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Jasmonate-responsive transcriptional regulation in *Catharanthus roseus*

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Citation

Zhang, H. (2008, November 6). *Jasmonate-responsive transcriptional regulation in Catharanthus roseus*. Retrieved from <https://hdl.handle.net/1887/13223>

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Chapter 1

Introduction

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Part of this introduction together with parts of the discussion (Chapter 5) were published in: Zhang H., Memelink J., Regulation of secondary metabolism by jasmonate hormones, In: *Plant-Derived Natural Products: Synthesis, Function and Application*, eds.: A.E. Osbourn, V. Lanzotti, Springer

Introduction

Secondary metabolites play diverse roles in plants. Some compounds play crucial developmental roles, such as the anthocyanin pigments in flowers to attract pollinating insects. The biosynthesis of such compounds is under tight developmental control (Grotewold, 2006). Other compounds, including alkaloids in many plant species (Facchini, 2001) and glucosinolates in the Brassicaceae (Halkier and Gershenzon, 2006), are toxins or yield toxins after plant attack as protection against micro-organisms or herbivores.

Biosynthesis of defensive secondary metabolites is often induced following attack of the plant. Primary signals specifying attack which are recognized by the plant are called elicitors, pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (Jones and Dangl, 2006). Elicitors include microbial molecules derived for example from the bacterial cell wall or the flagella, signalling compounds present in insect oral secretions, or molecules derived from the damaged plant cell walls. These elicitors activate signal transduction pathways that generate secondary signals produced by plants (Zhao et al., 2005). Three major secondary signalling molecules are jasmonates (JAs; Turner et al., 2002; Wasternack, 2007; Balbi and Devoto, 2008), ethylene (Wang et al., 2002) and salicylic acid (Shah., 2003). Production of these hormones generates a signal transduction network that leads to a cascade of events responsible for the physiological adaptation of the plant cell to the external stress. The JAs, ethylene and salicylic acid signal transduction pathways act synergistically or antagonistically in a variety of responses, leading to fine-tuning of the complex defense response (Kunkel and Brooks, 2002).

Among these three key regulatory signals, by far the most important molecules for induction of secondary metabolism are the JAs. JAs have been found to induce the biosynthesis of a variety of secondary metabolites in different plant species, including alkaloids, terpenoids, glucosinolates and phenylpropanoids (Memelink et al., 2001; Zhao et al., 2005). This chapter will review recent advances in our understanding of the mechanism of action of JAs in induction of alkaloid metabolism in tobacco and in *Catharanthus roseus*

(Madagascar periwinkle). Many of these advances have been enabled by pioneering research on signal transduction of JAs using the model plant *Arabidopsis thaliana* (Katsir et al., 2008a).

Jasmonates are essential for elicitor signal transduction

Addition of elicitors is a common method of enhancing secondary metabolism in plant cell cultures for metabolic, enzymatic or regulatory studies. In its broadest definition, an elicitor is any compound or a mixture of compounds that induces a plant defence reaction. Most elicitors used in plant research originate from microorganisms but others are derived from the plant cell wall. In addition, a variety of abiotic elicitors has been used, such as heavy metals.

Elicitors are often applied in the form of crude mixtures, such as a fungal cell wall extract. In a few cases, elicitors have been purified to homogeneity. In alkaloid research, an extract from baker's yeast is commonly used as an elicitor. Yeast extract contains several components that can elicit plant defence responses, including chitin, *N*-acetylglucosamine oligomers, β -glucan, glycopeptides and ergosterol. In addition, a low molecular weight component, which is probably a small peptide, induces the expression of terpenoid indole alkaloid biosynthesis genes in cells of *C. roseus* (Menke et al., 1999b).

Biotic elicitors induce a defence reaction in plant cells because they are recognized as "non-self" in the case of microbial elicitors or as "abnormally modified self" in the case of plant cell wall fragments. The recognition occurs via the same mechanisms by which plant cells recognize non-self molecules derived from microbial pathogens. In plant-microbe interactions such elicitors are called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (Jones and Dangl, 2006; Mackey and McFall, 2006). MAMPs are recognized by transmembrane receptors called pattern recognition receptors (PRRs) or MAMP-receptors. MAMP-receptors are often classified separately from R-protein receptors which recognize directly or indirectly effector proteins injected by microbial pathogens into the host cell

(Jones and Dangl, 2006). Many of these effector proteins modify target molecules in the host to promote the virulence of the pathogen. Conform to the experimentally confirmed “guard” model many R-proteins recognize such modifications of plant molecules without interacting directly with the pathogen-derived effector.

