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Jasmonate-responsive transcriptional regulation in *Catharanthus roseus*

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献给我的父母妻子和女儿

To my parents, wife and daughter

Contents

Chapter 1

Introduction

Hongtao Zhang and Johan Memelink

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 tendril 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-insensitive*3 (*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 SlCOI1 and SlJAZ1, 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.

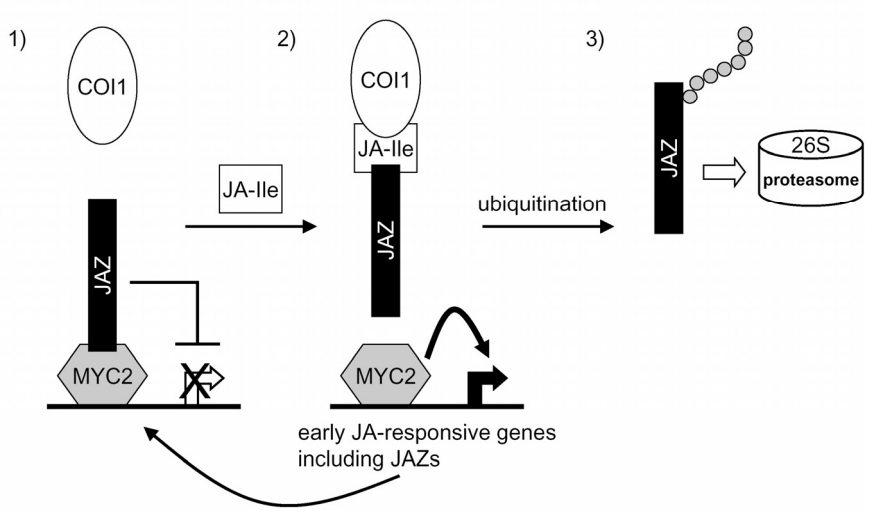

Figure 1. Model for jasmonate signal transduction leading to expression of AtMYC2-regulated genes.

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 *NtJAZ*1-3 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.

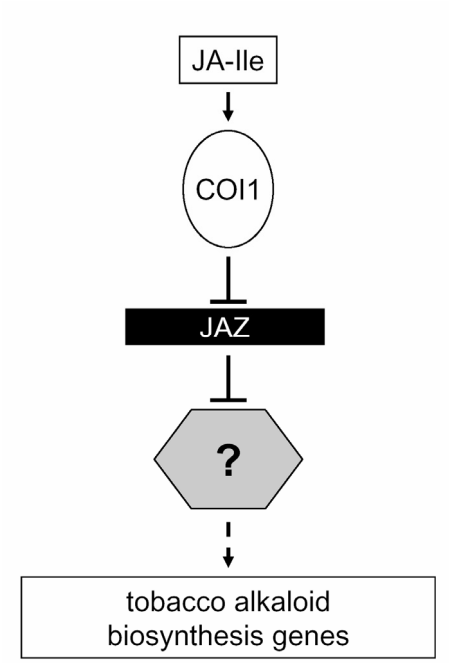

Figure 2. Model for jasmonate signal transduction leading to the expression of tobacco alkaloid biosynthesis genes.
As depicted in Figure 1, JA-Ile forms the molecular glue between NtCOI1 and NtJAZ, leading to degradation of the latter proteins. The unidentified transcription factor repressed by the 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 3α(S)-strictosidine. Strictosidine is deglucosylated 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 desacetoxyvindoline-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).

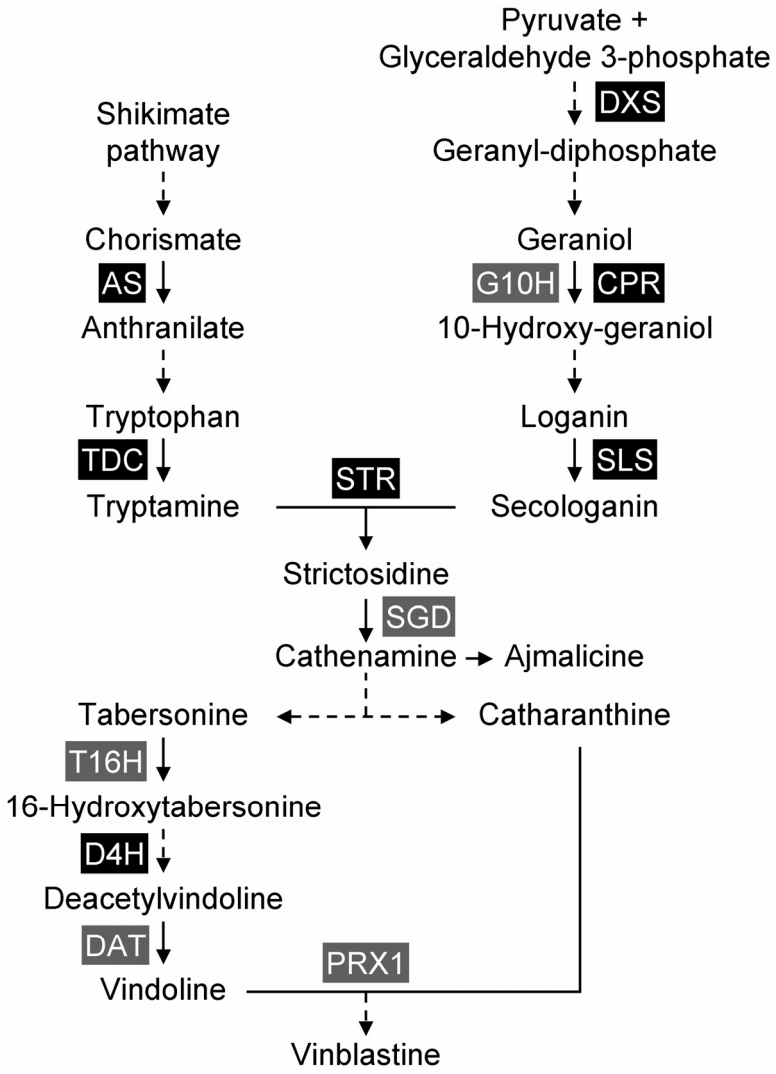

Figure 3. Biosynthetic pathway for terpenoid indole alkaloids in *Catharanthus roseus*.
Solid arrows indicate single enzymatic conversions, whereas dashed arrows indicate multiple enzymatic conversions. Indicated are enzymes, for which the corresponding genes were cloned. Enzymes encoded by genes regulated by ORCA3 are shown against a black background. AS: anthranilate synthase, CPR: cytochrome P450 reductase, D4H: desacetoxyvindoline 4-hydroxylase, DAT: acetyl-CoA:4-O-deacetylvindoline 4-O-acetyltransferase, DXS: D-1-deoxyxylulose 5-phosphate synthase, G10H: geraniol 10-hydroxylase, PRX1: peroxidase 1, SGD: strictosidine β-D-glucosidase, SLS: secologanin synthase, STR: strictosidine synthase TDC: tryptophan decarboxylase, T16H: tabersonine 16-hydroxylase.

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).

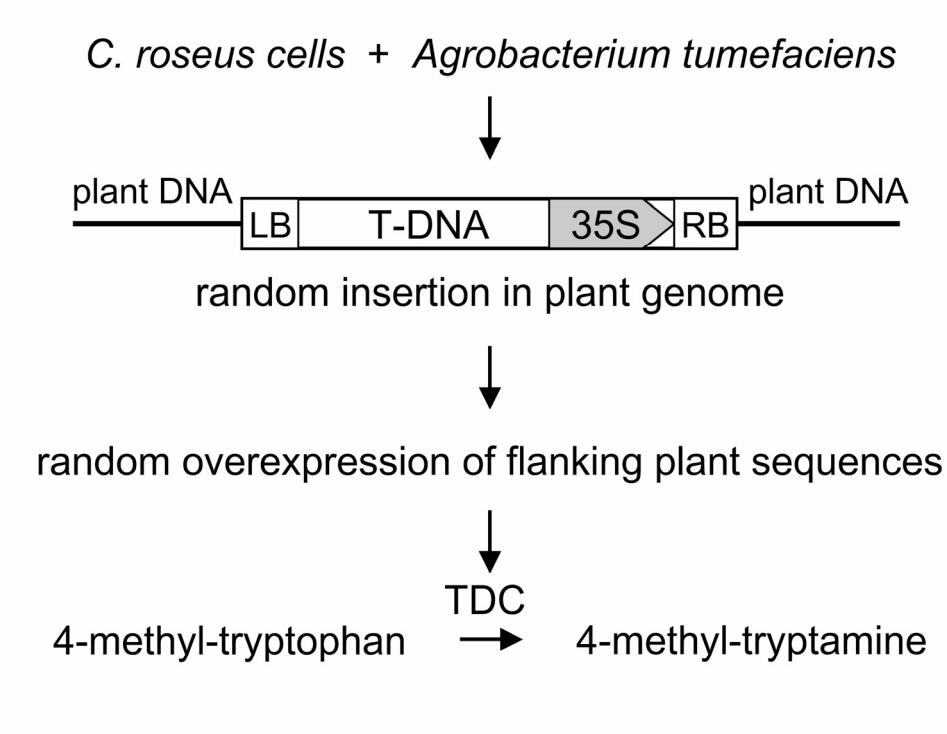

Figure 4. Schematic representation of the T-DNA tagging approach to identify regulators of TIA biosynthetic genes in *C. roseus*. For explanation, see the text. LB, left border, RB, right border; 35S, CaMV 35S promoter; TDC, tryptophan decarboxylase.

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.

References

Aerts RJ, Snoeijer W, Aerts-Teerlink O, van der Meijden E, Verpoorte R (1991) Control and biological implications of alkaloid synthesis in *Cinchona* seedlings. Phytochemistry 30: 3571-3577

Aerts RJ, Stoker A, Beishuizen M, van de Heuvel M, van der Meijden E, Verpoorte R (1992) Detrimental effects of Cinchona leaf alkaloids on larvae of the polyphagous insect *Spodoptera exigua.* J Chem Ecol 18: 1955-1964.

Austin MJ, Muskett P, Kahn K, Feys BJ, Jones JDG, Parker JE (2002) Regulatory role of SGT1 in early R gene-mediated plant defenses. Science 295: 2077-2080

Azevedo C, Sadanandom A, Kitagawa K, Freialdenhoven A, Shirasu K, Schulze-Lefert P (2002) The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. Science 295: 2073-2076

Balbi V and Devoto A (2008) Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. New Phytol 177: 301-318

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448: 666-671

Contin A, van der Heijden R, Lefeber AWM, Verpoorte R (1998) The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in *Catharanthus roseus* cell culture. FEBS Lett 434: 413-416

del Pozo JC and Estelle M (2000) F-box proteins and protein degradation: an emerging theme in cellular regulation. Plant Mol Biol 44:123-128

Devoto A, Nieto-Rostro M, Xie D, Ellis C, Harmston R, Patrick E, Davis J, Sherratt L, Coleman M, Turner JG (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. Plant J 32: 457-466

Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. Nature 435: 441-445

Facchini PJ (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. Ann Rev Plant Physiol Plant Mol Biol 52: 29-66

Feys BF, Benedetti CE, Penfold CN, Turner JG (1994) Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell 6: 751-759

Gagne JM, Downes BP, Shiu SH, Durski AM, Vierstra RD (2002) The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. Proc Natl Acad Sci USA 99: 11519-11524

Goddijn OJM, van der Duyn-Schouten PM, Schilperoort RA, Hoge JHC (1993) A chimaeric tryptophan decarboxylase gene as a novel selectable marker in plants. Plant Mol Biol 22: 907-912

Goossens A, Häkkinen ST, Laakso I, Seppänen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila AM, Söderlund H, Zabeau M, Inzé D, Oksman-Caldentey KM (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl Acad Sci USA 100: 8595-8600

Gray WM, Muskett PR, Chuang HW, Parker JE (2003) Arabidopsis SGT1b is required for SCF^{TIR1}-mediated auxin response. Plant Cell 15: 1310-1319

Grotewold E (2006) The genetics and biochemistry of floral pigments. Annu Rev Plant Biol 57: 761-780

Guilfoyle T (2007) Sticking with auxin. Nature 446: 621-622

Halitschke R, Baldwin IT (2003) Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata*. Plant J 36: 794-807

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol 57: 303-333

Jones JD and Dangl JL (2006) The plant immune system. Nature 444: 323-329

Katoh A, Ohki H, Inai K, Hashimoto T (2005) Molecular regulation of nicotine biosynthesis. Plant Biotechnology 22: 389-392

Katsir L, Chung HS, Koo AJ, Howe GA (2008a) Jasmonate signaling: a conserved mechanism of hormone sensing. Curr Opin Plant Biol 11: 428-435

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008b) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci USA105: 7100-7105

Kepinski S and Leyser O (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435: 446-451

Kunkel BN and Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Curr Opin Plant Biol 5: 325-331

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA (2004) The tomato homolog of *CORONATINE-INSENSITIVE1* is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell 16: 126-143

Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell 16: 1938-1950

Lorenzo O and Solano R (2005) Molecular players regulating the jasmonate signalling network. Curr Opin Plant Biol 8:532-540

Luijendijk TJC, van der Meijden E, Verpoorte R (1996) Involvement of strictosidine as a defensive chemical in *Catharanthus roseus*. J Chem Ecol 22: 1355-1366

Mackey D and McFall AJ (2006) MAMPs and MIMPs: proposed classifications for inducers of innate immunity. Mol Microbiol 61:1365-1371

Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ, Choi YD, Choi G, Browse J (2006) Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. Plant J 46: 984-1008

Melotto M, Mecey C, Niu Y, Chung HS, Katsir L, Yao J, Zeng W, Thines B, Staswick P, Browse J, Howe G, He SY (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interaction with the COI1 F-box protein. Plant J 55: 979-88

Memelink J, Verpoorte R, Kijne JW (2001) ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 6: 212-219

Menke FLH, Champion A, Kijne JW, Memelink J (1999a) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18: 4455-4463

