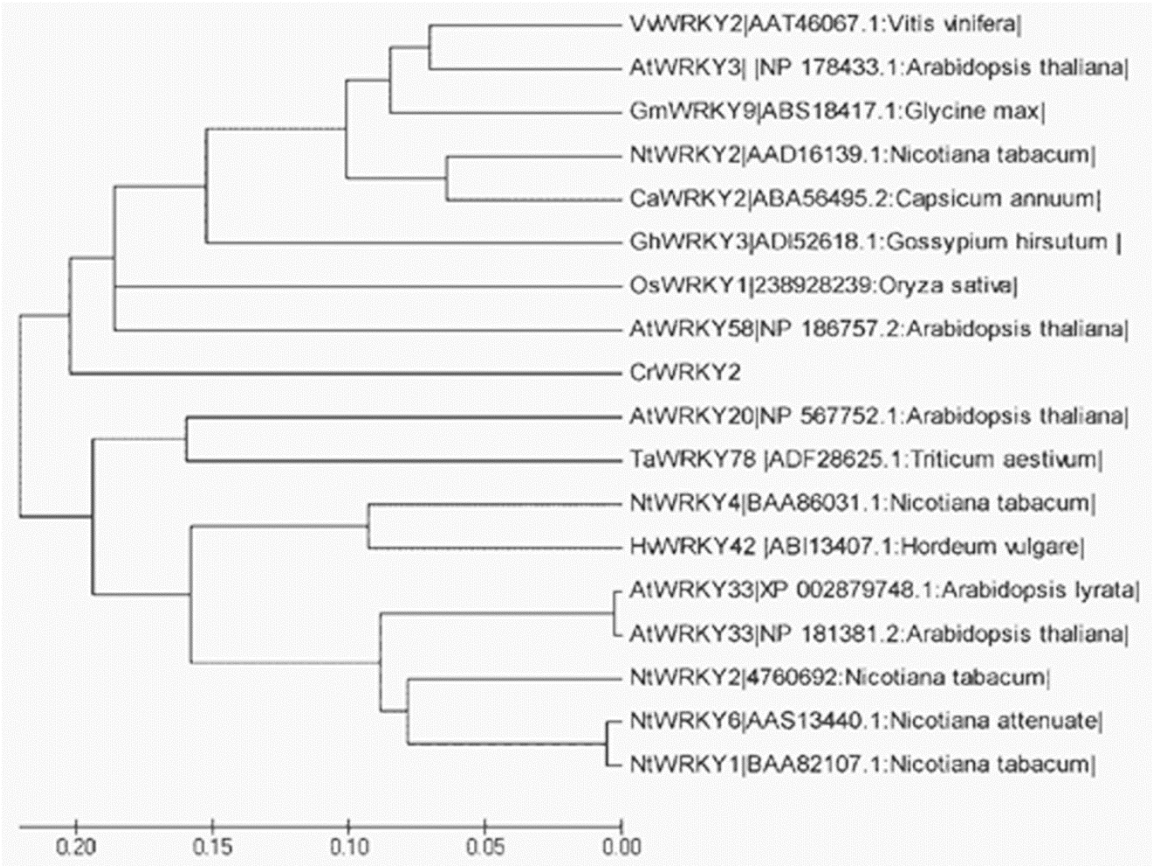


Figure 4.3 A neighbor-joining phylogenic tree of CrWRKY2 and selected WRKY protein from other plant species is constructed by MEGA5 software. The statistical reliability of individual nodes of the newly constructed tree is assessed by bootstrap analyses with 1,000 replications. The proteins, their respective plant species and GenBank accession numbers are indicated.

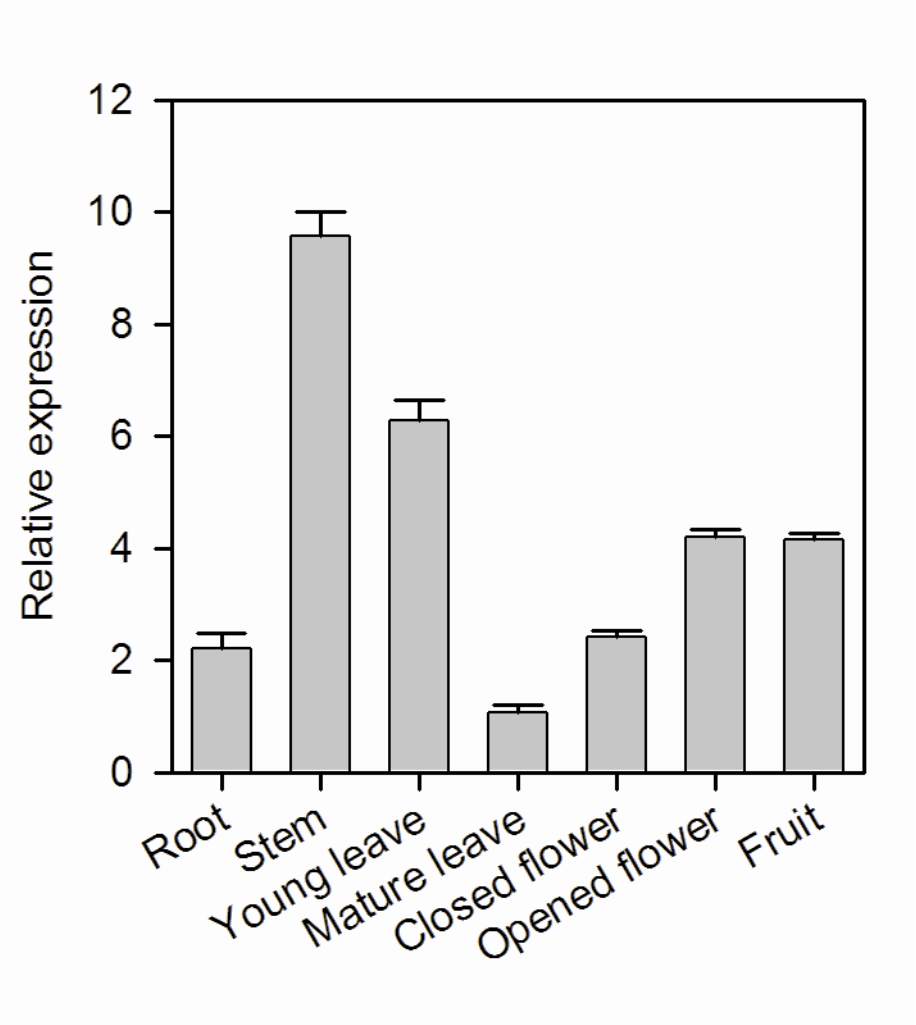


Figure 4.4 Expression profile of CrWRKY2 in roots, stems, young leaves, mature leaves, closed flowers and opened flowers of *C. roseus* plant. The level of CrWRKY2 transcript in plant tissues were measured by qRT-PCR. The *C. roseus* RPS9 was used as internal control. Values are presented as mean and standard derivation of triplicate experiments.