The widely accepted view of two separate branches in the plant immune system using either MAMP-receptors or R-proteins has been challenged (Mackey and McFall, 2006). One reason is that many R-proteins that directly interact with pathogen-derived effectors, such as the rice R-protein Pi-ta which interacts with AvrPita from the rice blast fungus *Magnaportha grisea*, are in fact MAMP-receptors. In addition MAMP-receptors and R-proteins cannot be distinguished on the basis of their protein structures, cellular localization, signalling mechanisms or their induced responses. Instead, it has been proposed to categorize receptors as MAMP-receptors or MIMP-receptors (Mackey and McFall, 2006). The latter class recognizes microbe-induced molecular patterns (MIMPs) which are the result of the activity of pathogen-derived effectors on host molecules, and which would include plant cell wall-derived molecules. According to this classification R-proteins are either MAMP-receptors or MIMP-receptors.

Intensive research efforts, including pharmacological studies, have uncovered components of the signal transduction pathway connecting elicitor or MAMP perception to induction of defence genes (Zhao et al., 2005). In several elicitor responses, including secondary metabolite production, (methyl(Me))jasmonic acid and some of its bioactive precursors and derivatives play key roles as intermediate signals. In different plant species, elicitors were shown to induce accumulation of endogenous jasmonic acid, and (Me)jasmonic acid itself increased secondary metabolite production (Menke et al., 1999b; Zhao et al., 2005). In addition, in several species it was shown that blocking jasmonate biosynthesis abolished elicitor-induced metabolite accumulation and the expression of biosynthesis genes (Menke et al., 1999b; Zhao et al., 2005). Elicitors induce many intracellular events, including an increase in cytoplasmic calcium concentration, ion transport, production of reactive oxygen species and protein phosphorylation. How these events are exactly coupled to induced

jasmonate biosynthesis remains largely unknown. A detailed review of intracellular events triggered by elicitors and their possible role in signal transduction is presented by Zhao et al. (2005).

The control points that govern the synthesis and accumulation of JAs remain to be identified. Timing and control of jasmonate biosynthesis suggest several ways in which jasmonate signalling might be modulated during stress perception. One level of control in jasmonate biosynthesis and/or signalling might be the sequestration of biosynthetic enzymes and substrates inside the chloroplasts (Stenzel et al., 2003). In this way, jasmonate biosynthesis and signalling would only be activated by the availability of substrate upon cellular decompartmentalization during wounding or pathogen attack. However, wounding induces the expression of several jasmonate biosynthesis genes (Turner et al., 2002), suggesting that, at least partly, the wound-induced production of JAs is a result of the increased transcription of genes encoding the jasmonate biosynthesis pathway enzymes and their subsequent *de novo* protein synthesis. In addition, JAs themselves induce the expression of genes involved in jasmonate biosynthesis (Turner et al., 2002), indicating the existence of a positive feedback regulatory mechanism for jasmonate biosynthesis in which JAs stimulate their own production.

Jasmonate biosynthesis

JAs, including jasmonic acid (JA) and several of its cyclic precursors and derivatives, constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues (Turner et al., 2002; Wasternack, 2007; Balbi and Devoto, 2008). These signalling molecules affect a variety of developmental processes including fruit ripening, production of viable pollen, root elongation, and tendrill coiling. In addition and more importantly for this thesis, JAs regulate responses to wounding and abiotic stresses, and defence against insects, and necrotrophic pathogens.

An important defence response is the induction of secondary metabolite accumulation, which depends on JAs as a regulatory signal. JAs are fatty acid

derivatives which are synthesized via the octadecanoid pathway. Most of the enzymes of this pathway leading to jasmonate biosynthesis have been identified by a combination of biochemical and genetic approaches (Wasternack, 2007). The enzymes leading to JA biosynthesis are located in two different subcellular compartments. The octadecanoid pathway starts in the chloroplasts with phospholipase-mediated release of α -linolenic acid from membrane lipids. The fatty acid α -linolenic acid is then converted to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The second part of the pathway takes place in the peroxisomes. OPDA is transported from the chloroplasts to the peroxisomes where it is reduced by OPDA reductase (OPR3) to give 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), followed by three rounds of beta-oxidation involving three enzymes to yield (+)-7-*iso*-jasmonic acid which equilibrates to the more stable (-)-JA.