Menke FLH, Parchmann S, Mueller MJ, Kijne JW, Memelink J (1999b) Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. Plant Physiol 119: 1289-1296

Paschold A, Halitschke R, Baldwin IT (2007) Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. Plant J 51: 79-91

Sasse F, Buchholz M, Berlin J (1983) Site of action of growth inhibitory tryptophan analogues in *Catharanthus roseus* cell suspension cultures. Z Naturforsch 38c: 910-915

Shah J (2003) The salicylic acid loop in plant defense. Curr Opin Plant Biol 6: 365-371

Shoji T, Ogawa T, Hashimoto T (2008) Jasmonate-induced nicotine formation in tobacco is mediated by tobacco *COI1* and *JAZ* genes. Plant Cell Physiol 49: 1003-1012

Shoji T, Yamada Y, Hashimoto T (2000) Jasmonate induction of putrescine N-methyltransferase genes in the root of *Nicotiana sylvestris*. Plant Cell Physiol 41: 831-839

Sottomayor M, Lopes Cardoso I, Pereira LG, Ros Barceló A (2004) Peroxidase and the biosynthesis of terpenoid indole alkaloids in the medicinal plant *Catharanthus roseus* (L.) G. Don. Phytochem Rev 3: 159-171

Staswick PE, Su W, Howell SH (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA 89: 6837-6840

Staswick PE and Tiryaki I (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell 16: 2117-2127

Stenzel I, Hause B, Miersch O, Kurz T, Maucher H, Weichert H, Ziegler J, Feussner I, Wasternack C (2003) Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. Plant Mol Biol 51: 895-911

Tan X, Calderon-Villalobos LI, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446: 640-645

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature 448: 661-665

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell 14 Suppl: S153-S164

Vanholme B, Grunewald W, Bateman A, Kohchi T, Gheysen G (2007) The tify family previously known as ZIM. Trends Plant Sci12: 239-244

van der Fits L, Hilliou F, Memelink J (2001) T-DNA activation tagging as a tool to isolate regulators of a metabolic pathway from a genetically non-tractable plant species. Transgenic Res 10: 513-521

van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295-297

van der Fits L, Memelink J (2001) The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J 25: 43-53

Vom Endt D, Soares e Silva M, Kijne JW, Pasquali G, Memelink J (2007) Identification of a bipartite jasmonate-responsive promoter element in the *Catharanthus roseus ORCA3* transcription factor gene that interacts specifically with AT-hook DNA-binding proteins. Plant Physiol 144: 1680-1689

Wang L, Allmann S, Wu J, Baldwin IT (2008) Comparisons of LIPOXYGENASE3- and JASMONATE-RESISTANT4/6-silenced plants reveal that jasmonic acid and jasmonic acid-amino acid conjugates play different roles in herbivore resistance of *Nicotiana attenuata*. Plant Physiol 146: 904-915

Wang KL, Li H, Ecker JR (2002). Ethylene biosynthesis and signaling networks. Plant Cell 14 suppl: S131-S151

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot 100: 681-697

Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) *COI1*: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280: 1091-1094

Xu L, Liu F, Lechner E, Genschik P, Crosby WL, Ma H, Peng W, Huang D, Xie D (2002) The SCF^{COI1} ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell 14: 1919-1935

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283-333

Chapter 2

Characterization of a novel putative regulator of plant secondary metabolism

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Abstract

Plant secondary metabolites are crucial among others as protectants against various forms of stress, including microbial pathogens, herbivores, and UV light. Jasmonates are stress signalling hormones, which play important roles in induction of secondary metabolite biosynthesis genes via largely unknown mechanisms. Using a T-DNA activation tagging approach with *Catharanthus roseus* cells, we isolated part of a novel gene called *CrA42*. The phenotype of the T-DNA tagged cell line A42 was that it displayed high mRNA levels of the genes encoding the terpenoid indole alkaloid (TIA) biosynthetic enzymes tryptophan decarboxylase (*TDC*) and strictosidine synthase (*STR*). Therefore, *CrA42* potentially encodes a novel regulator of alkaloid biosynthesis genes. This chapter describes the results of experiments aimed at establishing whether CrA42 is indeed a regulator of TIA biosynthesis genes. 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. In addition, other experiments investigating its subcellular localization, its gene activation potential and its gene expression response to jasmonates and fungal elicitors did not point to a role for CrA42 as a regulator of TIA biosynthesis genes. Therefore it is unclear whether CrA42 protein plays a role in expression of alkaloid biosynthesis genes, and if so, what its function could be.

Introduction

Plants distinguish themselves from most other organisms by their ability to form a repertoire of secondary metabolites. Collectively, these low molecular weight compounds from the plant kingdom show an enormous chemical diversity. Many of these compounds find industrial uses as pharmaceuticals, dyes, food additives, etc. For the plant itself, secondary metabolites play crucial roles in multiple aspects of the plant life cycle. One of the important functions is

protection against stress, including microbial pathogens, herbivores and UV light. Secondary metabolism is highly regulated, occurring only at specific stages of plant growth and development, and in response to specific environmental stimuli. How these different levels of regulation work at the molecular level is largely unknown.

Alkaloids are a prominent class of secondary metabolites. Biosynthesis of terpenoid indole alkaloids (TIAs) in the plant species *Catharanthus roseus* (Madagascar periwinkle) is induced by fungal elicitors and by the plant stress hormone jasmonate (JA, Memelink et al., 2001). This regulation occurs largely, if not completely, at the level of gene expression. Several biosynthetic genes were shown to be induced by fungal elicitors and methyljasmonate (Pasquali et al., 1992; Menke et al., 1999b, van der Fits and Memelink; Pauw et al., 2004; Memelink et al., 2001). JA serves as a second messenger for the elicitor signal transduction pathway (Menke et al., 1999b). Elicitor first induces JA biosynthesis, and JA then induces TIA biosynthetic gene expression. For elicitor-induced JA biosynthesis, protein phosphorylation and Ca^{2+} influx are important intermediate events (Menke et al., 1999b; Memelink et al., 2001). Upstream of JA, 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., 2004b; Pauw et al., unpublished results). Downstream of JA biosynthesis, protein phoshorylation is also an important step (Menke et al., 1999b). JA finally activates the ORCA transcription factors, which bind and transcriptionally activate the promoters of the *TDC* and *STR* genes (Menke et al., 1999a; van der Fits and Memelink, 2000; 2001).

At the start of this project, it was unknown how JA is perceived by plant cells and which signal transduction steps lead to transcription factor activation. The only Arabidopsis gene isolated at that moment involved in JA signal transduction based on the mutant phenotype, encoded the F-box protein COI1 (Xie et al., 1998). Based on the general function of F-box proteins and the mutant phenotype it was assumed that COI1 was involved in degradation of a repressor of JA responses.

T-DNA activation tagging is a method to generate dominant mutations in plants or plant cells by the insertion of a T-DNA carrying constitutive enhancer elements that can cause transcriptional activation of flanking plant genes. Tagged genes can then be isolated via plasmid rescue. We applied this approach to *C. roseus*, in an attempt to isolate regulators of genes that are involved in TIA biosynthesis (van der Fits and Memelink, 2000, van der Fits et al., 2001). The use of suspension-cultured cells enabled us to perform a nearly saturated screen of T-DNA-tagged cells for resistance to a toxic substrate of one of the TIA biosynthetic enzymes, tryptophan decarboxylase (TDC). Further screening of an estimated amount of 500,000 stable tagging events resulted in the isolation of three tagged cell lines with high expression levels of the *TDC* gene and the strictosidine synthase gene (*STR*). Plasmid rescue from one of the tagged cell lines led to the isolation of *ORCA3*, a gene encoding a JA-responsive AP2/ERF-domain transcription factor that regulates several TIA biosynthesis genes (van der Fits and Memelink, 2000), illustrating the usefulness and validity of this approach to isolate regulators of alkaloid biosynthesis genes.

Another one of the three selected tagged cell lines, called A42, also showed high *TDC* and *STR* expression levels. Southern blotting indicated that a single T-DNA copy was integrated (van der Fits et al., 2001). Plasmid rescue with XbaI resulted in the isolation of 4.8 kb of flanking plant DNA. Sequence comparisons with Arabidopsis genomic and EST databases showed that on this rescued DNA part of a gene is located that encodes a protein with similarity to a predicted Arabidopsis protein of unknown function.

The aim of the experiments described in this chapter 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.

Results

Isolation of a complete CrA42 genomic clone and cDNA

The genomic fragment rescued from T-DNA tagged cell line A42 had a size of 4.8 kb (van der Fits et al., 2001). Initially, no open reading frame or homology with sequences in public databases could be found. However, after release of the complete genomic sequence of Arabidopsis (The Arabidopsis Genome Initiative, 2000), homology was found with the gene with the AGI code At5g42620. This is a unique gene which is annotated as coding for a zinc ion binding metalloendopeptidase with homology with the major surface glycoprotein leishmanolysin from various species of Leishmania and which contains a domain conserved in the pfam01457 superfamily of peptidase M8 and leishmanolysin-like proteins. Alignment of the *C. roseus* and Arabidopsis sequences with TBLASTX showed that both genes have identical intron-exon structures with multiple introns, explaining why it was not possible in first instance to find an open reading frame. The partial *C. roseus* A42 protein and the corresponding part of the protein encoded by Arabidopsis At5g42620 are highly similar with 85 % amino acid identity over a length of 550 amino acids. Assuming that the genes in *C. roseus* and Arabidopsis have similar lengths, the rescued fragment was predicted to contain about half of the gene.

To isolate the complete gene a genomic library (Goddijn et al., 1994) was screened with a XhoI/EcoRI fragment derived from the rescued genomic DNA (Fig. 1). A single positive plaque was purified in two more screening rounds and the corresponding lambda DNA was isolated and characterized by restriction enzyme mapping and hybridization with the XhoI/EcoRI probe. This analysis showed that the lambda clone contained an insert of around 13 kb which probably carried the complete gene (Fig. 1). Restriction fragments were subcloned to sequence 10.9 kb of the genomic clone up to a BamHI site predicted to be located 3' of the end of the gene. Comparison of the obtained sequence with the Arabidopsis At5g42620 gene using TBLASTX showed that it was very likely that the gene was completely contained within the sequenced part. The TBLASTX analysis showed that *CrA42* gene consisted of 17 exons with consensus intron-exon borders. Twelve introns were quite small, which is a common property of plant introns, with lengths around 90 bp. The encoded

protein has a length of 841 amino acids with 79% identity to the Arabidopsis protein which has a length of 831 amino acids.

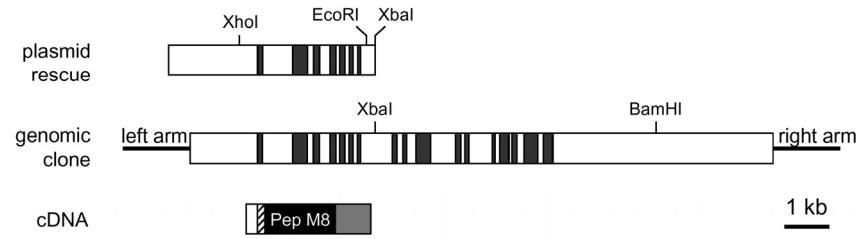

Figure 1. Schematic representation of *CrA42* DNA clones.
At the top is the DNA fragment rescued from T-DNA tagged *C. roseus* cell line A42 with XbaI. The left border flanked the T-DNA activation construct. A XhoI/EcoRI subfragment was used to screen a genomic library. The orientation of the genomic clone relative to the lambda arms is indicated. The intron-exon structure of the *CrA42* gene is shown with exons in grey. In the structure of the cDNA the positions and sizes of the 5' untranslated region, the N-terminal predicted transmembrane domain and the peptidase family M8 domain are indicated by white, hatched and black boxes respectively. The genomic fragment used for recapitulation of the phenotype of cell line A42 extended from the start codon to the BamHI site. The restriction sites are shown because of their roles in the experimental procedures and are not unique in the genomic fragment.

Attempts to recapitulate the phenotype of line A42 by overexpressing CrA42

As a first step we wanted to verify that overexpression of the *CrA42* gene was the cause for the phenotype of line A42, which was elevated *TDC* and *STR* expression compared to other lines (Fig. 2). We fused a genomic fragment extending from the predicted ATG start codon to a BamHI site predicted to be located 3' from the end of the gene (Fig. 1) to the CaMV 35S promoter and introduced the construct in cell line MP183L by particle bombardment. Control cell lines were generated by bombardment with the empty vector. Independent lines were then analyzed for gene expression.

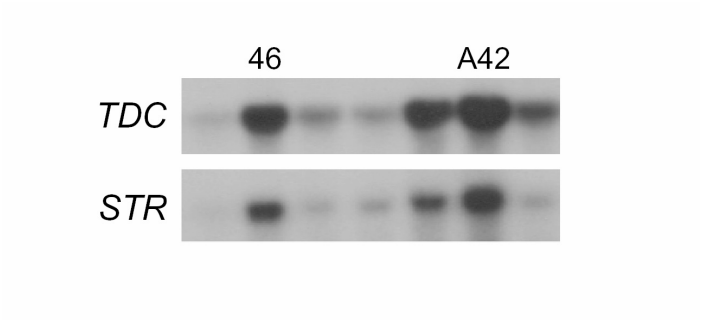

Figure 2. Expression analysis of independent T-DNA tagged *C. roseus* BIX lines. Northern blots containing identical amounts of total RNA from each line were hybridized with *TDC* or *STR* cDNA probes. Only relevant line numbers are indicated. In line 46 the *ORCA3* gene was T-DNA tagged (van der Fits and Memelink, 2000).