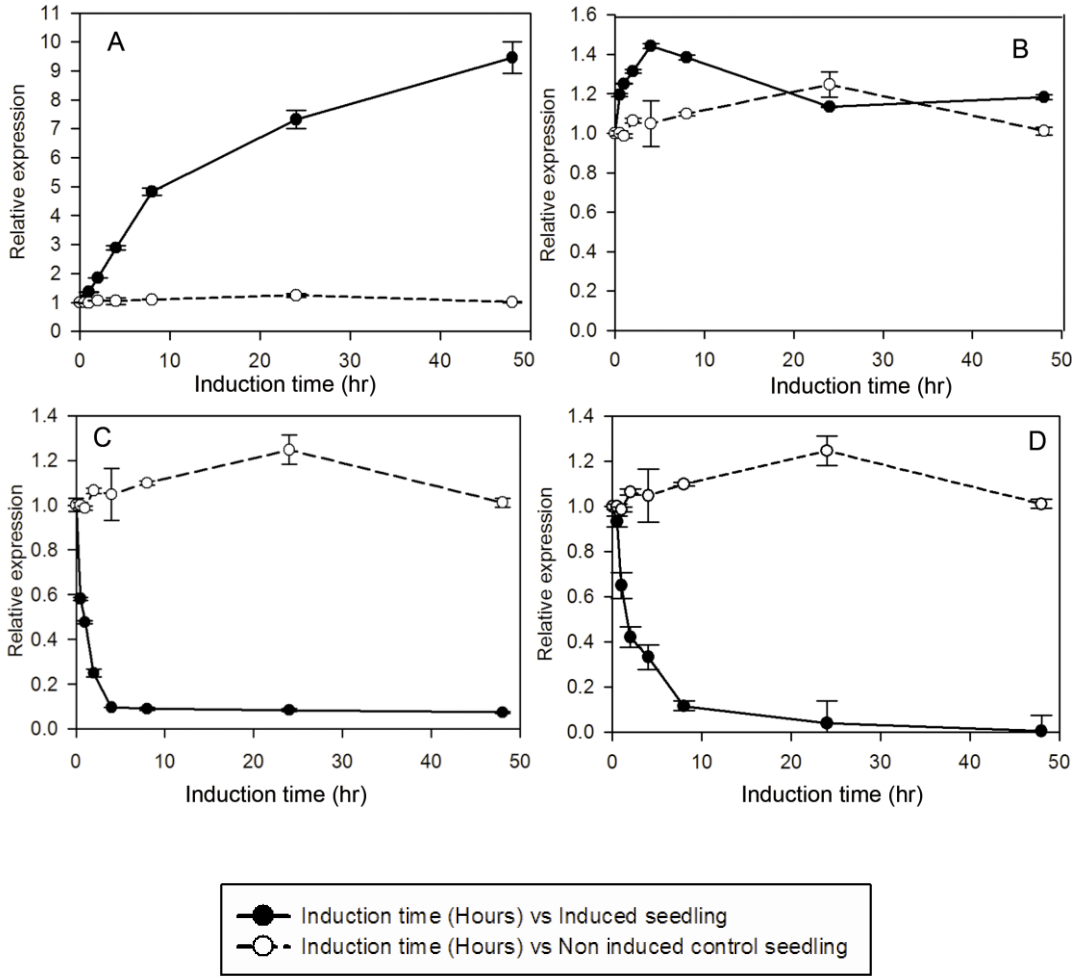


Figure 4.5 Expression profiles of *CrWRKY2* in *C. roseus* seedlings treatment with 100 µM MeJA (A) , 10 µM SA (B), 100 ppm ET (C) and wounding (D). The level of *CrWRKY*2 transcript in plant tissues were measured by qRT-PCR. The *C. roseus RPS9* was used as internal control. Values are presented as mean and standard derivation of triplicate experiments.

A

B

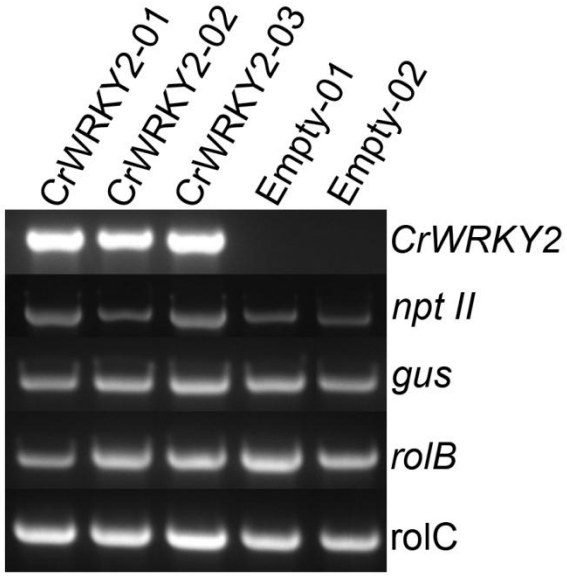


Figure 4.6 Plant expression vectors used for generating transgenic hairy roots and PCR confirmation of the presence of transgenes in transgenic hairy roots. (A) Modified pCAMBIA2301 vector containing the *CrWRKY2* cDNA under the control of *CaMV35S* promoter and *rbcS* terminator. (B) *CrWRKY2*, *nptII*, *GUS*, *rolB* and *rolC* gene were PCR amplified from genomic DNA isolated from transgenic CrWRKY1 and empty vector control hairy root lines using gene specific primer (Table 4.1). *GUS*; *β-glucuronidase*; LB, left T-DNA border; *npt II*, *neomycin phosphotransferase*; P, *CaMV35*S promoter; RB, right T-DNA border; T1, *CaMV35S* polyA; T2, *rbcS* polyA; T3, *nos* polyA.