Subsequently, JA can be metabolized in the cytoplasm by at least seven different reactions. Well-characterized reactions include methylation to methyl-jasmonate (MeJA) by *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyl transferase (JMT), conjugation to amino acids by JA amino acid synthase (JAR1) or hydroxylation to 12-hydroxyjasmonic acid (12-OH-JA). OPDA, JA, MeJA and JA-Ile are active signalling molecules, whereas 12-OH-JA is thought to be a biologically inactive derivative (Wasternack, 2007).

Jasmonate perception and signalling in Arabidopsis and tomato

How JAs induce gene expression has been mainly unravelled in studies using Arabidopsis and tomato (*Solanum lycopersicum*) (Katsir et al., 2008a). To identify molecular components of jasmonate signal transduction, screenings for Arabidopsis mutants that are insensitive to (Me)JA or to coronatine (a bacterial toxin which is a structural and functional analogue of JA-Ile) or that show constitutive jasmonate responses have been performed (Lorenzo and Solano, 2005). Several mutants have been characterized.

The *coronatine insensitive1 (coi1)* mutant was isolated in a screen for Arabidopsis mutants insensitive to root growth inhibition by coronatine (Feys et al., 1994). The *coi1* mutant is also insensitive to JAs (Feys et al., 1994), is defective in resistance to certain insects and pathogens and fails to express jasmonate-regulated genes (Turner et al., 2002). The *COI1* gene encodes an F-box protein (Xie et al., 1998). F-box proteins associate with cullin, Skp1 and Rbx1 proteins to form an E3 ubiquitin ligase known as the SCF complex, where the F-box subunit functions as the specificity determinant targeting proteins for ubiquitin-mediated proteolysis by the 26S proteasome (del Pozo and Estelle, 2000). Co-immunoprecipitation experiments showed that COI1 associates *in vivo* with Skp1, cullin and Rbx1 proteins to form the SCF^{COI1} complex (Devoto et al., 2002; Xu et al., 2002). Therefore, the requirement for COI1 in jasmonate-dependent responses indicates that ubiquitin-mediated protein degradation is a crucial event in jasmonate signalling. Plants that are deficient in other components or regulators of SCF complexes, including AXR1, COP9 and SGT1b, also show impaired jasmonate responses (Lorenzo and Solano, 2005). The existence of a *COI1* function that is conserved in species other than Arabidopsis was demonstrated by the identification of *COI1* homologues in tomato (Li et al., 2004), tobacco (Shoji et al., 2008) and *Nicotiana attenuata* (Paschold et al., 2007). COI1 is a component that is specific to the JA pathway, whereas SGT1b and AXR1 are shared by other signalling pathways. Mutations in *AXR1* or *SGT1b* have pleiotropic effects that impair plant responses not only to JA but also to auxin and pathogens, suggesting that both SGT1b and AXR1 are regulators of SCF complexes and are involved in several different signalling pathways (Austin et al., 2002; Azevedo et al., 2002; Gray et al., 2003).

A particularly effective screen for jasmonate signalling mutants has been described by Lorenzo et al. (2004). Screening for mutants affected in JA-induced root growth inhibition in an *ethylene-insensitive3 (ein3)* background resulted in the identification of five complementation groups identifying 5 loci called *JA-insensitive (JAI)* 1-5. The *JAI1* locus corresponds to the *AtMYC2* gene (Lorenzo et al., 2004), encoding a basic Helix-Loop-Helix (bHLH) transcription factor which regulates a subset of jasmonate-responsive genes

involved in wounding responses. The *JAI2* locus corresponds to the previously characterized *JAR1* gene (Staswick et al., 1992), encoding an enzyme that couples JA to amino acids with a preference for isoleucine (Staswick and Tiryaki, 2004). The *JAI4* locus corresponds to the *SGT1b* gene (Lorenzo and Solano, 2005). The *JAI5* locus corresponds to the *COI1* gene (Lorenzo et al., 2004).