This first recapitulation experiment looked very promising. Out of a total of 19 independent cell lines five showed elevated *CrA42* mRNA levels, four of which showed also elevated *TDC* and *STR* mRNA levels (Fig. 3A). Among 25 independent transgenic control cell lines none showed elevated *CrA42*, *TDC* or *STR* expression (data not shown). Analysis of the expression of several other genes showed slightly elevated levels of the gene encoding secologanin synthase (SLS), a cytochrome P450 enzyme catalyzing the last step in the biosynthesis of the terpenoid precursor of the TIAs (Fig. 3B). Another gene encoding geraniol 10-hydroxylase (G10H), a cytochrome P450 enzyme which catalyzes the first committed step in the biosynthesis of the terpenoid precursor, was not affected. The expression of the *ORCA* genes was not affected, suggesting that CrA42 worked independently of the ORCAs. These results strongly suggested that *CrA42* overexpression led to elevated expression of the TIA biosynthesis genes *TDC*, *STR* and possibly *SLS*, which encouraged us to continue with this project.

As a next step we isolated a cDNA clone corresponding to the *CrA42* gene to confirm the intron-exon structure and to be able to perform other experiments including production of recombinant protein and construction of GFP fusions. Analysis of the cDNA sequence showed that the intron-exon structure of the *CrA42* gene was correctly predicted based on the comparison with the

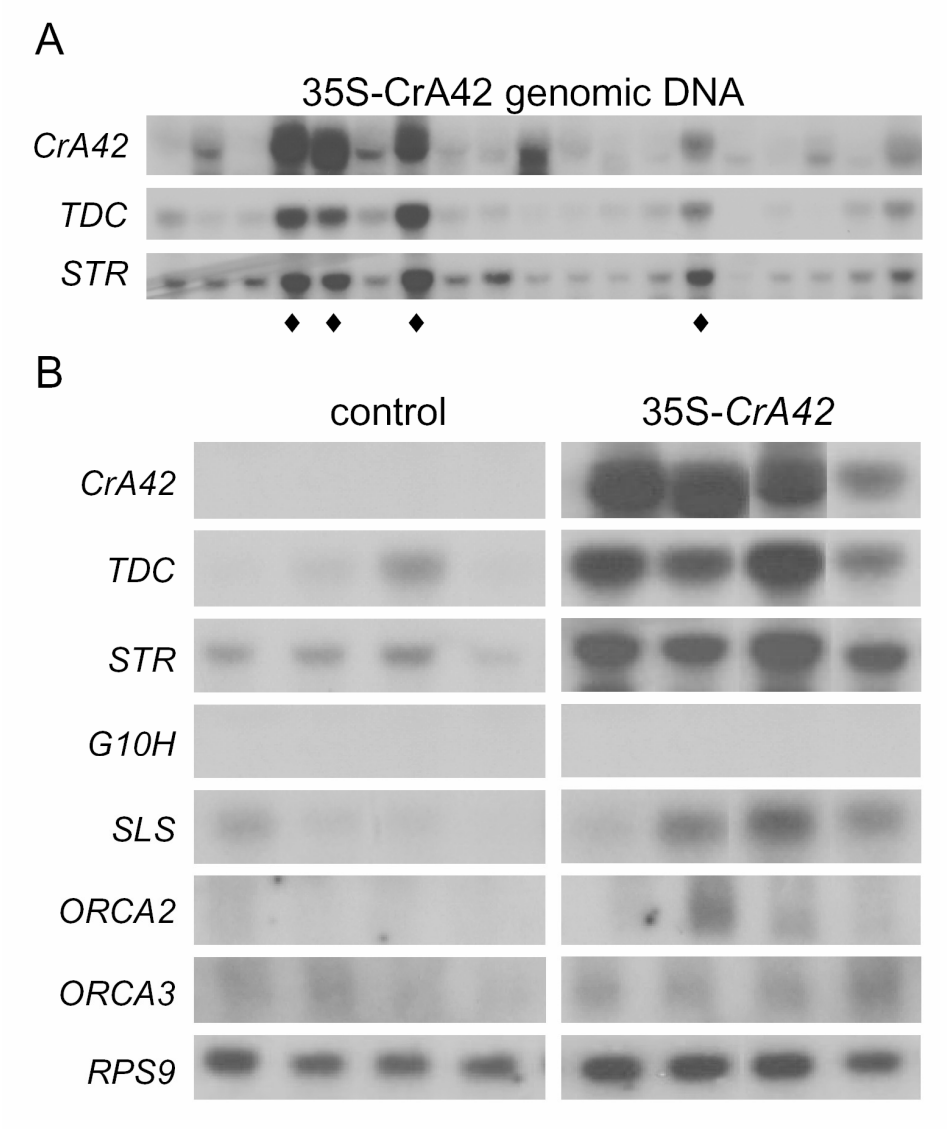

Figure 3. Gene expression analysis in *C. roseus* MP183L lines transformed with a genomic *CrA42* overexpression construct.

A. Northern blots containing identical amounts of total RNA from independent lines were hybridized with the indicated probes. The diamonds mark four lines with elevated mRNA levels for all three genes. B. Gene expression analysis of the four lines marked in Fig. 3A. mRNA levels were compared with those in four randomly selected transgenic control lines. See text for explanation of gene abbreviations.

Arabidopsis At5g42620 gene. In addition the ATG start codon turned out to be correctly predicted.We generated transgenic MP183L cell lines carrying the *CrA42* cDNA fused to the CaMV 35S promoter and in parallel control cell lines transformed with the empty vector. Gene expression analysis showed that *TDC* and *STR* gene expression levels did not differ from control cell lines and if anything were lower (Fig. 4). In addition, *TDC* and *STR* mRNA levels did no show any correlation with expression levels of the *CrA42* transgene. In a similar experimental set-up, estradiol-inducible expression of *CrA42* in a cell line transformed with a construct carrying the cDNA in the XVE expression module (Zuo et al., 2000) did not affect *STR* mRNA levels at 24 or 48 hours of treatment with 10 or 50 µM estradiol (results not shown).

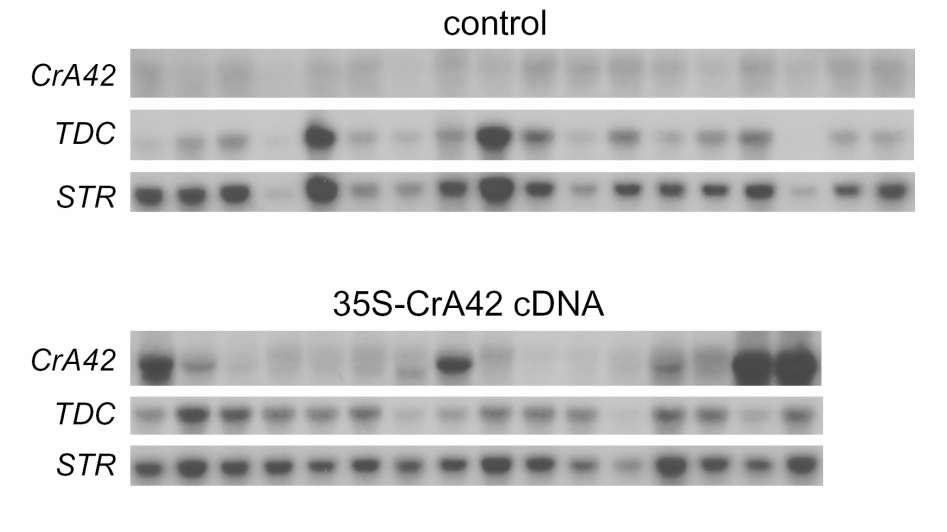

Figure 4. Gene expression analysis in *C. roseus* MP183L lines transformed with a *CrA42* cDNA overexpression construct.

Northern blots containing identical amounts of total RNA from independent control lines or lines transformed with the *CrA42* cDNA overexpression construct were hybridized with the indicated probes.

These negative results were unexpected and disappointing. A possible explanation was that overexpression of the *CrA42* genomic clone did not stimulate *TDC* and *STR* expression levels due to the action of the encoded protein, but instead worked at the RNA level for instance by serving as the precursor for an miRNA silencing a repressor. Therefore we decided to repeat

the recapitulation experiment with the genomic construct. As shown in Fig. 5, this experiment gave similar results as the cDNA recapitulation experiment. Cell lines transformed with the genomic construct did not show higher *TDC* and *STR* mRNA levels than control lines, and there was no correlation between the *CrA42* and *TDC* and *STR* mRNA levels.

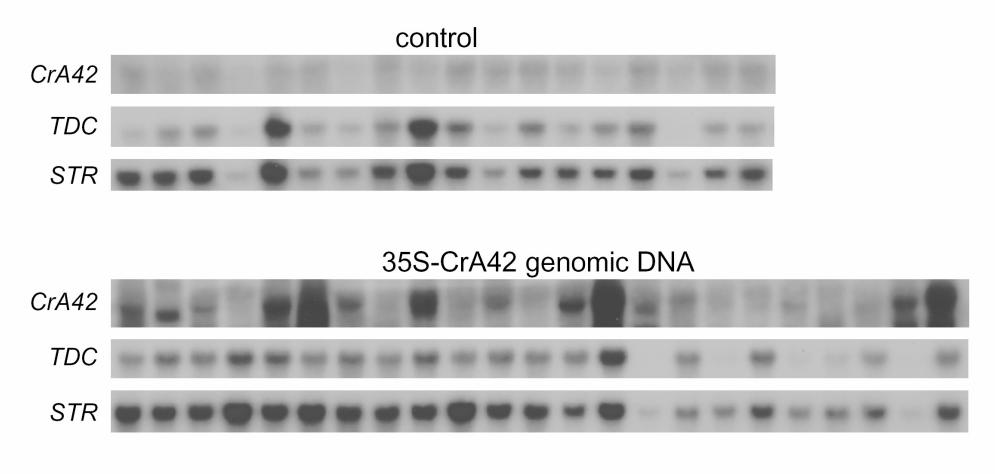

Figure 5. Gene expression analysis in *C. roseus* MP183L lines transformed with a genomic *CrA42* overexpression construct.
The results shown are from a repetition of the experiment shown in Fig. 3. Northern blots containing identical amounts of total RNA from independent control lines or lines transformed with the *CrA42* genomic overexpression construct were hybridized with the indicated probes.

CrA42 expression is not induced by MeJA or yeast extract

While the recapitulation experiments went on, several other experimental approaches were taken to understand CrA42 function. We will discuss each of these experiments below.

Based on the hypothesis that CrA42 is involved in *TDC* and *STR* expression, and given that the expression of TIA biosynthesis genes including *TDC* and *STR* is induced by methyljasmonate (MeJA) and yeast extract (YE) (van der Fits and Memelink, 2000; Pauw et al., 2004), we wondered whether

the *CrA42* gene would respond to these signals. *CrA42* mRNA levels in cells treated with the solvent DMSO were very low and just barely above the detection limit. *CrA42* expression was not induced by MeJA or YE, and if anything, was slightly repressed (Fig. 6).

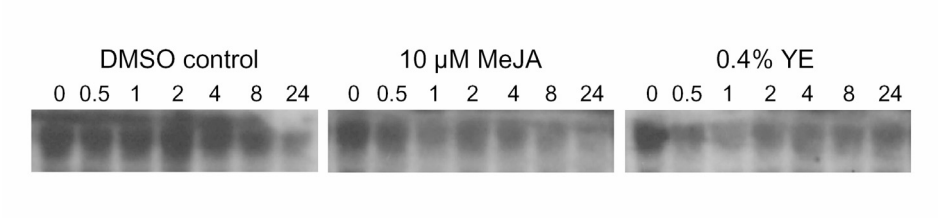

Figure 6. The *CrA42* gene is not induced by MeJA or yeast extract.
Cells from *C. roseus* cell line MP183L were treated with 0.1% (v/v) DMSO, 10 µM MeJA or 0.4% (w/v) yeast extract (YE) for the indicated number of hours. The Northern blot was hybridized with the *CrA42* cDNA.

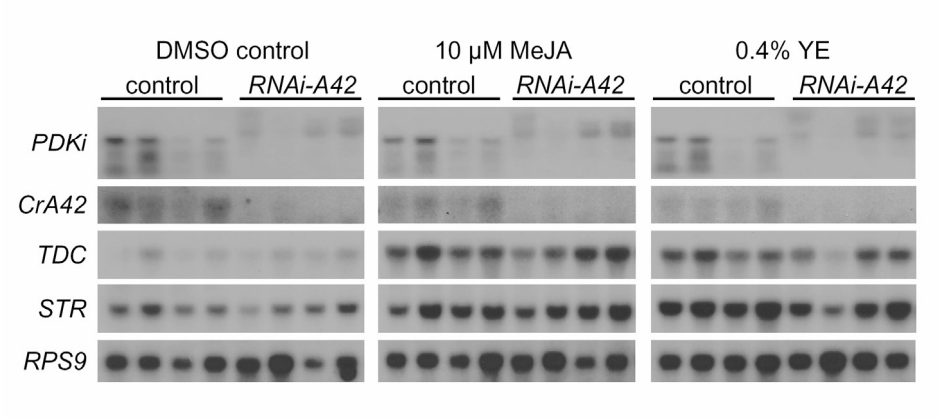

Figure 7. Silencing of *CrA42* does not affect MeJA- or YE-responsive *TDC* and *STR* expression.
Four independent control cell lines and four independent cell lines transformed with an RNAi construct targeting *CrA42* mRNA were treated for 6 hours with 0.1% (v/v) DMSO, 10 µM MeJA or 0.4% (w/v) yeast extract (YE). Northern blots were hybridized with the indicated probes. *PDKi* is the intron from the *PDK* gene contained in the silencing vector (Wesley et al., 2001). *RPS9* encodes the ribosomal protein S9 and was used as a loading control.

Silencing CrA42 does not affect TDC and STR expression

Based on the first recapitulation experiment suggesting that overexpression of *CrA42* stimulated *TDC* and *STR* expression, we wondered whether silencing of *CrA42* gene expression by RNA interference (RNAi) would negatively affect *TDC* and *STR* expression. Independent cell lines transformed with either the empty vector pHannibal (Wesley *et al*., 2001) or pHannibal carrying an inverted repeat of a part of the *CrA42* cDNA separated by the *PDK* (pyruvate dehydrogenase kinase) intron were analysed for gene expression after treatment with 10 µM MeJA or 0.4% YE for 6 hours (Fig. 7). In the silencing lines *CrA42* mRNA was undetectable. Hybridization with the *PDK* intron showed that all lines expressed the intron sequence. No significant differences were observed in *TDC* and *STR* mRNA levels between control lines and the RNAi-*CrA42* lines. This indicated that CrA42 was not required for MeJA- or YE-responsive *TDC* and *STR* expression.