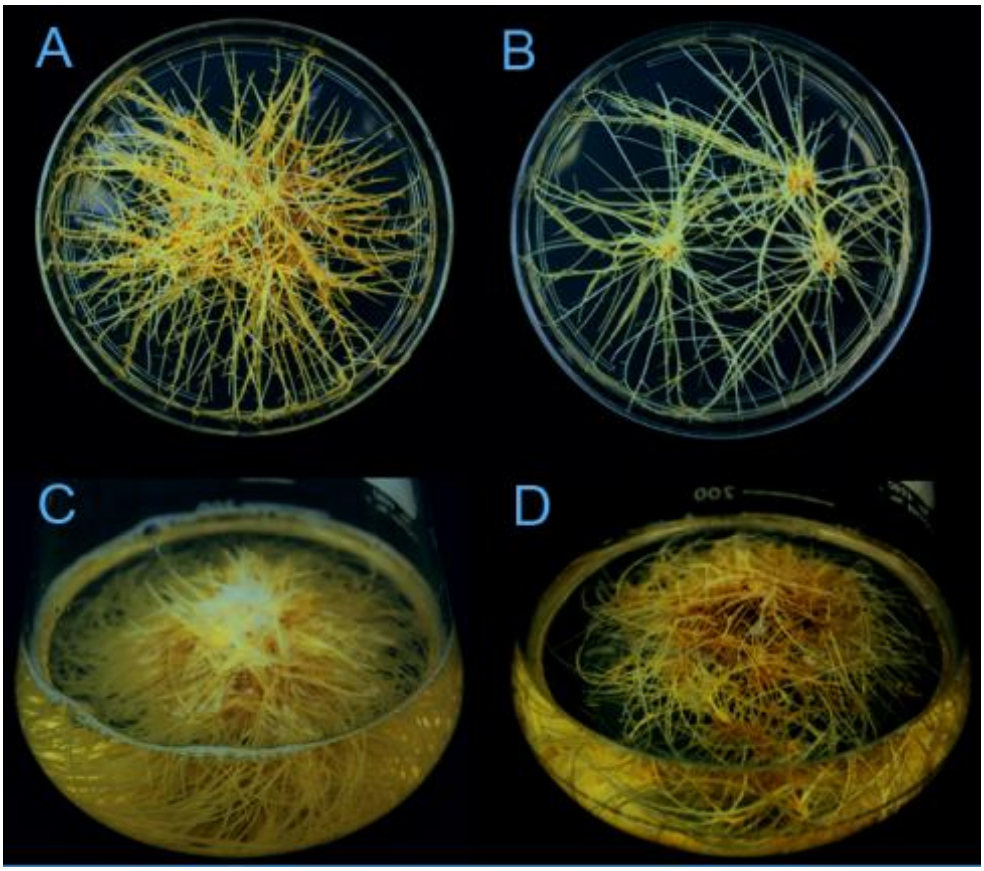
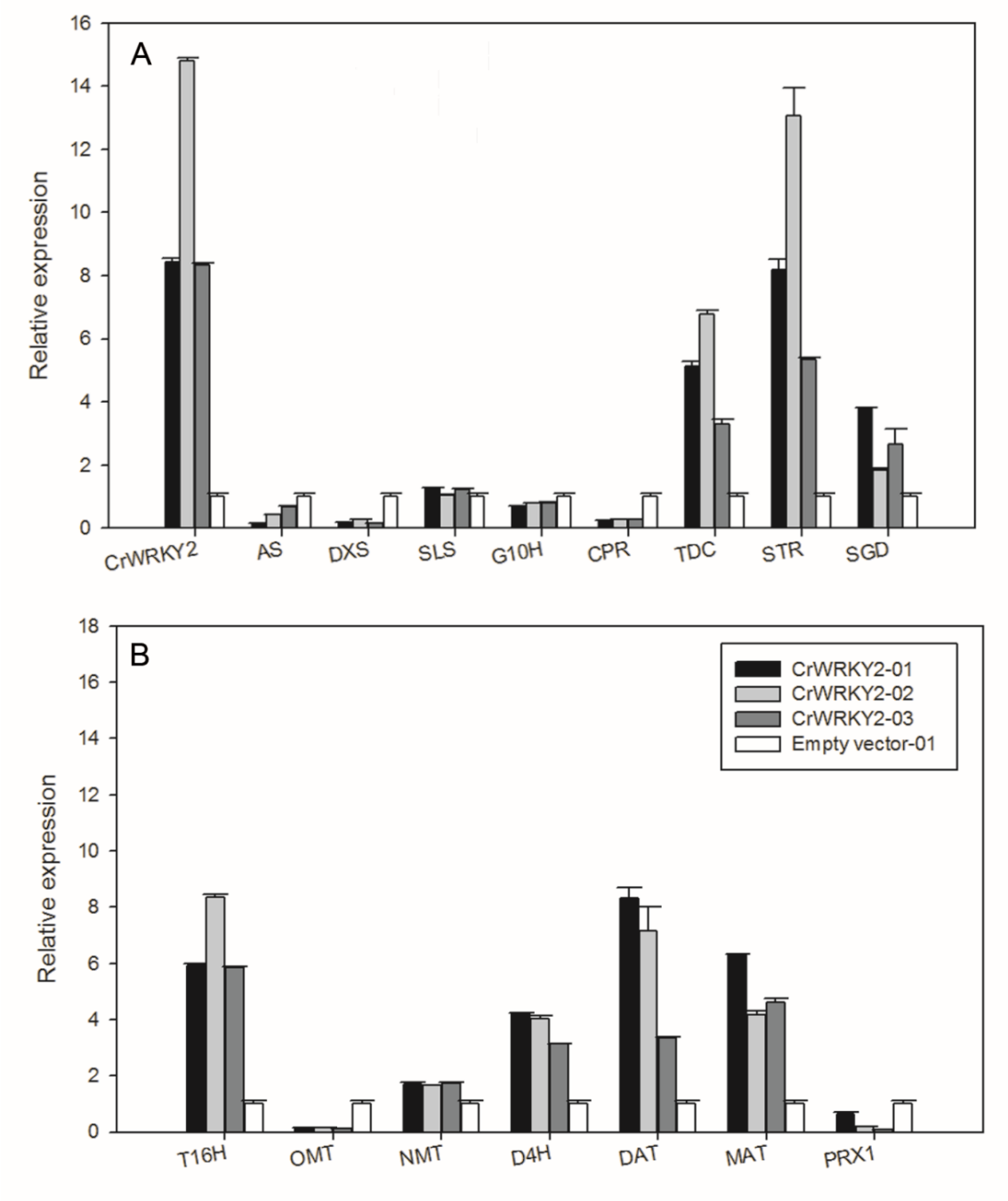