Recently, the gene affected in the *jai3* mutant was identified. It encodes a protein with a zinc finger-like ZIM motif (Chini et al., 2007). There are several related genes in Arabidopsis forming a gene family called ZIM or TIFY (Vanholme et al., 2007). The members that are induced at the gene expression level by JAs are called Jasmonate ZIM domain (JAZ) proteins (Chini et al., 2007; Thines et al., 2007). They contain in addition to the highly conserved central ZIM motif a highly conserved C-terminal Jas motif and a less conserved N-terminal region. In the *jai3* mutant an aberrant protein is expressed with a deletion of the C-terminal domain including the Jas motif. The wild-type JAI3 (or JAZ3) protein is rapidly degraded in response to JA in a COI1-dependent manner, whereas the *jai3* mutant protein is stable. The JAI3 protein was shown to interact in vitro and in yeast with AtMYC2. Based on these findings it was postulated that JAI3 is a repressor of AtMYC2 which is rapidly degraded in response to JA thereby activating AtMYC2 (Figure 1; Chini et al., 2007).

In independent studies, members of the JAZ gene family in Arabidopsis were characterized as being predominant among genes induced in anthers after 30 minutes of JA treatment (Mandaokar et al., 2006). Subsequent study of the family member JAZ1 demonstrated that it is rapidly degraded in response to JA in a COI1-dependent manner (Thines et al., 2007). On the other hand a deletion derivative of JAZ1 lacking the C-terminal domain is stable.

Interestingly, these authors were able to detect interaction between JAZ1 and COI1 in a yeast two-hybrid assay in the presence of JA conjugated to Ile (JA-Ile) in the yeast growth medium or in an in vitro pull-down assay in the presence of JA-Ile. No interaction was detected in the presence of OPDA, JA, MeJA or JA conjugated to Trp or Phe, whereas JA-Leu was about 50-fold less effective in promoting interaction between COI1 and JAZ1 than JA-Ile. JA-Ile and JA-Leu are products of the JAR1-mediated conjugation reaction (Staswick

and Tiryaki, 2004). JA-Ile and coronatine also promote the interaction between JAZ3 and JAZ9 in a yeast two-hybrid assay, whereas JA or MeJA are ineffective (Melotto et al., 2008).

Using tomato SICO1 and SIJAZ1, it was shown that the complex binds radiolabeled coronatine (Katsir et al., 2008b). Binding could be displaced with unlabeled coronatine or JA-Ile. These experiments showed that COI1 is the receptor for at least certain JAs including JA-Ile, as well as for the microbial JA-Ile mimic coronatine.

The expression of the JAZ genes in Arabidopsis is induced by JA (Mandaokar et al., 2006; Chini et al., 2007; Thines et al., 2007) and is controlled by AtMYC2 (Chini et al., 2007). AtMYC2 and JAZ proteins therefore form a jasmonate-responsive oscillator, where JAZ proteins negatively regulate AtMYC2 activity at the protein level, JAs cause JAZ degradation and AtMYC2 activation, and AtMYC2 switches on the expression of JAZ repressors at the gene level (Figure 1).

The picture that emerges for jasmonate signal transduction is highly reminiscent of auxin signal transduction, which involves auxin-responsive degradation of AUX/IAA repressor proteins via the F-box protein TIR1 (Guilfoyle, 2007). TIR1 is the auxin receptor (Kepinski and Leyser, 2005; Dharmasiri et al., 2005) with auxin acting as the molecular glue between TIR1 and AUX/IAA proteins (Tan et al., 2007). COI1 is the closest relative to TIR1 that is not related to auxin perception among the about 700 members of the Arabidopsis F-box protein family (Gagne et al., 2002). JA-Ile forms the molecular glue between COI1 and JAZ1, JAZ3, JAZ9 (Chini et al., 2007; Melotto et al., 2008) and possibly other JAZ family members (Figure 1). It was proposed that different biologically active JAs could form the molecular glue between COI1 and specific JAZ family members, and that these family members could act as repressors of specific downstream targets, presumably other transcription factors (Thines et al., 2007).

Challenges are to determine whether different JAs can indeed act as molecular glues with specific JAZ family members, and to find out what are the specific targets of each member of the JAZ family of repressors. It is also

conceivable that biologically active JAs other than JA-Ile form the molecular glues between COI1 and hitherto unidentified repressors distinct from the JAZ proteins.