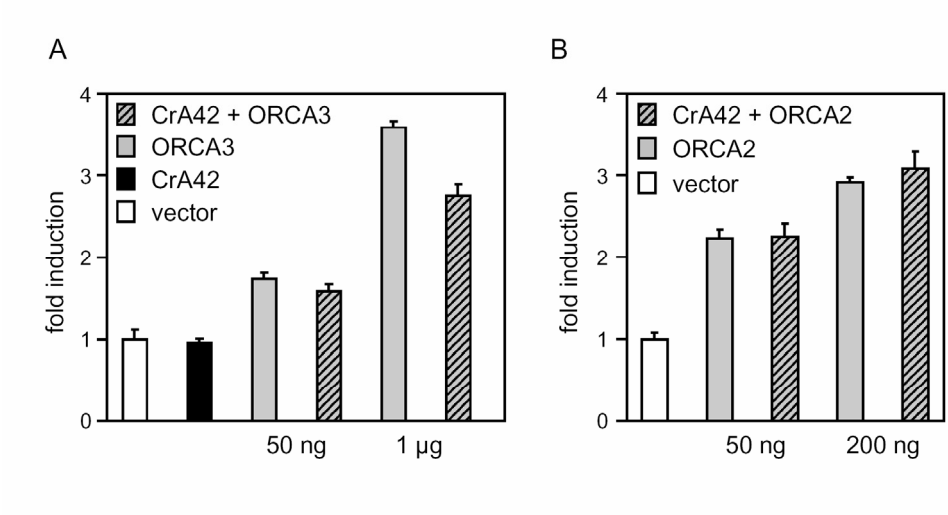

Figure 8. CrA42 does not trans-activate *STR* promoter activity alone or with the ORCAs. A. *C. roseus* MP183L cells were transiently co-transformed with an *STR-GUS* reporter construct and 6 µg of *CrA42* effector plasmid or the indicated amount of *ORCA3* effector plasmid alone or combined as indicated. B. *C. roseus* MP183L cells were transiently co-transformed with an *STR-GUS* reporter construct and the indicated amount of *ORCA2* effector plasmid alone or combined with 6 µg of *CrA42* effector plasmid. Total effector amount was adjusted to 6 µg in all transformations using empty vector plasmid pRT101. Bars represent means + SE (n = 3). GUS activities are shown as fold induction compared with the vector control.

CrA42 does not activate the STR promoter in transient assays

Based on the observation that *CrA42* overexpression increased *TDC* and *STR* mRNA levels, we asked the question whether CrA42 would activate the *STR* promoter in transient assays. In addition we investigated the possibility that CrA42 might amplify the activating effects of the ORCA transcription factors.

As shown in Fig. 8A, CrA42 did not trans-activate the *STR* promoter derivative XH (-531 to +52 relative to the transcription start site), whereas ORCA3 and ORCA2 (Fig. 8) did. CrA42 also did not work as a co-activator of the ORCA transcription factors even when the latter were expressed at suboptimal amounts (Fig. 8).

CrA42 is localized in speckles in the cytoplasm

The CrA42 protein has a predicted N-terminal transmembrane domain, and the rest of the protein is predicted to be localized at the cytoplasmic side of the membrane.

To establish the subcellular localization of CrA42, various derivatives were transiently expressed in *C. roseus* MP183L cells as GFP fusions. In contrast to GFP, which was localized both in the cytoplasm and in the nucleus, the CrA42-GFP fusion was excluded from the nucleus and only present in the cytoplasm in large speckles (Fig. 9).

The localization of CrA42 was not at the membrane as predicted. To directly test the activity of the N-terminal domain it was fused to GFP. The resulting protein had a subcellular distribution similar to GFP alone. An N-terminal deletion derivative of CrA42 lacking the predicted transmembrane domain was also expressed as a GFP fusion protein and was found to have a similar subcellular localization as the full-length protein. Searching for proteins with similar localization patterns, we found that the Arabidopsis protein encoded by gene At1g07310 with unknown function had a very similar subcellular distribution (supplemental results from Koroleva et al., 2005, at

http://data.jic.bbsrc.ac.uk/gfp/). The subcytoplasmic organelles labelled by GFP were not identified by these authors.

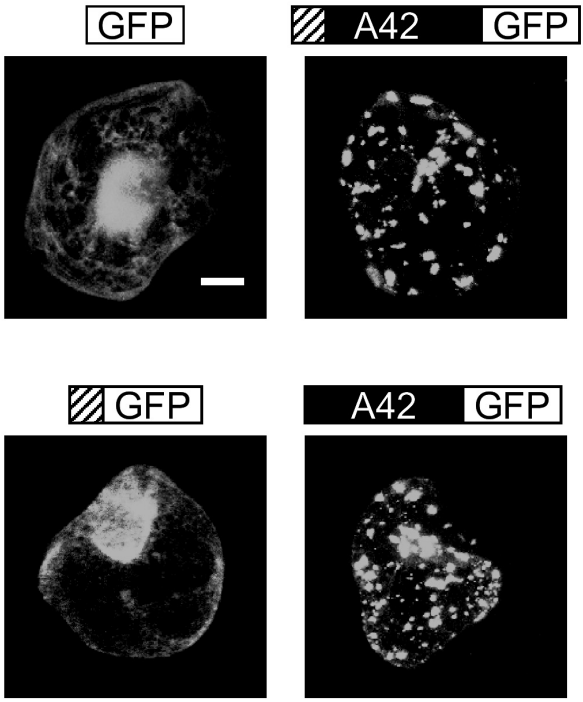

Figure 9. CrA42 protein is localized in cytoplasmic speckles in *C. roseus* cells.
Confocal laser scanning microscopy at 63x magnification of *C. roseus* MP183L cells transiently transformed with plasmids carrying *GFP* or *GFP* fusions with *CrA42* as indicated. The hatched box indicates the N-terminal predicted transmembrane domain. Images shown consist of stacked optical sections taken each 1 µm. The size bar in the upper left picture corresponds to 10 µm.

Discussion

Part of the *CrA42* gene was isolated by plasmid rescue from T-DNA tagged cell line A42 (van der Fits et al., 2001). A complete gene was isolated by screening a genomic library of *C. roseus*. The T-DNA turned out to be integrated 2118 bp upstream of the ATG start codon of the gene. This position indicated that the

phenotype of line A42 could be due to overexpression of the full-length CrA42 protein. The phenotype of line A42 was that it contained higher *TDC* and *STR* mRNA levels than other lines isolated during the screening procedure. *TDC* and *STR* mRNA levels were even slightly higher than in line 46, in which the AP2/ERF-domain transcription factor ORCA3 was T-DNA-tagged (van der Fits and Memelink, 2000). In line 46 the T-DNA tag was integrated around 600 bp upstream of the *ORCA3* gene. ORCA3 has been validated as an activator of *TDC* and *STR* expression both in stable recapitulation experiments (van der Fits and Memelink, 2000) as well as in transient promoter activation assays (van der Fits and Memelink, 2000; 2001).

As a first priority we wanted to verify that the phenotype of line 42 is caused by overexpression of the *CrA42* gene. We were unable to reliably establish that overexpression of *CrA42* leads to elevated *TDC* and *STR* mRNA levels. In a first experiment using a genomic clone there was a very good correlation between the *CrA42* mRNA levels and those of *TDC* and *STR*. However in two other experiments using either the genomic clone or a cDNA clone, lines transformed with *CrA42* constructs did not differ from controls and no correlation between *CrA42*, *TDC* and *STR* mRNA levels was observed. One problem with the latter experiments was that the *TDC* and *STR* mRNA levels were highly variable even between control lines, whereas in the first experiments control lines consistently showed low expression levels.

Other experimental approaches taken to study CrA42 function did not yield results that were consistent with a role of CrA42 as a regulator of the expression of alkaloid biosynthesis genes. CrA42 was unable to trans-activate the *STR* promoter in transient assay. The expression of the *CrA42* gene was not affected by YE or MeJA, signal molecules that have a strong effect on *TDC* and *STR* expression. CrA42 was not required for MeJA- or YE-responsive *TDC* and *STR* expression. Finally, CrA42 was localized in cytoplasmic speckles. If this localization is not an artefact of overexpression, it is not easily reconcilable with a function as a gene regulator.

What caused the phenotype of T-DNA tagged line A42, if it was not overexpression of *CrA42*? First, the phenotype of line A42 might be due to

(epi)genetic variations not linked to the T-DNA. The variable expression of *TDC* and *STR* in control lines (Figs. 4 and 5) indicate that considerable variation can exist in the expression of alkaloid biosynthesis genes in transgenic lines unrelated to T-DNA activation tagging. In this context, it may be of interest to note that repeated RNA analysis of line A42 showed reproducibly high *TDC* expression, whereas variation was observed for *STR* expression ranging from very high to normal expression levels (van der Fits et al., 2001). Second, the phenotype might be linked to the integrated T-DNA, but not to *CrA42*. For example, a gene located distally from *CrA42* might be responsible for the phenotype. The complex phenotype of the T-DNA activation tagged Arabidopsis *let* mutant was shown to be caused by overexpression of the *LEAFY PETIOLE* (*LEP*) gene located proximal to the T-DNA as well as overexpression of the *VASCULAR TISSUE SIZE* gene located distal from the T-DNA (van der Graaff et al., 2002). Although the *LEP* gene is quite small compared to the *CrA42* gene, activation tags have been shown to work over a distance of at least 3.8 kb (Weigel et al., 2000). Another possibility is that the gene responsible for the phenotype of line A42 is located next to the left T-DNA border, since the CaMV 35S enhancer elements work independent of their orientation (Fang et al., 1989), although our T-DNA is quite large with a size over 6 kb.

A last option is that the *CrA42* allele which was T-DNA-tagged in cell line BIX differs from the genomic clone picked up from a library made from leaves of *C. roseus* variety Morning Mist and from the cDNA sequence amplified from cell line MP183L. The varieties used to generate the cell lines are unknown. Differences in the amino acid sequences of the proteins encoded by alleles from different varieties could account for differences in function. Given the high conservation of the *A42* gene between *C. roseus* and Arabidopsis, such sequence differences between *C. roseus* varieties seems unlikely, but they cannot be excluded.

Materials and Methods

Cell Cultures, Stable Transformation, Treatments, and GFP analysis

Catharanthus roseus cell suspension line MP183L was maintained by weekly 10-fold dilution in 50 mL of Linsmaier and Skoog (LS) medium containing 88 mM sucrose, 2.7 µM 1-NAA and 0.23 µM kinetin and was grown at 28°C in a 16/8 hour light/dark regime at 200 µE m^{-2} s^{-1} at 70% relative humidity on a rotary shaker at 120 rpm. Treatments were 4 d after transfer. MeJA was diluted in DMSO. Yeast extract (Difco) was dissolved in water, autoclaved, passed through an ultra-filter with a molecular weight cut-off of 3 kD (Millipore) to remove chitin, and was added at a final concentration of 400 µg/ml to cells. Control cultures were treated with DMSO at a final concentration of 0.1% (v/v). For stable transformations of cell line MP183L, plasmid constructs of interest were co-transformed with the plasmid pGL2 (Bilang et al., 1991) carrying a hygromycin selection gene driven by the CaMV 35S promoter in a ratio of 4 to 1 by particle bombardment (van der Fits and Memelink, 1997). Transgenic cells were selected on solid medium containing 50 µg/mL hygromycin-B and individual transgenic calli were converted to cell suspensions. GFP fluorescence was examined with a Leica inverted microscope (DM IRBE) equipped with a Leica SP1 confocal scanhead with an argon laser at an excitation wavelength of 488 nm and collection of emitted fluorescence after passage through a broad band pass filter (500-550 nm). The resulting signal was amplified, digitalized and the consistent picture reconstituted by Leica software.

Screening of the genomic library

A genomic library from *C. roseus* variety Morning Mist (Goddijn et al., 1994) consisting of Sau3AI partial fragments of sizes between 9 to 13 kb cloned in lambda GEM11 (Promega) was transfected to *Escherichia coli* strain KW251 (Promega). A total of 0.5 million plaques were transferred to nylon filters (Hybond-N, Amersham-GE Healthcare) and hybridized as described (Memelink

et al., 1994). The probe was a 3 kb XhoI/EcoRI fragment corresponding to the 3' part of the rescued genomic fragment (Fig. 1).