Figure 4.7 The transgenic hairy root cultures. CrWRKY2 hairy roots (A) and empty vector control hairy roots (B) grow in one third-strength SH solid medium. CrWRKY2 hairy roots grow in one third-strength SH medium in 250 Erlenmeyer flask for three week


A
Relative expression
0
2
4
6
8
10
12
14
16
CrWRKY2
AS
DXS
SLS
G10H
CPR
TDC
STR
SGD
B
Relative expression
0
2
4
6
8
10
12
14
16
18
CrWRKY2-01
CrWRKY2-02
CrWRKY2-03
Empty vector-01
T16H
OMT
NMT
D4H
DAT
MAT
PRX1

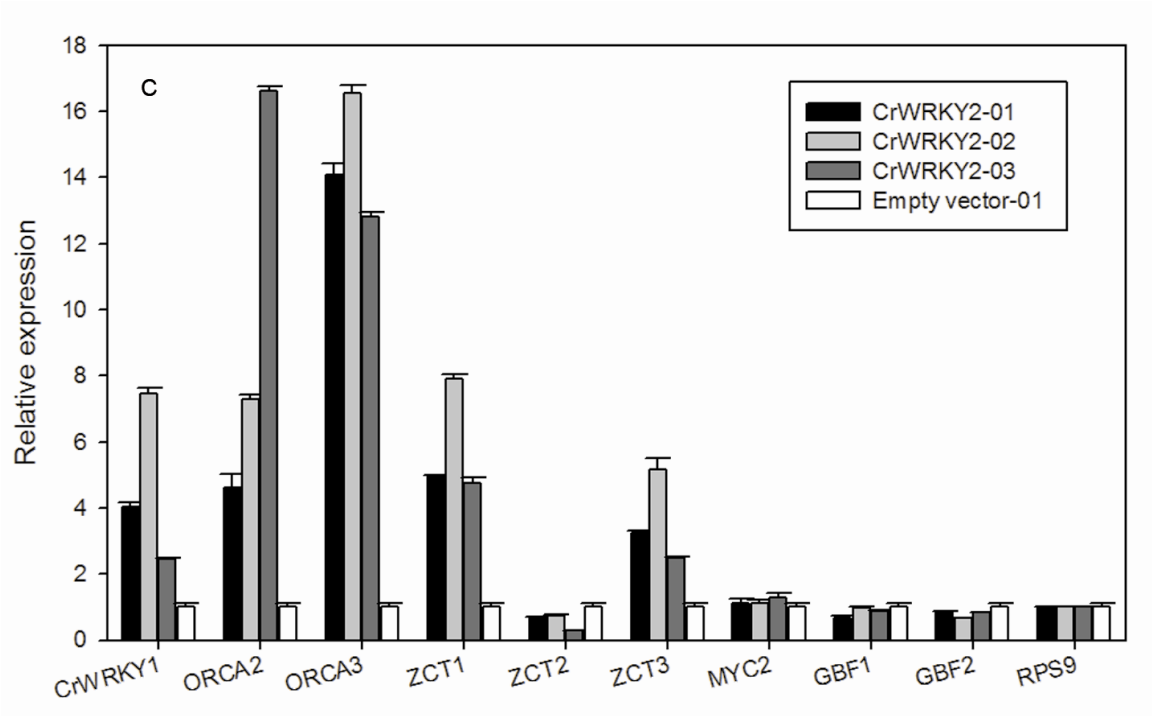


Figure 4.8 Expression of TIA pathway and regulatory genes in CrWRKY2 over-expressing hairy root in comparison with empty vector control hairy root. The transcription level of CrWRKY2 and genes in upstream pathway (A), gene in vidoline pathway branch (B) and regulatory genes (C) in pathway are presented in relative expression ($2^{-\Delta\Delta CT}$). Values are mean and standard derivation of triplicate experiments.