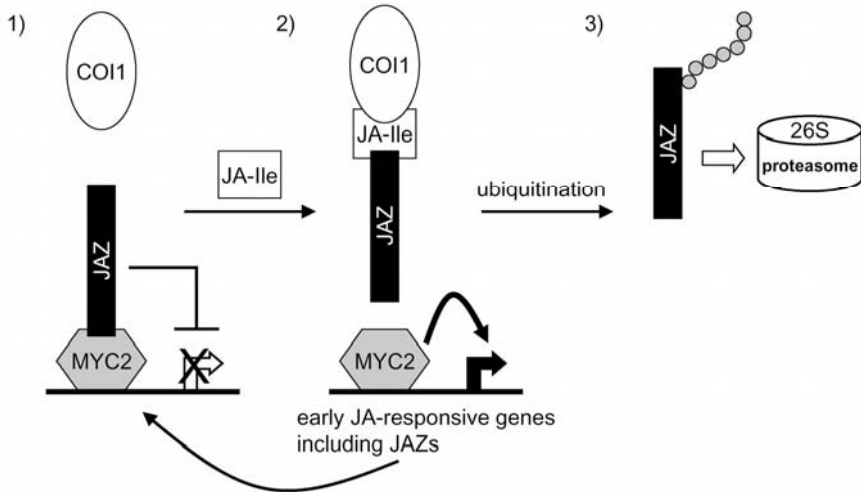


Figure 1. Model for jasmonate signal transduction leading to expression of AtMYC2-regulated genes.

1) In the absence of JA-Ile, JAZ interacts with AtMYC2 maintaining this transcription factor inactive. 2) JA-Ile forms the molecular glue between JAZ and COI1. 3) SCF^{COI1} promotes the ubiquitination of bound JAZ proteins resulting in their subsequent degradation by the 26S proteasome. AtMYC2 is liberated and activates transcription of target genes, including genes encoding JAZ proteins, resulting in a negative feedback loop. Although depicted as a single protein, COI1 forms part of the E3 ubiquitin ligase SCF^{COI1}.

Jasmonate signalling in tobacco alkaloid biosynthesis

The main alkaloid found in tobacco plants, nicotine, is composed of a pyrrolidine ring and a pyridine ring. The pyrrolidine moiety is derived from *N*-methylputrescine, which is formed from putrescine by putrescine *N*-methyltransferase (PMT) (Katoh et al., 2005). The pyridine moiety of nicotine is derived from nicotinic acid. Nicotine is exclusively synthesized in the roots and is translocated to the leaves via the xylem. Multiple genes encoding nicotine biosynthesis enzymes, including *PMT*, are transcriptionally activated by exogenous application of jasmonates in tobacco roots and in cultured tobacco cells (Goossens et al, 2003; Katoh et al., 2005). Promoter regions of ~250 base pairs from three *PMT* genes from *Nicotiana sylvestris* could confer jasmonate-

responsive expression on a *GUS* reporter gene in transgenic hairy roots, showing that the jasmonate signal converges on relatively small promoter regions to confer transcriptional responses (Shoji et al., 2000).

In *Nicotiana attenuata* JA accumulates in response to attack by the herbivore *Manduca sexta* (tobacco hornworm) or in response to wounding and application of caterpillar oral secretions (a mimic of herbivore attack). This is likely caused by induction of jasmonate biosynthesis genes, since the *NaLOX3* gene was induced by these treatments (Halitschke and Baldwin, 2003). Silencing the expression of *NaLOX3* reduced nicotine accumulation in response to JA. Simultaneous silencing of *NaJAR4* and *NaJAR6* (the *N. attenuata* orthologues of *AtJAR1*) also reduced JA-responsive nicotine accumulation (Wang et al., 2008). Application of JA-Ile restored nicotine accumulation, indicating that JA-Ile is an important signalling molecule for nicotine production in *Nicotiana attenuata*.

Silencing of the *COI1* gene in tobacco plants abolished the MeJA-responsive expression of nicotine biosynthesis genes including *PMT*, as well as MeJA-responsive nicotine accumulation (Shoji et al., 2008). The same report describes the isolation of three members of the tobacco *JAZ* gene family and their involvement in nicotine biosynthesis. The *NtJAZ1-3* genes were induced by MeJA. For *NtJAZ1* it was shown that the protein was rapidly degraded via the 26S proteasome in response to MeJA, whereas a derivative lacking the C-terminal Jas domain was stable. Overexpression of C-terminal deletion derivatives of *NtJAZ1* or *NtJAZ3* abolished MeJA-responsive *PMT* gene expression as well as nicotine accumulation.

These observations show that MeJA-responsive nicotine biosynthesis is controlled by the jasmonate receptor *COI1* and depends on degradation of members of the *JAZ* repressor family (Figure 2). There are no published data yet about the nature of the transcription factor(s) repressed by the *JAZ* proteins in tobacco.