Plasmid Constructs

As a first step in the construction of the genomic recapitulation plasmid, the C-terminal part of the *CrA42* gene contained on a 5.9 kb BamHI fragment isolated from the genomic lambda clone was cloned into pRT101 (Töpfer et al., 1987). A central part of the *CrA42* gene contained on a 2.8 kb HindIII/XhoI fragment isolated from the genomic lambda clone was cloned in pIC-20R (Marsh et al., 1984). The N-terminal part of the *CrA42* gene contained on a 4.1 kb SacI/XbaI fragment isolated from the genomic lambda clone (SacI site derived from lambda GEM11 polylinker) was cloned into pBluescript II SK+, resulting in clone SK-A42XS4. The N terminal part starting from the ATG start codon was then amplified using SK-A42XS4 as a template with the primers 5'-CGC GTC GAC ATG GAG TTA AGG ATT CAG TC-3' and T7, digested with SalI/HindIII, and cloned in the pIC-20R plasmid clone digested with SalI/HindIII. From the resulting plasmid, a SalI/XhoI fragment carrying the N-terminal part of the *CrA42* gene was cloned into the pRT101 derivative carrying the C-terminal BamHI fragment digested with XhoI to reconstitute the complete *CrA42* gene in pRT101. To isolate a full-length cDNA clone, 5' sequences were isolated by PCR with a gene-specific primer and a vector primer using a pACTII cDNA library of YE-treated MP183L cells (Menke et al., 1999) as a template. The *CrA42* open reading frame (ORF) was PCR amplified with the primers 5'-CGC GTC GAC ATG GAG TTA AGG ATT CAG TC-3' and 5'-ACG CGT CGA CGG ATC CTC TAG ACA TTT GGC TCT GGC AAG AAT C-3' using the pACTII cDNA library as a template, digested with SalI and BamHI and cloned in pRT101 digested with XhoI and BamHI. The *CrA42* RNAi construct consisted of an inverted repeat of an EcoRI fragment (positions 1313-2081 in the cDNA sequence relative to the start codon) in pHannibal (Wesley et al., 2001). The EcoRI fragment fragment was cloned in pHannibal digested with EcoRI such that the 5' end flanked the *PDK* intron and in pBluescript II SK+ digested with EcoRI such that the 5' end flanked the HindIII site. The fragment was then excised as a HindIII/BamHI fragment from pBluescript II SK+ and cloned in the

pHannibal derivative digested with HindIII/BamHI to create the inverted repeat. To construct GFP fusions, the *CrA42* ORF lacking the stop codon was PCR amplified with the primers 5'-CGG GAT CCG GTC GAC CAT ATG GAG TTA AGG ATT CAG TCTG-3' and 5'-AAC TGC AGC GTC TAG AAA ACC AAG AAT CGA GTT CAC C -3' using the *CrA42* ORF cloned in pGEM-T Easy (Promega) as a template, digested with XbaI and BamHI fragment and cloned in pIC-20R digested with XbaI/BamHI. From the resulting plasmid, a SalI fragment was cloned in pTH2 (Chiu et al., 1996; Niwa et al., 1999) digested with SalI. The *CrA42* transmembrane domain was amplified with the primers 5'-CGC GTC GAC ATG GAG TTA AGG ATT CAG TC-3' and 5'-CAT GCC ATG GAA ATA ACA TAA TCA TCC CCA TT-3', digested with SalI and NcoI fragment and cloned in pTH2 digested with SalI/NcoI. A *CrA42* derivative lacking the transmembrane domain was fused to GFP by replacing the NdeI/XhoI fragment from pTH2-CrA42 with a PCR product amplified with the primers 5'-GGA ATT CCA TAT GTA TGG AAC ACT CCA AGA ACA C-3' and 5'-GTG AAA TTC TCC GAG AAT GC-3' using the *CrA42* ORF in pGEM-T Easy as a template and digested with NdeI/XhoI. The *GUS* reporter plasmid carrying the *STR* promoter derivative XH was described by Pasquali *et al*. (1999). The *ORCA2* and *ORCA3* overexpression constructs in pMOG184 (Menke *et al*., 1999a) and pRT101 (van der Fits and Memelink, 2001), respectively, were previously described.

Transient Expression Assays

Cell line MP183L was transformed by particle bombardment as described (van der Fits and Memelink, 1997) using a home-made helium gun and 1.8 µm tungsten particles (Pioneer Hi-Bred). Cells were co-bombarded in triplicate with 2 µg of a *STR-GusSH* reporter construct carrying the *STR* XH promoter derivative (Pasquali et al., 1999), effector plasmids as indicated in the figure legends, and 1 µg of the p2rL7 reference plasmid (De Sutter et al., 2005). Co-transformations with the empty overexpression vectors served as controls. Cells were harvested 20 hours after bombardment. GUS activities measured as described (van der Fits and Memelink, 1997) were corrected for RLUC activities

(Dyer et al., 2000) and expressed as relative activities compared with the vector control.

RNA Extraction and Northern Blot Analyses

Total RNA was extracted from frozen cells by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% (v/v) ethanol, and resuspended in water. Ten µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose/1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences). Probes were ^{32}P-labeled by random priming. (Pre-) hybridization and subsequent washings of blots were performed as described (Memelink et al., 1994) with minor modifications.

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References

Bilang R, Iida S, Peterhans A, Potrykus I, Paszkowski J (1991) The 3'-terminal region of the hygromycin-B-resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*. Gene 100: 247-250

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6: 325-330

De Sutter V, Vanderhaeghen R, Tilleman S, Lammertyn F, Vanhoutte I, Karimi M, Inzé D, Goossens A, Hilson P (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. Plant J 44: 1065-1076

Dyer BW, Ferrer FA, Klinedinst DK, Rodriguez R (2000) A noncommercial dual luciferase enzyme assay system for reporter gene analysis. Anal Biochem 282: 158-161

Fang RX, Nagy F, Sivasubramaniam S, Chua NH (1989) Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1: 141-150

Goddijn OJ, Lohman FP, de Kam RJ, Schilperoort RA, Hoge JH (1994) Nucleotide sequence of the tryptophan decarboxylase gene of *Catharanthus roseus* and expression of *tdc-gusA* gene fusions in *Nicotiana tabacum*. Mol Gen Genet 242:217-225

Koroleva OA, Tomlinson ML, Leader D, Shaw P, Doonan JH (2005) High-throughput protein localization in Arabidopsis using Agrobacterium-mediated transient expression of GFP-ORF fusions. Plant J 41: 162-174

Marsh JL, Erfle M, Wykes EJ (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32: 481-485

Memelink J, Swords KMM, Staehelin LA, Hoge JHC (1994) Southern, Northern and Western blot analysis. In Plant Molecular Biology Manual, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds (Dordrecht: Kluwer Academic Publishers), pp. F1-F23

Memelink J, Verpoorte R, Kijne, JW (2001) ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 6: 212-219

Menke FLH, Champion A, Kijne JW, Memelink, J (1999a) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18: 4455-4463

Menke FLH, Parchmann S, Mueller MJ, Kijne JW, Memelink J (1999b) Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. Plant Physiol 119: 1289-1296

Niwa Y, Hirano T, Yoshimoto K, Shimizu M, Kobayashi H (1999) Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. Plant J 18: 455-463

Pasquali G, Erven ASW, Ouwerkerk PBF, Menke FLH, Memelink J (1999) The promoter of the strictosidine synthase gene from periwinkle confers elicitor-inducible expression in transgenic tobacco and binds nuclear factors GT-1 and GBF. Plant Mol Biol 39: 1299-1310

Pasquali G, Goddijn OJ, de Waal A, Verpoorte R, Schilperoort RA, Hoge JH, Memelink J (1992) Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors. Plant Mol Biol 18: 1121-1131

Pauw B, Hilliou FAO, Sandonis Martin V, Chatel G, de Wolf CJF, Champion A, Pre M, van Duijn B, Kijne JW, van der Fits L, Memelink J (2004a) Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in *Catharanthus roseus*. J Biol Chem 279: 52940-52948

Pauw B, van Duijn B, Kijne JW, Memelink J (2004b) Activation of the oxidative burst by yeast elicitor in *Catharanthus roseus* cells occurs independently of the activation of genes involved in alkaloid biosynthesis. Plant Mol Biol 55: 797-805

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss H-H (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res 15: 5890

van der Fits L, Hilliou F, Memelink J (2001) T-DNA activation tagging as a tool to isolate regulators of a metabolic pathway from a genetically non-tractable plant species. Transgenic Res 10: 513-521

van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. Plant Mol Biol 33: 943-946

van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295-297

van der Fits L, Memelink, J (2001) The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J 25: 43-53

van der Graaff E, Hooykaas PJJ, Keller B (2002) Activation tagging of the two closely linked genes *LEP* and *VAS* independently affects vascular cell number. Plant J 32: 819-830

Weigel D, Ahn JH, Blázquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrándiz C, Kardailsky I, Malancharuvil EJ, Neff MM, Nguyen JT, Sato S, Wang ZY, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Activation tagging in Arabidopsis. Plant Physiol 122: 1003-1013

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27: 581-590

Zuo J, Niu QW, Chua NH (2000) Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J 24: 265-273

Chapter 3

The basic helix-loop-helix transcription factor CrMYC2 controls the jasmonate-responsive expression of the *ORCA* genes regulating alkaloid biosynthesis in *Catharanthus roseus*

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Summary

Jasmonates are plant signalling molecules that play key roles in defence against insects and certain pathogens, among others by controlling the biosynthesis of protective secondary metabolites. In *Catharanthus roseus*, the AP2/ERF-domain transcription factor ORCA3 controls the jasmonate-responsive expression of genes encoding enzymes involved in terpenoid indole alkaloid biosynthesis. *ORCA3* gene expression is itself induced by jasmonate. The *ORCA3* promoter contains an autonomous jasmonate-responsive element (JRE) composed of a quantitative sequence responsible for a high level of expression and a qualitative sequence that acts as an on/off switch in response to methyl-jasmonate (MeJA). Here we identify the basic Helix-Loop-Helix (bHLH) transcription factor CrMYC2 as the major regulator of MeJA-responsive *ORCA3* gene expression. The *CrMYC2* gene is an immediate-early jasmonate-responsive gene. CrMYC2 binds to the qualitative sequence in the *ORCA3* JRE *in vitro*, and trans-activates 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. Our 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 regulate in turn a subset of alkaloid biosynthesis genes.

Introduction

Jasmonates, including jasmonic acid (JA) and several of its cyclic precursors and derivatives, constitute a family of bioactive oxylipins that are involved in the regulation of a number of processes in plants, including certain developmental processes, senescence, and responses to wounding and pathogen attack (Turner *et al*., 2002; Balbi and Devoto, 2008). An important defence response that depends on jasmonates as regulatory signals is the induction of secondary metabolite accumulation (Gundlach *et al*., 1992; Memelink *et al*., 2001). Jasmonates induce secondary metabolism at the transcriptional level by switching on the coordinate expression of a set of biosynthesis genes (Memelink *et al*., 2001).

Methyl-jasmonate (MeJA) stimulates terpenoid indole alkaloid (TIA) metabolism in cell suspensions of *Catharanthus roseus* (Gantet *et al*., 1998) and induces the expression of all of the TIA biosynthesis genes tested (van der Fits and Memelink, 2000). The MeJA-responsive expression of a number of these biosynthesis genes, including the strictosidine synthase (*STR*) gene, is controlled by the transcription factor Octadecanoid-derivative Responsive Catharanthus AP2-domain protein 3 (ORCA3) (van der Fits and Memelink, 2000). ORCA3 and the related transcription factor ORCA2 (Menke *et al*., 1999) contain a DNA-binding domain of the APETALA2/Ethylene Response Factor (AP2/ERF) type. ORCA2 and ORCA3 *trans*-activate the *STR* promoter via sequence-specific binding to a jasmonate- and elicitor-responsive element (JERE) (Menke *et al*., 1999; van der Fits and Memelink, 2001).

The expression of the *ORCA* genes themselves is rapidly induced by MeJA (Menke *et al*., 1999; van der Fits and Memelink, 2001), which implies that ORCAs either auto-regulate their own gene expression level or alternatively that the *ORCA* genes are regulated by one or more upstream transcription factors. The latter option, a transcriptional cascade regulating plant stress-responsive gene expression, has been proposed for ethylene (Solano *et al*., 1998) and cold signalling (Chinnusamy *et al*., 2003).

Functional studies of the *ORCA3* promoter identified an autonomous jasmonate-responsive element (JRE) (Vom Endt *et al*., 2007), which is

composed of 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. The *ORCA3* JRE does not contain a sequence with similarity to the *STR* JERE, and the ORCA proteins do not bind to the *ORCA3* promoter *in vitro* or trans-activate it in transient expression assays (Vom Endt *et al*., 2007). This suggests that the *ORCA* genes are regulated by one or more upstream transcription factors. In a search for such transcription factors using the *ORCA3* JRE as bait in a yeast one-hybrid screening, a group of related proteins bearing a single AT-hook DNA-binding motif was isolated (Vom Endt *et al*., 2007). The AT-hook proteins specifically bind to the quantitative sequence in the *ORCA3* JRE, suggesting that they are involved in determining the level of *ORCA3* gene expression.

The yeast one-hybrid screening did not result in the isolation of proteins binding to the qualitative sequence. Here we identify the basic Helix-Loop-Helix (bHLH) transcription factor CrMYC2 as the major regulator of MeJA-responsive *ORCA* gene expression.

Results

Basic Helix-Loop-Helix proteins from Catharanthus roseus

The qualitative sequence in the JRE from the *ORCA3* promoter contains a G-box with one mismatch called a T/G-box, which may interact with bHLH proteins. A prominent bHLH transcription factor controlling JA-responsive gene expression in Arabidopsis is AtMYC2 (Lorenzo *et al*., 2004), which binds *in vitro* to G- and T/G-boxes (de Pater *et al*., 1997; Chini *et al*., 2007). Therefore we focused our search for the regulator of MeJA-responsive *ORCA3* gene expression on bHLH proteins from *C. roseus*.

Previously we isolated 5 partial cDNA clones encoding distinct bHLH proteins called CrMYC1-5 from *C. roseus* in a yeast one-hybrid screen using a tetramer of a G-box from the promoter of the *STR* gene as bait (Pré *et al*., 2000). Characterization of a full-length sequence of *CrMYC1* was reported previously (Chatel *et al.,* 2003). We isolated full-length cDNA clones for the

other CrMYC proteins via PCR using Catharanthus cDNA libraries as templates. Analysis of the encoded proteins (Table 1) showed that CrMYC2 is a close homologue of AtMYC2.

Table 1 *C. roseus* CrMYC proteins and their most similar counterparts in Arabidopsis

C. roseus protein	*A. thaliana* protein	bHLH group	function in *A. thaliana*	reference
CrMYC1	AtbHLH031 (BIGPETAL)	XII	flower development	Szécsi *et al*., 2006
CrMYC2	AtbHLH006 (AtMYC2)	IIIe	jasmonate response	Lorenzo *et al*., 2004
CrMYC3	AtbHLH121	IVb	unknown	
CrMYC4	AtbHLH080	IX	unknown	
CrMYC5	AtbHLH024 (SPATULA)	VIIb	flower development	Alvarez *et al*., 1999

Most similar Arabidopsis proteins were found by searching the Arabidopsis protein database using the BLASTP (Basic Local Alignment Search Tool for Proteins) algorithm (Altschul *et al*., 1997). Systematic naming and group classification of the Arabidopsis proteins are according to Heim *et al*. (2003).