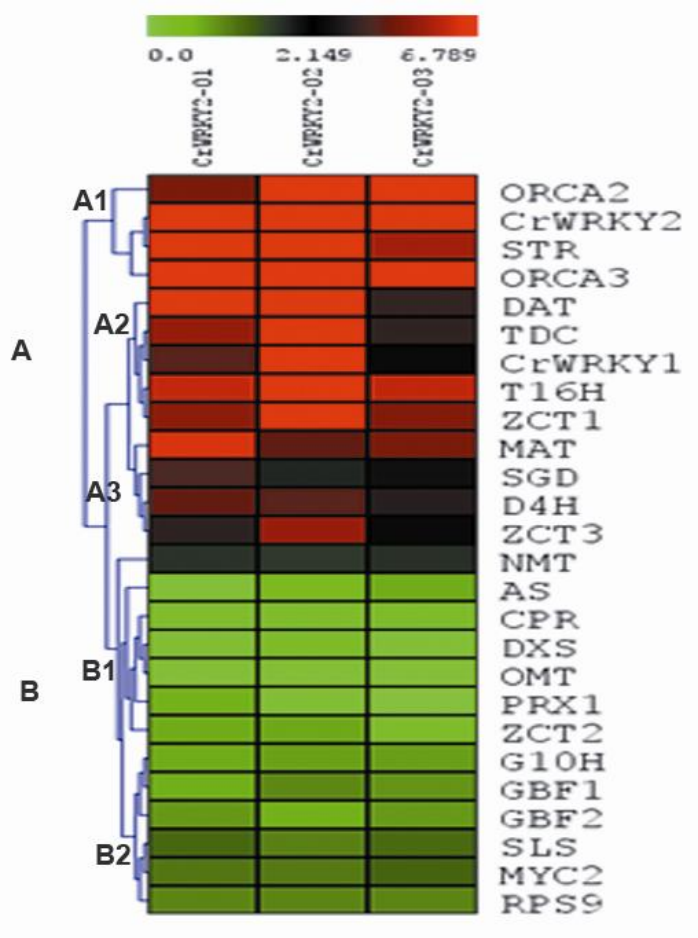


Figure 4.9 Cluster diagram of TIA pathway and regulatory genes in CrWRKY2 overexpressed hairy root. Grouping was performed using hierarchical clustering, which represent in four different colors. The intensity of the colors enhance with increases in the expression profile of a particular gene as indicates on the top of figure. Up-regulated gene clusters are indicated by red blocks. Down-regulated gene clusters are indicated by green block.

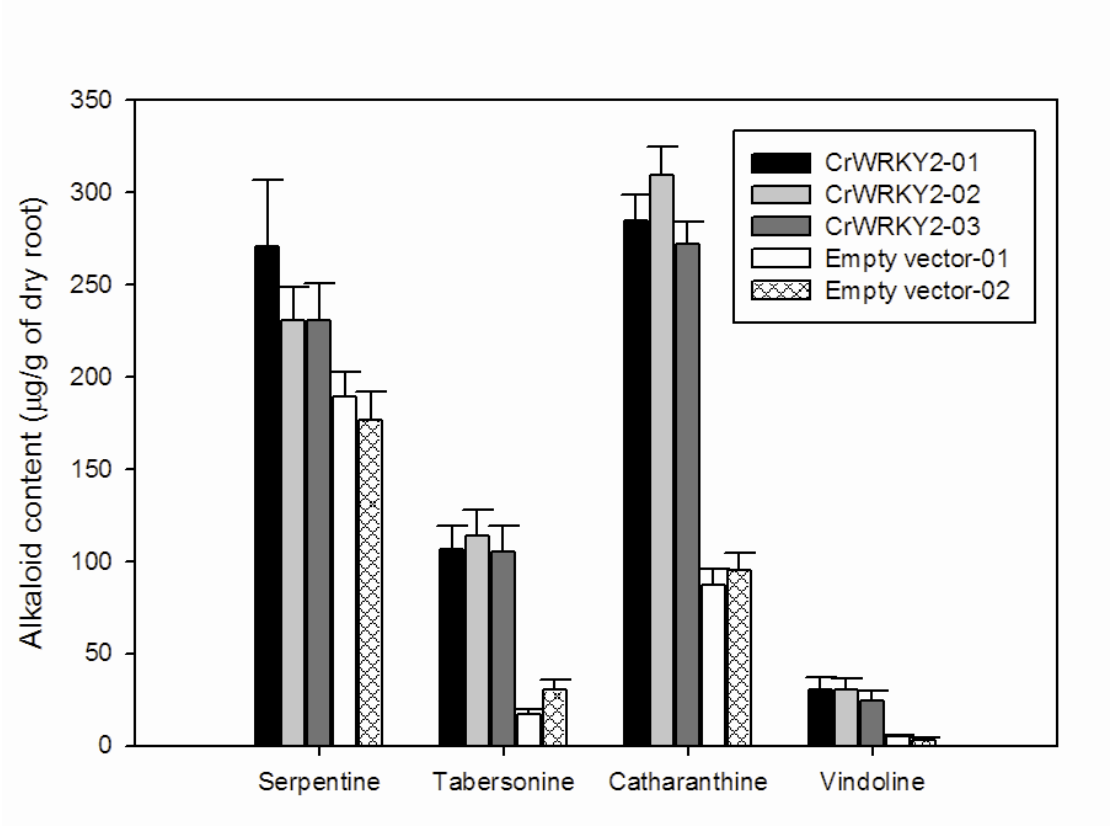


Figure 4.10 Accumulation of serpentine, cathranthine, vindoline and tabersoline in CrWRKY2-overexpressing hairy roots compared with empty vector hairy root. Values are presented as mean and standard derivation of triplicate analysis.