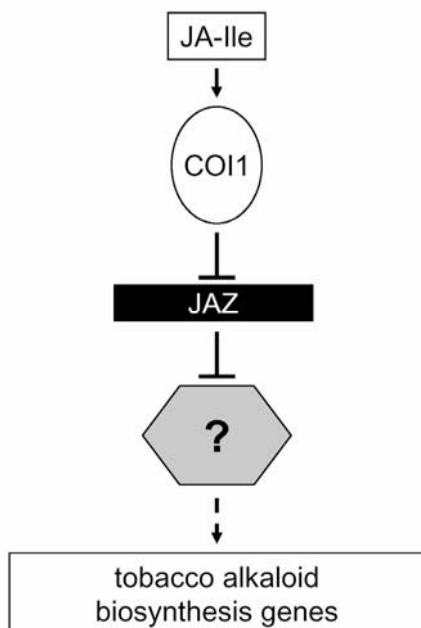


Figure 2. Model for jasmonate signal transduction leading to the expression of tobacco alkaloid biosynthesis genes.

As depicted in Figure 1, JA-Ile forms the molecular glue between NtCOI1 and NtJAZ, leading to degradation of the latter proteins. The unidentified transcription factor repressed by the NtJAZ proteins is then liberated and activates directly or indirectly transcription of the genes encoding tobacco alkaloid biosynthesis enzymes. Solid lines indicate interactions between proteins and broken lines indicate interactions between proteins and genes.

Terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*

Research on the terpenoid indole alkaloids (TIAs) is mainly primed by the pharmaceutical applications of several of the compounds. The monomeric alkaloids serpentine and ajmalicine are used as tranquilizer and to reduce hypertension, respectively. The dimeric alkaloids vincristine and vinblastine are potent antitumor drugs. In plants, TIAs are thought to be involved in defence responses. Several reports show that physiological concentrations of TIAs can have antifeeding activity or can delay growth of insect larvae, fungi and microbes (Aerts et al., 1991, 1992; Luijendijk et al., 1996). TIAs are found in a limited number of plant species belonging to the plant families Apocynaceae, Loganiaceae, Rubiaceae and Nyssaceae. The best progress on molecular characterization of the pathway has been made with *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle), a member of the Apocynaceae family. *C.roseus* cells have the genetic potential to synthesize over a hundred terpenoid indole alkaloids.

The initial step in TIA biosynthesis is the condensation of tryptamine with the iridoid glucoside secologanin (Figure 3). This condensation is

performed by the enzyme strictosidine synthase (STR) and results in the synthesis of 3 α (S)-strictosidine. Strictosidine is deglycosylated by strictosidine β -D-glucosidase (SGD). Further enzymatic and spontaneous conversions result in the biosynthesis of numerous TIAs. Tryptamine, providing the indole moiety of TIAs, is formed by decarboxylation of tryptophan by the enzyme tryptophan decarboxylase (TDC). Secologanin, providing the terpenoid part of the TIAs, is synthesized via multiple enzymatic conversions from geraniol.

The terpenoid precursors that constitute the backbone of geraniol are produced via the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway (Contin et al., 1998). Most steps involved in the conversion of geraniol to secologanin are unknown. Geraniol 10-hydroxylase (G10H) catalyses the first committed step in the formation of secologanin by 10-hydroxylation of geraniol, whereas secologanin synthase (SLS) catalyses the last conversion of loganin into secologanin. G10H and SLS are both cytochrome P450 monooxygenases, which require the co-enzyme NADPH:cytochrome P450 reductase (CPR).

Dimeric alkaloids are formed by peroxidase-catalyzed condensation of vindoline and catharanthine (Sottomayor et al., 2004). Many monomeric TIAs are found in all plant organs, but vindoline and vindoline-derived dimeric alkaloids are only found in chloroplast-containing plant tissues. Vindoline is derived via a number of steps from tabersonine. The first step is catalyzed by the P450 enzyme tabersonine 16-hydroxylase (T16H), which also requires CPR as a co-enzyme. The two final steps are catalyzed by acetyl CoA:deacetylvindoline 4-O-acetyltransferase (DAT) and the 2-oxoglutarate-dependent dioxygenase desacetoxylvindoline-4-hydroxylase (D4H).

Over ten genes have now been cloned from the estimated total number of around 25 genes encoding enzymes involved in TIA biosynthesis. In addition, genes acting in primary precursor pathways leading to the formation of tryptophan and geraniol have been cloned (Figure 3).