The deduced amino acid sequence of CrMYC2 (Figure 1) is characterized by the basic helix-loop-helix region possessing typical features of the B group of bHLH transcription factors, known to bind preferentially to the G-box sequence. A domain rich in acidic residues is present and has been suggested to be a transcriptional activation domain in other bHLH transcription factors.

CrMYC2 is an immediate-early jasmonate-responsive gene

To get a first indication whether CrMYC2 may be involved in jasmonate-responsive gene expression in *C. roseus*, we analysed gene expression in response to MeJA. In cell line MP183L, *CrMYC2* expression was strongly and transiently induced by MeJA with a peak around 30 min and re-establishment of basal levels at 24 hours (Figure 2a). The expression of the *ORCA3* gene was

Fig.1

```
1          CCCTCGCCCTATCACCACTTTTCTCTCCCCACCACTAGCCGCCCTCCCATTTCTCTCTCT
61         CTCTTCCTTCAATTTTTCACTCACTCAAACAGTCTTCTTCTTCTCGAATTCAACAATCAC
121        TGCCATACCCAGAAACAAGAACCCTTTTTGAGTCTTCAATTTCTTTTTTTTTTTTTGTTT
181        GATGGAATGACGGACTATAGGCTACAACCGAAAATGAACCTATGGGGTACGACGACCAAC
1                 M  T  D  Y  R  L  Q  P  K  M  N  L  W  G  T  T  T  N

241        ACCGCAGCTTCACCAATAATTACTTCAGATGATAATAGTTCGATGATGGAGGCTTTTATG
19          T  A  A  S  P  I  I  T  S  D  D  N  S  S  M  M  E  A  F  M

301        ACCTCATCAGATCCGATTTCTTTGTGGCCGCCGTCAATGTCTGTGAATCATCACCATCCA
39          T  S  S  D  P  I  S  L  W  P  P  S  M  S  V  N  H  H  H  P

361        CCAACTCCTACTTCTTCCGCCGTAACAACTGCGGTGGACTCCGCTAAATCTATGCCTGCC
59          P  T  P  T  S  S  A  V  T  T  A  V  D  S  A  K  S  M  P  A

421        CAACCTGCTTTTTTCAATCAAGAAAATCTCCAACAGCGCCTTCAAACTCTAATTGATGGT
79          Q  P  A  F  F  N  Q  E  N  L  Q  Q  R  L  Q  T  L  I  D  G

481        GCTAGGGAGAGTTGGACTTATGCCATATTTTGGCAGTCGTCTGTTGTCGAATTCGCCGGT
99          A  R  E  S  W  T  Y  A  I  F  W  Q  S  S  V  V  E  F  A  G

541        CCTTCGGTCTTGGGTTGGGGCGATGGATATTATAAGGGAGAAGAAGATAAAGGGAAGAGG
119         P  S  V  L  G  W  G  D  G  Y  Y  K  G  E  E  D  K  G  K  R

601        AAGAATTCGTCTTCCGCGAGTTCTTTTGCAGAACAGGAACACAGAAAGAAAGTCCTTAGA
139         K  N  S  S  S  A  S  S  F  A  E  Q  E  H  R  K  K  V  L  R

661        GAGCTCAATTCTTTGATTGCTGGGCCACAAGGCACCGCCGATGATGCAGTTGATGAAGAG
159         E  L  N  S  L  I  A  G  P  Q  G  T  A  D  D  A  V  D  E  E

721        GTGACCGATACCGAATGGTTTTTCTTAATTTCAATGACTCAGTCATTTGTTTCCGGGAGC
179         V  T  D  T  E  W  F  F  L  I  S  M  T  Q  S  F  V  S  G  S

781        GGTCTTCCAGGGCAGGCCTTATACAATTCAAACCCGGTATGGGTTACCGGAGCAGGGAGG
199         G  L  P  G  Q  A  L  Y  N  S  N  P  V  W  V  T  G  A  G  R

841        CTGGCGGTTTCACACTGCGACCGGGCCAGGCAGGCTCAAAGTTTTGGGCTTCAGACCTTA
219         L  A  V  S  H  C  D  R  A  R  Q  A  Q  S  F  G  L  Q  T  L

901        GTTTGTATTCCCTCCGCAAACGGCGTTGTGGAGCTGGGTTCAACGGAATTGATTTTTCAG
239         V  C  I  P  S  A  N  G  V  V  E  L  G  S  T  E  L  I  F  Q

961        AGCTCCGATCTCATGAATAAGGTTAGGATACTGTTCAATTTTAATAATATAGATTTGGGT
259         S  S  D  L  M  N  K  V  R  I  L  F  N  F  N  N  I  D  L  G

1021       TCGAGCTCTGGACCTTGGCCTGAGAACGATCCTTCTTCTCTGTGGCTTACTGATCCATCG
279         S  S  S  G  P  W  P  E  N  D  P  S  S  L  W  L  T  D  P  S

1081       CCCTCAGGGGTAGGGGTTAAGGAGGGGGTGAATACTAATAATAATACTAGTGTTCAAGGG
299         P  S  G  V  G  V  K  E  G  V  N  T  N  N  N  T  S  V  Q  G

1141       AATTCAATTCCTTCTGGTAATAAGCAGCAACTTGTGTTTGGAAATAATGATAATCATCCA
319         N  S  I  P  S  G  N  K  Q  Q  L  V  F  G  N  N  D  N  H  P

1201       ACCACGAGTACTTTGACTGATCATCCCGGGGCTGGGGCTGTAAATAGTTATAATAATTCA
339         T  T  S  T  L  T  D  H  P  G  A  G  A  V  N  S  Y  N  N  S

1261       TCTCAGAATGCCCAGCAGCCTCAAGGTAGTTTCTTTACAAGGGAATTGAATTTTTCAGAA
359         S  Q  N  A  Q  Q  P  Q  G  S  F  F  T  R  E  L  N  F  S  E
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1321    TACGGGTTTGAAAGGAGTAGTGTAAAGAATGGGAATTGTAAGCCAGAATCGGGAGAAATA
379      Y  G  F  E  R  S  S  V  K  N  G  N  C  K  P  E  S  G  E  I

1381    TTGAACTTTGGTGGTGAATCTGTTACCAAGAAGAATTCTGTAAGTGGAAATGGGAACTTG
399      L  N  F  G  G  E  S  V  T  K  K  N  S  V  S  G  N  G  N  L

1441    TTTTCAGTACAATCACAGTTTGGAGCTGGGGAGGAGAACAAGAACAAGAAAAGGCCATCT
419      F  S  V  Q  S  Q  F  G  A  G  E  E  N  K  N  K  K  R  P  S

1501    CCTGTGTCAAGGGGGAGCAATGATGAGGGGATGCTTTCTTTTACTTCTGGGGTAGTTTTG
439      P  V  S  R  G  S  N  D  E  G  M  L  S  F  T  S  G  V  V  L

1561    CCCTCTACTGGTGTAGTGAAGTCTAGTGGTGGTGGTGGTGGTGGAGACTCCGATCATTCT
459      P  S  T  G  V  V  K  S  S  G  G  G  G  G  G  D  S  D  H  S

1621    GATCTTGAGGCCTCAGTGGTGAAGGAGGCAGAGAGTAGTAGGGTTGTGGATCCCGAGAAA
479      D  L  E  A  S  V  V  K  E  A  E  S  S  R  V  V  D  P  E  K

1681    CGGCCTAGGAAGAGGGGGAGAAAGCCTGCAAATGGAAGGGAAGAGCCATTGAATCATGTG
499      R  P  R  K  R  G  R  K  P  A  N  G  R  E  E  P  L  N  H  V

1741    GAGGCAGAGAGGCAGAGGAGGGAGAAGTTGAACCAGAGGTTCTATGCTCTTAGAGCCGTG
519      E  A  E  R  Q  R  R  E  K  L  N  Q  R  F  Y  A  L  R  A  V

1801    GTTCCTAATGTTTCAAAAATGGATAAAGCTTCACTTCTTGGTGATGCTATTTCTTATATC
539      V  P  N  V  S  K  M  D  K  A  S  L  L  G  D  A  I  S  Y  I

1861    AATGAGTTGAAAGCTAAACTTCAAACTACAGAAACAGATAAAGATGAATTGAAGAACCAA
559      N  E  L  K  A  K  L  Q  T  T  E  T  D  K  D  E  L  K  N  Q

1921    TTGGACTCATTAAAGAAGGAATTAGCAAGTAAAGAATCTCGGCTTTTATCATCACCAGAT
579      L  D  S  L  K  K  E  L  A  S  K  E  S  R  L  L  S  S  P  D

1981    CAAGATCTCAAGTCCTCAAACAAGCAGTCAGTGGGTAACTTGGATATGGATATTGATGTG
599      Q  D  L  K  S  S  N  K  Q  S  V  G  N  L  D  M  D  I  D  V

2041    AAAATCATTGGTCGGGAAGCAATGATTCGAGTCCAATCCAGTAAAAACAACCACCCTGCA
619      K  I  I  G  R  E  A  M  I  R  V  Q  S  S  K  N  N  H  P  A

2101    GCAAGAGTAATGGGAGCACTAAAGGATCTTGATCTTGAATTACTCCATGCTAGTGTCTCA
639      A  R  V  M  G  A  L  K  D  L  D  L  E  L  L  H  A  S  V  S

2161    GTGGTAAATGATTTGATGATCCAGCAAAATACAGTGAGAATGGGGAGCCGATTTTATACT
659      V  V  N  D  L  M  I  Q  Q  N  T  V  R  M  G  S  R  F  Y  T

2221    CAGGAGCAGCTTAGAATAGCATTGACATCCAGAATAGCCGGAAACTCGATGAGGCTCTTG
679      Q  E  Q  L  R  I  A  L  T  S  R  I  A  G  N  S  M  R  L  L

2281    GTATGAAGGGTAGATTAAACTCAAGAAAAGTTGGTAGGACTGAGAGTTTCTTTGCAAATT
699      V  *

2341    AGTTTTAGGCTCTAGGATATTTTAAGGCTCCCTGGAAGAATCCCTGTAAACTATTTCCTT
2401    TTCAACTAGGGTTTCCTAAAATTCTAGCAAGAGTCAGAAGTTGAGGTCTCTCTTAGTTGA
2461    AAACCTAAGAGAATTCAGCTTTCATCAAATTTTTGTCTTTTAACTTTTGGGGTTTATTTT
2521    TATGTCTAGATTGTATATATATTTGTAATCAATCAATAAACCTTGTCTACTCTGGTGCTT
2581    AGTTGTGCTGTTAGATTGGAGGTTTGCTAAAAAAAAAAAAAAA
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Figure 1. Nucleotide sequence of *CrMYC2* cDNA from *C. roseus* and deduced amino acid sequence. (To be continued on the next page)

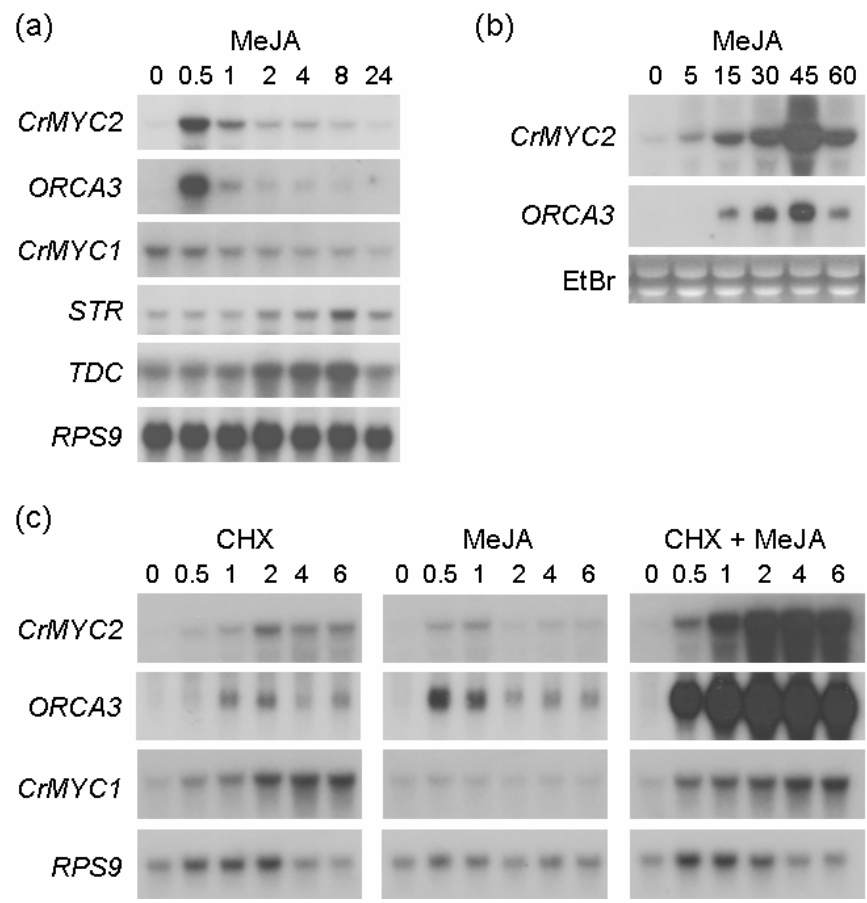

Figure 2. *CrMYC2* is an immediate-early jasmonate-responsive gene.

(a) *C. roseus* MP183L cells were exposed to 10 µM MeJA for the number of hours indicated. Northern blots were hybridized with cDNAs as indicated.

(b) *C. roseus* MP183L cells were exposed to 10 µM MeJA for the number of minutes indicated. The ethidium bromide (EtBr) stained gel is shown as a loading control.

(c) *C. roseus* BIX cells were treated with 100 µM cycloheximide (CHX), 10 µM MeJA, or both compounds for the number of hours indicated.

Figure 1. (Continued from previous page) The bHLH domain is represented in bold letters with the helices in italics and the loop underlined with a broken line. A region homologous to various R proteins is underlined with a solid line and a potential acidic activation domain is underlined with a bold solid black line. A putative bi-partite nuclear localization signal is highlighted in shaded grey, putative mono-partite nuclear localization signals are highlighted in grey and the region homologous to the Lc nuclear localization signal is outlined in black.

similar to *CrMYC2* with the same kinetics. Expression of the *CrMYC1* gene was slightly repressed by MeJA in cell line MP183L, in contrast to our earlier report of induction at the two later time points in cell line C20D (Chatel *et al*., 2003). The alkaloid biosynthesis genes *STR* and *TDC* were induced at later time points than *CrMYC2* and *ORCA3*. A control time course of treatment with the solvent DMSO showed no effect on the expression of any of the genes tested (results not shown).