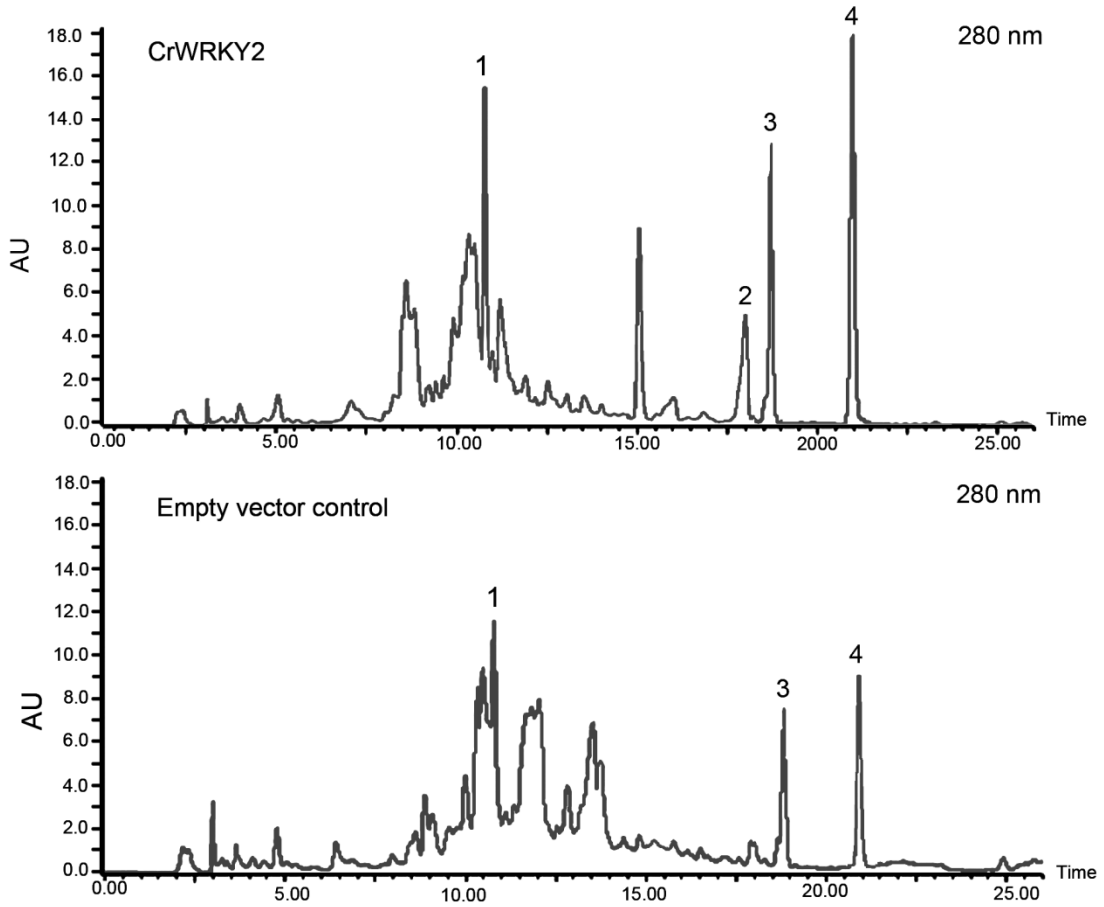


Figure 4.11 Alkaloid productions in CrWRKY1 (A) and empty vector control hairy (B) roots measured by reverse-phase HPLC-DAD. Peak 1 to 4 were identified by their retention time, UV-spectra (Figure 4.12) and indicated as, serpentine, vindoline,catharanthine and tabersonine. AU: absorbance unit.

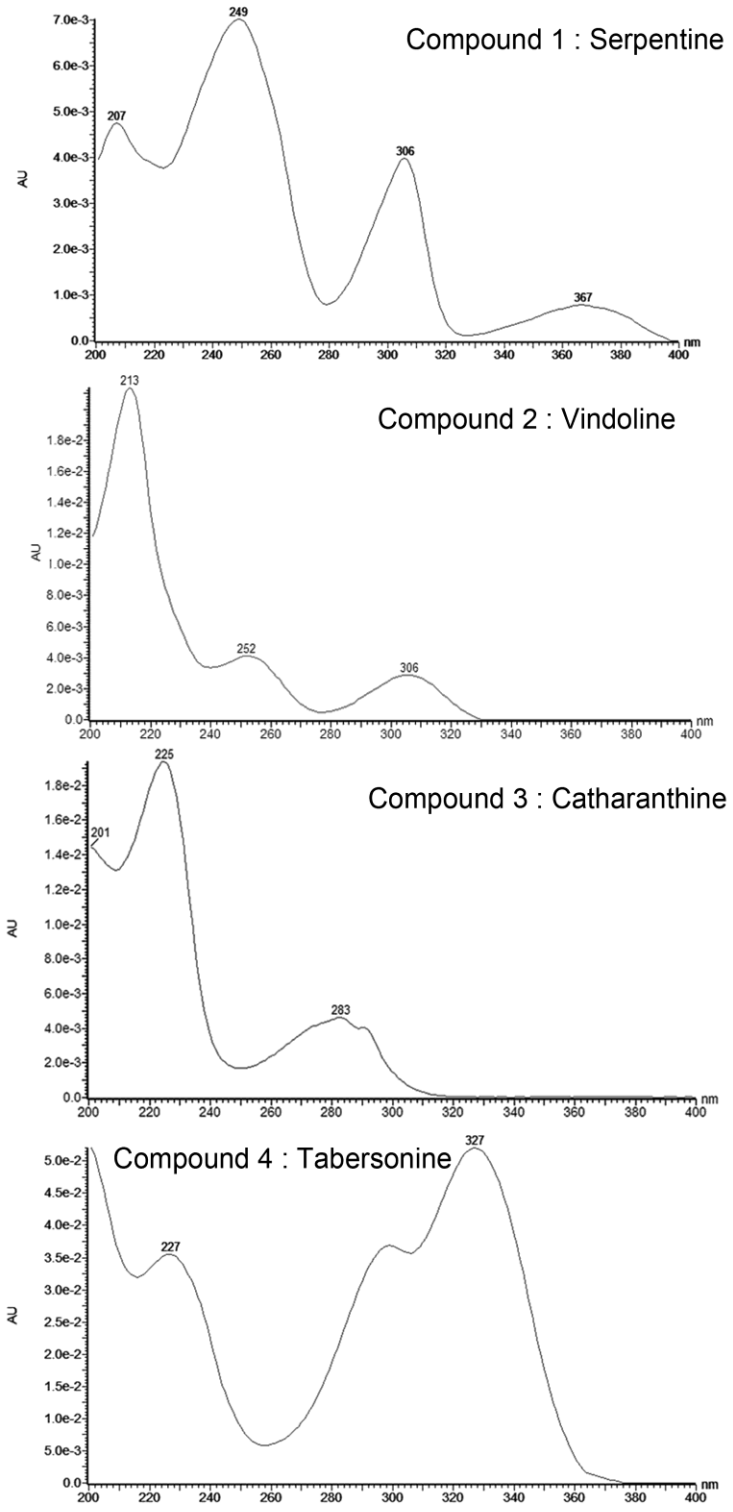


Figure 4.12 UV-spectra of sepentine, vindoline, catharanthine, and tabersonine. AU: absorbance unit.