Jasmonate signalling in TIA biosynthesis in *Catharanthus roseus*

All TIA biosynthesis genes tested are induced by MeJA in *Catharanthus* suspension-cultured cells (van der Fits and Memelink, 2000). In addition, MeJA induces genes in primary metabolism leading to the formation of TIA precursors. This presents a good example of the profound effect of JAs on plant metabolism at the level of gene expression.

The promoter of the *STR* gene has been studied in great detail to identify elicitor- and jasmonate-responsive sequences. A region close to the TATA box called jasmonate- and elicitor-responsive element (JERE) was found to dictate elicitor- and jasmonate-responsive reporter gene activation (Menke et al., 1999a). The JERE interacts with two transcription factors called octadecanoid-responsive *Catharanthus* AP2-domain proteins (ORCAs). ORCA2 was isolated by yeast one-hybrid screening of a *Catharanthus* cDNA library with the JERE as bait (Menke et al., 1999a) and ORCA3 was isolated by a genetic T-DNA activation tagging approach (van der Fits and Memelink, 2000). Both belong to the ERF family of transcription factors, which are unique to plants and are characterized by the APETALA2/Ethylene Response Factor (AP2/ERF) DNA-binding domain. In transient assays both ORCA proteins transactivate *STR* promoter activity via specific binding to the JERE. Overexpression of ORCA3 (van der Fits and Memelink 2000) or ORCA2 (unpublished results) in stably transformed *Catharanthus* cells leads to elevated expression levels of *TDC*, *STR* and several other TIA biosynthesis genes.

Importantly, the expression of the *ORCA2* and *ORCA3* genes themselves is induced by JAs (Menke et al., 1999a; van der Fits and Memelink, 2000; van der Fits and Memelink, 2001). This suggests that JAs induce alkaloid metabolism by increasing the amount of the ORCA regulatory proteins. To study how the *ORCA3* gene is regulated, its promoter was used in loss- and gain-of- function experiments to identify a 74 bp D region containing a jasmonate-responsive element (JRE; Vom Endt et al., 2007). The JRE is composed of two important sequences, a quantitative sequence responsible for a high level of expression, and a qualitative sequence that acts as an on/off switch in response to MeJA. Using the JRE in yeast one-hybrid screening of

Catharanthus cDNA libraries, several proteins belonging to the AT-hook class of DNA-binding proteins were isolated, which were found to interact specifically with the quantitative sequence within the JRE (Vom Endt et al., 2007). No proteins interacting with the qualitative sequence were found in this screening.

T-DNA activation tagging to dissect regulatory mechanisms in alkaloid metabolism

One of the most direct ways of dissecting complex biological processes in plants is generation and analysis of genetic mutants. Mutations resulting from T-DNA or transposon tagging or chemical mutagenesis usually cause loss of function, and are therefore recessive. Consequently, the mutant phenotype can only be visualised following selfing of the mutated plants. This demands a substantial amount of effort, and is not possible for all plant species. Another drawback of classical mutagenesis is that mutation of functionally redundant genes does not lead to phenotypically altered plants.

Many of these disadvantages are circumvented by an alternative approach to generate mutants, called T-DNA activation tagging. A T-DNA carrying a promoter reading towards its border is introduced into plant cells via *Agrobacterium*-mediated transformation. Upon random T-DNA integration into the genome, flanking plant sequences can be transcribed, which can result in a dominant mutation. Therefore, in contrast to classical mutagenesis, mutants generated by T-DNA activation tagging allow direct selection for the desired phenotype in the primary transformants. Furthermore, a phenotype can result from T-DNA activation tagging of a functionally redundant gene, allowing its analysis and cloning.

The T-DNA activation tagging approach was successful in isolating ORCA3, a regulator of TIA biosynthesis genes in *C. roseus* (van der Fits and Memelink, 2000). The gene encoding the TIA biosynthesis enzyme TDC was used as marker for mutant selection (Goddijn et al., 1993). TDC converts L-tryptophan into tryptamine, one of the first steps in TIA biosynthesis. TDC can use certain L-tryptophan derivatives as a substrate, such as 4-methyl-tryptophan (4-mT) (Sasse et al., 1983). This compound is toxic for plant cells,

and is converted by TDC into the non-toxic 4-methyl-tryptamine. *C. roseus* suspension-cultured cells were transformed with a T-DNA construct carrying enhancer elements from the CaMV 35S promoter located near the right border, and subsequently selected on 4-mT (Figure 4). Resistant cell lines were further screened for high *TDC* and *STR* expression by Northern blot analysis. From one hundred and eighty independent T-DNA-tagged 4-mT-resistant cell lines, twenty showed increased *TDC* expression (van der Fits et al., 2001). In three of them (lines 46, A42 and V-8) *STR* expression was also significantly increased, whereas in another three lines a slight elevation of *STR* expression was observed. Lines 46, A42 and V-8 had single T-DNA insertions according to Southern blot analysis. Isolation of flanking plant sequences via plasmid rescue was successful with lines 46 and A42. In line 46, the *ORCA3* gene was T-DNA tagged (van der Fits and Memelink, 2000).