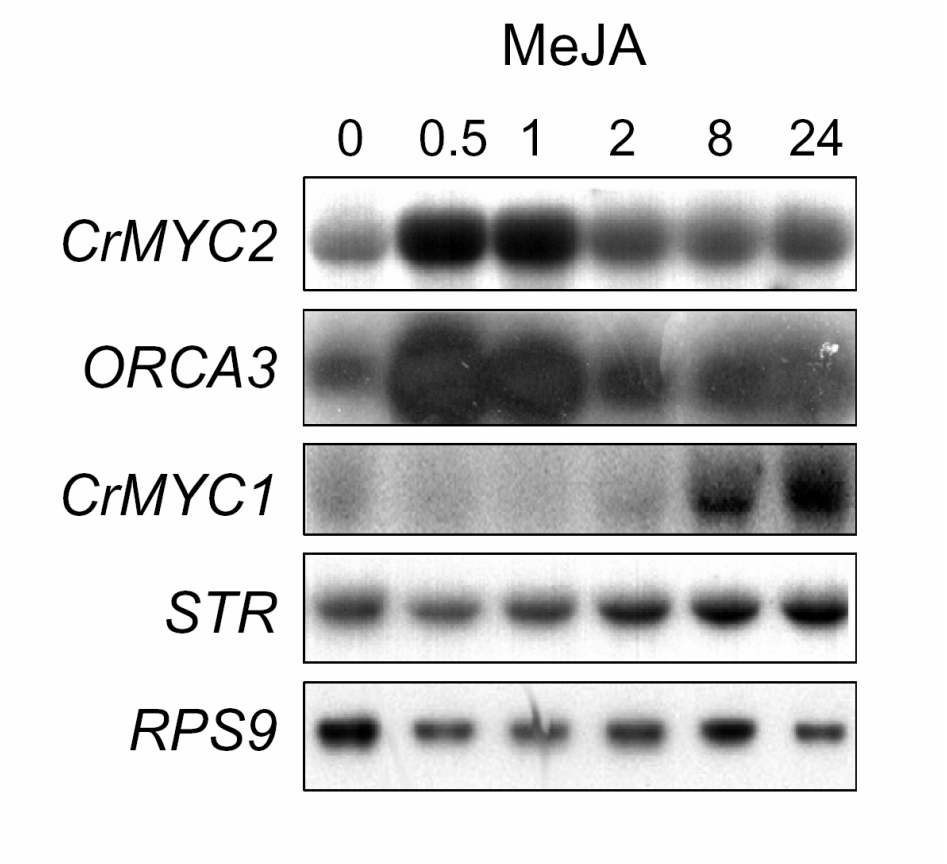

Figure 3. *CrMYC2* is rapidly induced by MeJA in *C. roseus* cell line C20D.
C20D cells were treated 4 days after transfer to medium without hormones with 100 µM MeJA for the number of hours indicated. Northern blots were hybridized with cDNAs as indicated on the left.

The early response of *CrMYC2* was analysed at shorter time points. As shown in Figure 2b, induction of *CrMYC2* by MeJA was already observed at the

shortest time point of 5 min after treatment. Short-time kinetics of *ORCA3* induction were similar to those of *CrMYC2*.

In cell line C20D *CrMYC2* was also rapidly induced by MeJA co-ordinately with the *ORCA3* gene with similar kinetics as in cell line MP183L (Figure 3). *CrMYC1* expression was also induced in this cell line but only at the two later time points as reported earlier (Chatel *et al*., 2003).

Immediate-early response genes are defined as those that respond to a signal without requiring *de novo* protein synthesis. To obtain evidence that *CrMYC2* is an immediate-early response gene, we treated cell line BIX with MeJA, the protein synthesis inhibitor cycloheximide (CHX) or both. MeJA induced *CrMYC2* expression rapidly as in the other two cell lines (Figure 2c). CHX alone had an inducing effect on *CrMYC2* expression. Together with MeJA, CHX caused super-induction of *CrMYC2* mRNA accumulation. Super-induction by CHX is commonly observed with immediate-early response genes (Edwards and Mahadevan, 1992). The expression of *ORCA3* was similar to *CrMYC2*. Although CHX also induced *CrMYC1* expression, it had no super-inducing effect together with MeJA, consistent with the notion that *CrMYC1* is not an early response gene.

The expression analysis showed that *CrMYC2* is an immediate-early jasmonate-responsive gene, with kinetics of induction that are similar to those of the *ORCA3* gene.

CrMYC2 binds in vitro to the qualitative sequence within the ORCA3 JRE

To test whether CrMYC2 can interact with the G-box-like sequence which corresponds to the qualitative sequence within the JRE from the *ORCA3* promoter, we performed an electrophoretic mobility shift assay (EMSA) with recombinant CrMYC2 protein and wild-type D fragment and different mutant derivatives. The D fragment is an *Ava*II/*Dde*I restriction fragment from the *ORCA3* promoter which contains the JRE (Figure 6a; Vom Endt *et al*., 2007). Mutant derivatives contained a block mutation changing 5 or 6 nucleotides in their complementary nucleotides. Block mutations M2 and M3 define the

quantitative sequence which binds AT-hook proteins (Vom Endt *et al*., 2007), whereas block mutations M6 and M7 cover the qualitative sequence containing the G-box-like sequence AACGTG (Figure 4a).

Analysis of recombinant CrMYC2 protein produced in *Escherichia coli* by SDS-PAGE and Coomassie Brilliant Blue staining or Western blotting and immuno-probing with anti-His antibodies showed the presence of a major band of the expected size of 81.5 kDa as well as minor smaller bands (Figure 4b).

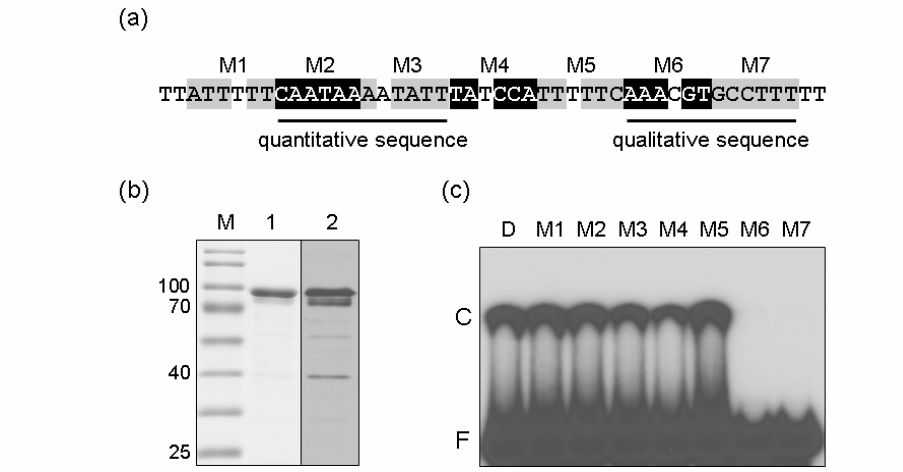

Figure 4. CrMYC2 binds *in vitro* to the qualitative sequence within the *ORCA3* JRE.

(a) The wild-type sequence of part of the D fragment from the *ORCA3* promoter is shown. Numbering of mutations is given above the sequence. In each mutant, boxed nucleotides were changed into their complementary nucleotide. Mutations affecting the quantitative and the qualitative sequences are underlined.

(b) Analysis of recombinant CrMYC2 protein. The protein was separated by 10% SDS-PAGE and either stained with Coomassie Brilliant Blue (lane 1) or visualized after Western blotting using anti-His antibodies (lane 2). Sizes of relevant marker (M) bands are indicated in kDa.

(c) *In vitro* binding of recombinant CrMYC2 to wild-type or mutated derivatives of fragment D. Fragments indicated at the top were used as probes in *in vitro* binding. F indicates free probes, whereas C indicates DNA-CrMYC2 complexes.

EMSA experiments using the recombinant protein showed that it bound the wild-type D fragment as well as all mutants except M6 and M7. These results show that CrMYC2 bound specifically to the D fragment by interaction with the qualitative sequence.

CrMYC2 activates gene expression via interaction with the qualitative sequence

Next we investigated whether CrMYC2 works *in vivo* as an activator of the expression of genes containing the *ORCA3* JRE in their promoter. First we tested artificial promoters consisting of a tetramer of the wild-type D fragment or mutant derivatives fused to the TATA box of the CaMV 35S promoter. In this configuration, the wild-type D fragment confers a high level of MeJA-responsive gene expression, whereas mutant derivatives M6 and M7 do not confer a response to MeJA (Vom Endt *et al*., 2007).

Co-bombardment of MP183L cells with a *GUS* reporter gene driven by the 4D tetramer and increasing amounts of a plasmid carrying the *CrMYC2* open reading frame fused to the CaMV 35S promoter showed that CrMYC2 indeed was able to activate gene expression via the D tetramer with the highest gene expression levels at 500 and 1000 ng of plasmid DNA (Figure 5). Subsequently 500 ng of *CrMYC2* effector plasmid was co-bombarded with reporter plasmids carrying the wild-type and mutant D tetramers. As shown in Figure 6b, CrMYC2 trans-activated all reporter genes, except those containing the M6 and M7 tetramers.

Next we investigated whether CrMYC2 also functioned as an activator of reporter genes carrying native *ORCA3* promoter derivatives. CrMYC2 activated reporter genes carrying the ∆828 (-826 to -53 relative to the start codon) or ∆264 (-264 to -53) promoter derivatives to similar levels as the D tetramer construct (Figure 6c). Deletion of 33 nucleotides including the qualitative sequence within the ∆264 promoter context caused a significant reduction in the level of trans-activation by CrMYC2.

These results show that CrMYC2 functioned as an activator of gene expression via interaction with the G-box-like qualitative sequence from the *ORCA3* promoter.

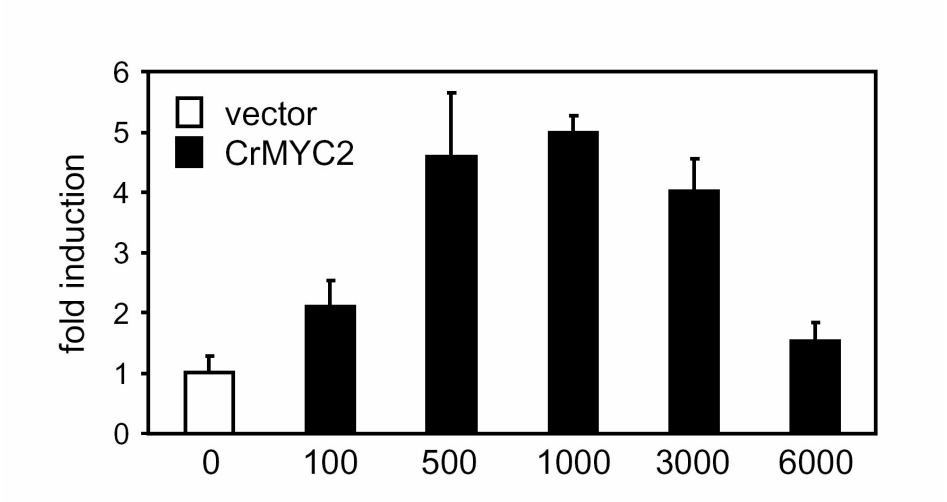

Figure 5. Dose-response relationship for trans-activation of gene expression via the D tetramer by CrMYC2.

C. roseus MP183L cells were transiently co-transformed with a *GUS* reporter construct carrying the *ORCA3* D tetramer, and increasing amounts indicated in ng of an effector plasmid carrying *CrMYC2* under control of the CaMV 35S promoter. Total effector amount was adjusted to 6 µg in all transformations using empty vector plasmid pRT101. Bars represent means + SE (n = 3). GUS activities are shown as fold induction compared with the vector (V) control.

CrMYC2 activates gene expression synergistically with AT-hook proteins but not with other CrMYCs

The quantitative sequence within the *ORCA3* JRE is important for maximum MeJA-responsive expression levels conferred by D tetramers, and this sequence interacts *in vitro* and in yeast with certain members of the AT-hook family of DNA-binding proteins (Vom Endt *et al*., 2007). By themselves, two of these AT-hook proteins trans-activated gene expression via D tetramers very weakly (Vom Endt *et al*., 2007). We asked the question whether AT-hook proteins might function synergistically with CrMYC2 to activate gene expression.

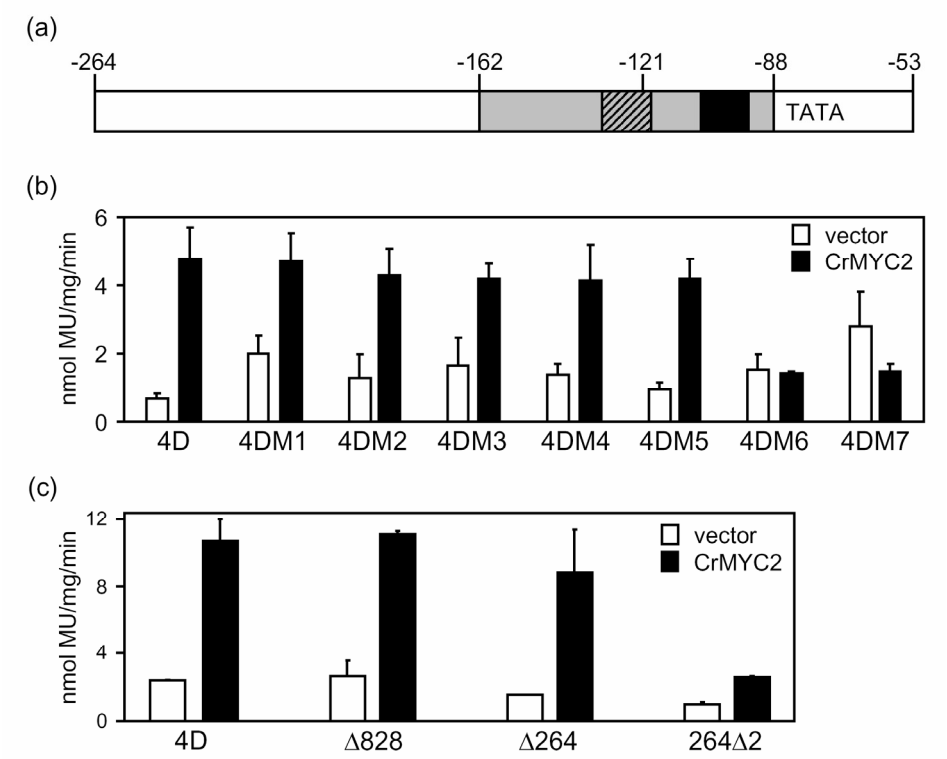

Figure 6. CrMYC2 activates gene expression via interaction with the qualitative sequence within the *ORCA3* JRE.

(a) Schematic representation of the *ORCA3* promoter derivative Δ264. Numbering is relative to the ATG start codon. The D fragment is shaded and the quantitative and qualitative sequences are indicated by striped and black boxes respectively. The position of the TATA box is indicated.

(b) Effect of block mutations in the D tetramer context on CrMYC2 trans-activation. *C. roseus* MP183L cells were transiently co-transformed with *GUS* reporter constructs carrying D wild-type or mutated tetramers, and 500 ng of effector plasmids. Mutations are as in Figure 2a.