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Chapter 5

Conclusions and Future Areas of Research

Catharanthus roseus is an important medicinal plant, that is a source of terpenoid indole alkaloids (TIAs) and an ideal system for molecular and enzymological studies (Facchini and De Luca,2008). Many attempts have been made to use plant cell culture technologies to enhance TIA production in *C. roseus*, most have been unsuccessful (Zhao and Verpoorte,2007). This can, in part, be explained by the requisite precise spatial and temporal regulation in TIA biosynthesis (Memelink and Gantet,2007). Over-expression of rate-limiting enzymes in cell cultures have been used to improve TIA production(Capell and Christou,2004; Aharoni and Galili,2011). However, up-regulation of one TIA pathway gene only enhanced at the enzymatic level, but was not able to achieve high levels of alkaloid accumulations (Goddijn et al.,1995; Canel et al.,1998). The propensity for high level alkaloid production may be complicated by the complex interactions between the biosynthesis pathways where several steps in the network share flux control (Shanks et al.,1998). A more promising approach to enhance the production of TIAs would be the induction of whole biosynthetic pathways by overexpressing regulatory transcription factors that are involved in switching on genes within the pathway (Broun,2004; Petersen,2007). At present, several transcription factors have been identified and characterized, however, other potential key transcription factors undoubtedly exist, including those belonging to the WRKY superfamily. The current knowledge of TIA biosynthesis and its transcriptional regulation is discussed in **Chapter1.**

In Chapter 2, we describe the isolation and characterization of the *C. roseus G10H* promoter. An approximately 533 bp genomic DNA fragment of the *G10H* promoter was obtained by genome walking. The major TSS maps to an adenine 40 bp upstream of the *G10H* start codon. Sequence analysis revealed the presence of several potential regulatory elements of gene expression in the G10H promoter, including a W-box, MYB-transcription factor recognition sequence, AT-box and P-box-like sequences. Comparison of known promoter sequences of *C. roseus* TIA pathway genes reveals the *G10H* promoter contains unique binding sites for several transcription factors; therefore, it may be regulated by a different transcriptional cascade. To investigate *G10H* promoter function, the promoter fragments with 5' and/or 3'-deletions were fused to the *GUS* reporter gene and GUS expression was analyzed by transient expression in tobacco protoplasts. The *GUS* reporter gene under the control of the *G10H* promoter was expressed in both transgenic tobacco plants and in a *C. roseus* hairy root system. In tobacco

seedlings, *GUS* expression was restricted to leaves and actively growing cells around the root tip. *GUS* expression was not detected in hypocotyl, root cap and older developing areas of the root. Induction of the *G10H* promoter by MeJA and fungal elicitor was observed in transgenic tobacco plants and *C. roseus* composite roots. Moreover, accumulating evidence show that, in *C. roseus*, MeJA induces the expression of a majority genes of the TIA pathway, including *STR*, *TDC*, *G10H*, *TDC* and *DAT* (El-Sayed and Verpoorte,2005; Memelink,2009). The JERE in the *STR* promoter was demonstrated to associate with AP2/ERF domain transcription factors; ORCA2 and ORCA3. Overexpression of *ORCA3* increased the expression levels of several genes in the TIA pathway(Memelink and Gantet,2007). *G10H* expression, however, is not regulated by ORCA3, suggesting that response to MeJA in *G10H* is controlled by different JA-responsive transcription factors that recognize other elements of the *G10H* promoter (Wang et al.,2010). Our analysis of the *G10H* promoter advances our understanding of the complex regulatory mechanism underlying TIA biosynthesis.

A major objective of our research is to investigate the transcriptional regulation of the *C. roseus* TIA pathway. Therefore, the promoters of *G10H*, *CPR*, *TDC*, *STR*, *DAT*, and *PRX1* were scanned with PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html). Almost all TIA pathway gene promoters examined, with the exception of *STR*, contain the typical W-box elements frequently found to be the binding sites for WRKY transcription factors implicating WRKY proteins are involved in the transcriptional regulation of TIA pathway genes in *C. roseus*. We designed degenerate primers, based on conserved WRKY domain sequences of WRKY transcription factors. Six WRKY cDNA fragments were obtained from methyl jasmonate (MeJA)-induced *C. roseus* seedlings. RT-PCR was used to examine the expression patterns of the six putative WRKY genes in MeJA-induced seedlings. The expression profile of one of them, CrWRKY1, correlated with TDC expression pattern. As described in **Chapter 3**, this led to the identification of CrWRKY1, a protein that was classified to group III WRKY transcription factors. CrWRKY1 is significantly expressed in roots and induced by MeJA, ethylene (ET) and giberrellic acid (GA). Transient expression analysis of the CrWRKY1–GFP reporter *in C. roseus* protoplasts revealed that CrWRKY1 is targeted to nuclei. Electromobility shift analysis (EMSA) indicated that the CrWRKY1 protein is capable of binding to the W-box *cis*- acting elements of the *TDC* promoter, and was demonstrated to have transactivation activity in yeast. Furthermore, transient expression experiments in *C. roseus* protoplasts confirmed that CrWRKY1 transactivate *TDC* promoter activity by binding to the W-boxes of the promoter; disruption of the W-boxes abolished the activation. The stable transformation of *CrWRKY*1 in *C. roseus* hairy roots

activated many TIA pathway and regulatory genes, in particular the *tryptophan decarboxylase* (*TDC*) gene, and repressed *ORCA2* and *ORCA3*. The up-regulation of *TDC* in CrWRKY1 hairy roots augmented TDC activity and increased resistance to 4-methyl tryptophan. In hairy roots, the overexpression of a dominant-repressive form of CrWRKY1, created by fusing the SRDX-repressor domain to C-terminus of CrWRKY1, resulted in down-regulation of the *TDC* gene. In contrast to *ORCA3* up-regulation was observed. Alkaloid accumulation in both CrWRKY1- and CrWRKY1-SRDX-hairy roots was analyzed by high performance liquid chromatography (HPLC). Significantly higher levels of serpentine, ajmalicine and catharanthine accumulated in CrWRKY1 hairy roots. These results strongly suggest the involvement of the CrWRKY1 transcription factor in the regulation of TIA biosynthesis, and indicate that *TDC* is a target gene of CrWRKY1 in *C. roseus*.