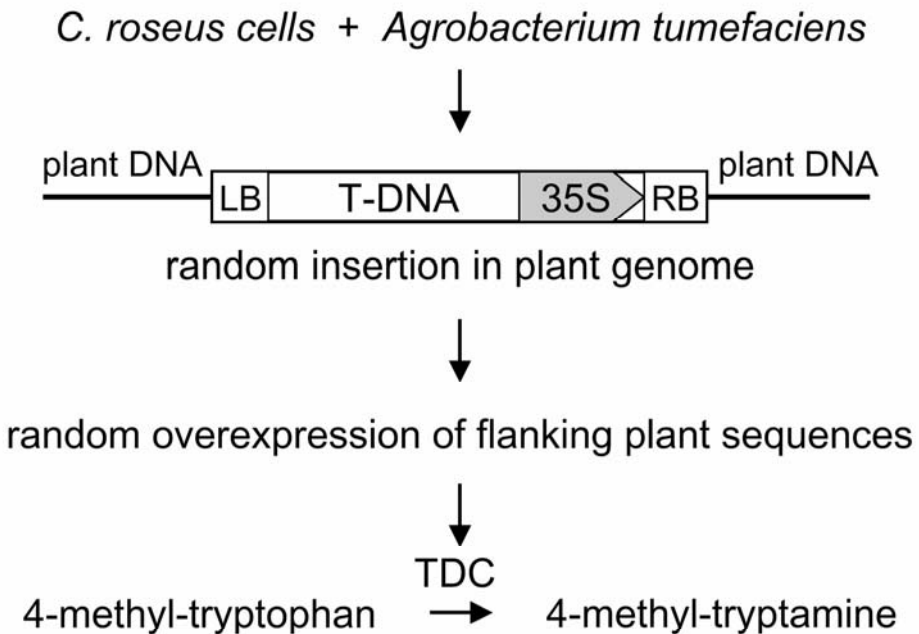


Figure 4. Schematic representation of the T-DNA tagging approach to identify regulators of TIA biosynthetic genes in *C. roseus*. For explanation, see the text. LB, left border, RB, right border; 35S, CaMV 35S promoter; TDC, tryptophan decarboxylase.

Outline of this thesis

The original aim of the thesis project was to functionally characterize the gene that was tagged in T-DNA activation tagged *C. roseus* line A42, based on the hypothesis that this gene could encode an important regulator of TIA biosynthesis.

Chapter 2 describes experiments to determine the function of the so-called *CrA42* gene. The first step in analysis of T-DNA-tagged genes is to recapitulate the phenotype of the original T-DNA tagged line by overexpression of the candidate gene. Overexpression of *CrA42* did not reproducibly result in enhanced *TDC* and *STR* mRNA levels. Therefore it was decided to abort this research project.

Instead studies were performed on other putative regulators of TIA biosynthesis that were closely linked to *ORCA3*. **Chapter 3** describes the function of the bHLH transcription factor *CrMYC2*. *CrMYC2* is the *Catharanthus* orthologue of *Arabidopsis AtMYC2*. It turned out that *CrMYC2* controls the MeJA-responsive expression of the *ORCA2* and *ORCA3* genes, and that it interacts with the qualitative sequence in the JRE from the *ORCA3* promoter.

Chapter 4 describes functional studies of two JAZ proteins from *C. roseus*. It was found that the JAZ proteins repress *CrMYC2* activity and thereby the expression of the *ORCA* genes and of the *TDC* and *STR* genes. In response to jasmonate, *CrJAZ1* was shown to be rapidly degraded via the 26S proteasome.

In **Chapter 5**, a general discussion of the results from Chapters 3 and 4 is presented, and a unifying model for the regulation of jasmonate-responsive alkaloid biosynthesis in tobacco and *C. roseus* is presented.

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