The CrWRKY2 protein was also isolated along with CrWRKY1. The details of the isolation and characterization of CrWRKY2 are described in **Chapter 4**. The full-length cDNA of *CrWRKY2* is 1002 bp in length and encodes a protein with 334 amino acids. CrWRKY2 contains two typical WRKY domains and two zinc finger motifs, categorizing it into group I of the WRKY superfamily. CrWRKY2 was highly expressed in leaves and stem of *C. roseus*. The overexpression of CrWRKY2 in hairy roots up-regulated TIA pathway genes. In particular, the genes involved in vindoline biosynthesis were up-regulated and the accumulation of vindoline was observed in CrWRKY2 hairy roots. Direct interaction of CrWRKY2 with W-box element(s) in the TIA pathway gene promoters have not yet been confirmed and will need to be addressed in future research endeavors.

WRKY proteins are plant-specific transcriptional factors and have been demonstrated to associate with regulating defense responses to wounding, pathogen infection or abiotic stresses in many plant species (Ulker and Somssich,2004). However, Ulker and Somssich (2004) reported that 440 WRKY knock-out lines rarely showed phenotypic alterations and the exact biological functions of most WRKY genes remain undefined. Our isolation of CrWRKY1 and CrWRKY2 provides useful information regarding the biological function of WRKY proteins in secondary metabolism. CrWRKY1 and CrWRKY2 were found to regulate the biosynthesis of the TIA pathway and affect TIA accumulation patterns in hairy roots. The analysis of *TDC*, *G10H*, *CPR*, *DAT* and *PRX1* demonstrates the presence of W-boxes in their promoters, therefore we can assume that as more TIA gene promoters are analyzed other *C. roseus* transcription factor will be discovered. Understanding TIA pathway regulation controls will aid the metabolic engineering

of transcription factors intended to enhance the production of pharmaceutically important TIA products such as vincristine and vinblastine at the industrial scale (Verpoorte et al.,2000).

Jasmonic acid (JA) is a plant signaling molecule that plays an important role in defense against wounding, insects and necrotrophic pathogens (Halim et al.,2006). Depending on the stress conditions and on the simultaneous induction of ET and SA biosynthesis, JA induces the expression of a specific set of genes encoding defense-related proteins and/or enzymes involved in biosynthesis of many secondary metabolites(Robert-Seilaniantz et al.,2007; Bari and Jones,2009). Many aspects concerning the mode of action of JA on the regulation of gene expression are poorly understood (Vom Endt et al.,2007; Ramamoorthy et al.,2008; Wei,2010). Several transcription factors have been identified that appear to be involved in JA-responsive gene expression, including CrWRKY1 and CrWRKY2. Dissection of the mechanisms by which JA activates these transcription factors at the protein and molecular levels are of major importance to understand how JA regulate the TIA pathway through CrWRKY1 and CrWRKY2. The promoter analysis of CrWRKY1 and CrWRKY2 provides some insight into the interactions, between signal molecules with transcription factors, underlying the regulatory cascade from JA to CrWRKY transcription factors and TIA pathway genes.

Regulation of TIA biosynthesis is accomplished spatially and temporally, by restricting product biosynthesis and accumulation to particular cells and at defined stages of plant development (Broun et al.,2006; El-Sayed and Verpoorte,2007). In the whole plant, the presence of different alkaloids and expressed genes within the above-ground and underground tissues of the plant confirms the expression of distinct tissue-specific pathways responsible for the precise tissue- and organ-specific compartmentation of TIA biosynthesis (Murata et al.,2008). The significant differences in the spectrum of alkaloids reflect the differences in the expression patterns of structural, as well as, regulatory factors in the aerial and underground tissues of the plant (Laflamme et al.,2001). The different patterns of tissue specificity in *C. roseus* plants and TIA accumulation in hairy roots of CrWRKY1 and CrWRKY2 indicate that the expressions of both are controlled by specific cell type or developmental stage. Further elucidations of these regulatory controls require expression of the transcription factors in whole plants. Theoretically, the genes can be introduced into *C. roseus* either by transformation using *Agrobacterium* or up-take by biolistic bombardment. Once the gene is introduced, the cell would be regenerated into a full transgenic plant (Veena and Taylor,2007). Currently, only Choi et al.,2004 have reported success in re-generating *C. roseus* plant from hairy roots. The degree of success varied depending on plant cultivars and the re-generating method has not been well established or well

studies. Recently, Liscombe and O'Connor,2011 reported using a virus-induced gene silencing (VIGS) approach to characterize the function of TIA pathway genes in *C. roseus*. VIGS is a method that exploits the RNAi pathway in plants to induce transient gene knock-downs. This process begins with the *Agrobacterium*-mediated introduction of modified virus-based cDNA constructs containing fragments of target gene sequences. Once expressed *in vivo*, dsRNAs are generated from an encoded viral polymerase as the virus replicates and spreads through the plant. These dsRNAs are then degraded into siRNA by DICER and RNase-like enzymes, and incorporated into a RNA-induced silencing complex (RISC). This complex targets the homologous RNA for degradation. Silencing persists until proliferation of viral RNAs is overcome by the silencing response (Unver and Budak,2009). VIGS is a useful alternative method to the difficult and laborious process of generating stably transformed plants, and offers the ability to overcome functional redundancy by suppressing all or most members of a gene family (Purkayastha and Dasgupta,2009). Besides the success development method of VIGS in *C. roseus*, the uses of this approach have also been proved in other medicinal plants such as *Papaverum soniferum* (Hileman et al., 2005), *Eschszlchozia calififonica* (Wege et al.,2007), *Thailctrum spp*.(Di Stillio et al.,2010), *Aquilegia spp*.(Gould and Kramer,2007) and *Hyoscymus niger* (Li et al.,2006). For further experiments, the ability to use VIGS as a method to investigate the regulatory functions of CrWRKY1 and CrWRKY2 in *C. roseus* should provide a better understanding of the complex regulation of TIA biosynthesis.

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