(c) *C. roseus* MP183L cells were transiently co-transformed with *GUS* reporter constructs carrying the D tetramer or native *ORCA3* promoter derivatives and 500 ng of effector plasmids. Construct 264Δ2 has a deletion from positions -121 to -88 removing the qualitative sequence. Bars represent means + SE (n = 3).

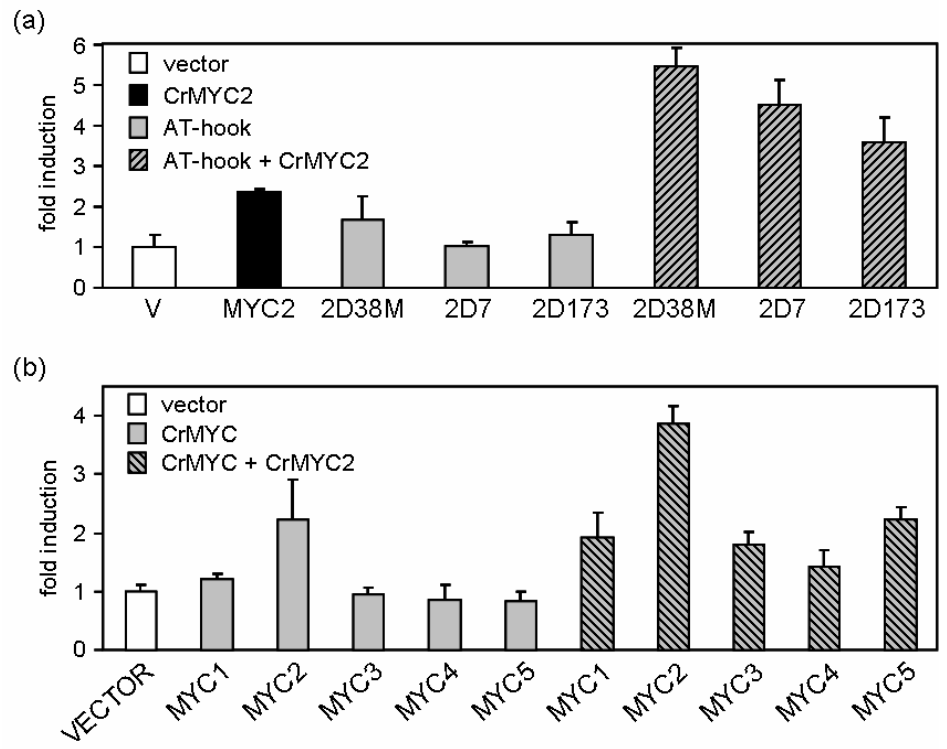

Figure 7. CrMYC2 activates gene expression synergistically with AT-hook proteins but not with other CrMYCs.

(a) *C. roseus* MP183L cells were transiently co-transformed with a *GUS* reporter construct carrying the D tetramer and 100 ng of *CrMYC2* or *AT-hook* (2D38M, 2D7, 2D173) effector plasmids alone or combined as indicated.

(b) *C. roseus* MP183L cells were transiently co-transformed with a *GUS* reporter construct carrying the D tetramer and 100 ng of *CrMYC* (M) effector plasmids alone or combined with 100 ng of *CrMYC2* effector plasmid as indicated. Total effector amount was adjusted to 6 µg in all transformations using empty vector plasmid pRT101. Bars represent means + SE (n = 3). GUS activities are shown as fold induction compared with the vector (V) control.

To be able to observe possible synergistic trans-activation effects, a suboptimal amount of 100 ng of *CrMYC2* effector plasmid was co-bombarded with or without 100 ng of *AT-hook* effector plasmid and the 4D-*GUS* reporter gene. Three different AT-hook proteins for which we had putative full-length cDNA clones were tested. The amount of *CrMYC2* effector plasmid used in this

experimental setup caused an intermediate level of reporter gene expression (Figure 7a). The *AT-hook* effector plasmids caused either no or weak trans-activation of the reporter gene. However, combinations of *CrMYC2* and *AT-hook* effector plasmids caused strong trans-activation to levels that were similar to maximum levels obtained with non-limiting amounts of *CrMYC2* effector plasmid (Figure 5).

bHLH proteins can homo- and hetero-dimerize to form active or inactive complexes (Adhikary and Eilers, 2005). In addition, different bHLH proteins can compete for the same binding site. We wondered whether other CrMYC proteins might form heterodimers with CrMYC2 or might compete with CrMYC2 to modulate gene expression levels. In a similar setup as in Figure 7a, 100 ng amounts of *CrMYC* effector plasmids were co-bombarded with the 4D-*GUS* reporter gene with or without 100 ng of *CrMYC2* effector plasmid. Figure 7b shows that CrMYC2 was the only CrMYC protein that trans-activated the reporter gene at these effector plasmid amounts. Mixing the *CrMYC* effector plasmids with the *CrMYC2* effector plasmid showed that none of the other CrMYCs had a strong positive or negative effect on the level of reporter gene trans-activation by CrMYC2, whereas doubling the amount of *CrMYC2* effector doubled the expression level of the reporter gene.

These results show that limiting amounts of CrMYC2 functioned synergistically with the tested AT-hook family members, whereas there appeared to be no significant positive or negative effects of other CrMYC family members due to possible heterodimerization or competition with CrMYC2.

CrMYC2 does not activate expression of the STR promoter

The *CrMYC* clones including *CrMYC2* were originally isolated in a yeast one-hybrid screen using a tetramerized G-box (CACGTG) from the *STR* promoter as bait (Pré *et al*., 2000). Therefore we wondered whether CrMYC2 may perform a dual role in regulation of JA-responsive expression of this TIA

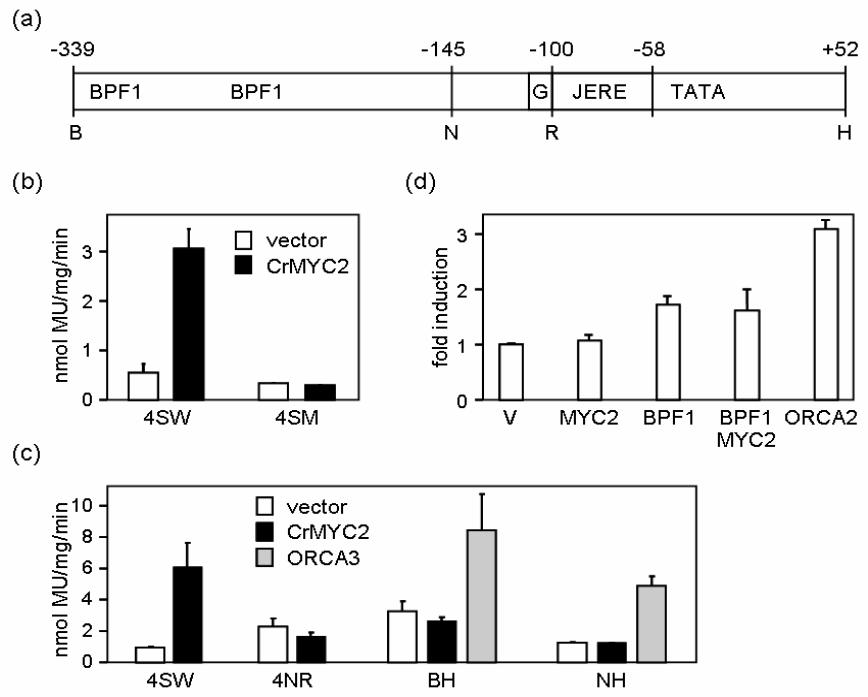

Figure 8. CrMYC2 does not activate expression of the *STR* promoter.

(a) Schematic representation of the *STR* promoter. Letters underneath indicate restriction sites. Numbering is relative to the transcriptional start site. The positions of the TATA box, the G-box, the jasmonate-and elicitor-responsive element (JERE) interacting with the ORCA proteins, and *in vitro* BPF1 binding sites (van der Fits *et al*., 2000) are indicated.

(b) *C. roseus* MP183L cells were transiently co-transformed with a *GUS* reporter construct carrying a tetramer of the wild-type G-box from the *STR* promoter (4SW) or a mutated derivative (4SM) and 500 ng of *CrMYC2* effector plasmids. Bars represent means + SE (n = 3).

(c) *C. roseus* MP183L cells were transiently co-transformed with *GUS* reporter constructs carrying tetramers of the *STR* G-box or the NR fragment or native *STR* promoter derivatives BH and NH and 500 ng of effector plasmids. Bars represent means + SE (n = 3).

(d) *C. roseus* C20D cells were transiently co-transformed with a *GUS* reporter gene carrying the *STR* promoter derivative BH and 3 µg of effector plasmids alone or combined as indicated. Total effector amount was adjusted to 6 µg in all transformations using empty vector plasmid. GUS activities are shown as fold induction compared with the vector (V) control. Bars represent means + SE (n = 4).

biosynthesis gene, indirectly by activating the expression of the *ORCA3* gene and directly by binding to the G-box.

First we tested whether CrMYC2 can trans-activate gene expression via the G-box tetramer. As shown in Figure 8b, this was indeed the case. Trans-activation occurred via specific interaction with the G-box and not via binding to sequences flanking or separating the individual G-boxes, since mutation of 2 nucleotides in the G-box abolished trans-activation. Next we tested whether CrMYC2 can trans-activate gene expression via a tetramer of a larger *STR* promoter fragment of 45 nucleotides containing the G-box (Figure 8a). Surprisingly a *GUS* reporter gene carrying 4 copies of this NR fragment was not trans-activated by CrMYC2 (Figure 8c). Next we tested -339 (BH) and -145 (NH) versions of the native *STR* promoter for trans-activation by CrMYC2. As a positive control we used an *ORCA3* effector plasmid. Whereas ORCA3 trans-activated gene expression via these BH and NH derivatives as reported earlier (Pauw *et al*., 2004; van der Fits and Memelink, 2001), CrMYC2 did not trans-activate these *STR* promoter-*GUS* constructs (Figure 8c).

In activation of promoters from genes encoding enzymes involved in anthocyanin biosynthesis, specific bHLH transcription factors work together with certain members from the MYB family of transcription factors (Mol *et al*., 1998). A MYB-type protein from Catharanthus has been reported to interact with the *STR* promoter (van der Fits *et al*., 2000). Although this BoxP-binding Factor1 (BPF1) is a member of the family of single-repeat (1R) MYBs which are structurally quite different from the R2R3 MYBs which interact with bHLH proteins to activate anthocyanin biosynthesis genes, we nevertheless wanted to test the possibility that BPF1 functions together with CrMYC2 in activating *STR* promoter activity. Cells of cell line C20D were co-bombarded with the BH-*GUS* reporter gene and *BPF1* or *CrMYC2* effector plasmids separately or combined. An *ORCA2* effector plasmid was used as a positive control (Menke *et al*., 1999). The results in Figure 8d show that ORCA2 strongly trans-activated reporter gene activity via the BH promoter, whereas BPF1 gave an intermediate level of trans-activation. Also in this cell line CrMYC2 did not trans-activate gene expression via the *STR* promoter, and the combination with BPF1 had no effect compared to BPF1 alone.

Thus, although CrMYC2 can interact with a tetramer of the G-box from the *STR* promoter in yeast (Pré *et al*., 2000), and could trans-activate gene expression via this tetramer, it did not trans-activate gene expression via native *STR* promoter derivatives containing this same G-box either alone or in combination with the 1R-MYB protein BPF1.

CrMYC2 is essential for MeJA-responsive ORCA expression

To determine whether CrMYC2 is required for MeJA-responsive expression of the *ORCA3* gene, we tried to knock down *CrMYC2* expression via RNA interference (RNAi).

Independent cell lines transformed with either the empty vector pHannibal (Wesley *et al*., 2001) or pHannibal carrying an inverted repeat of a central part of the *CrMYC2* sequence were screened for *CrMYC2* and *ORCA3* expression after treatment with MeJA for 30 min (Figure 9a). Levels of *CrMYC2* and *ORCA3* mRNA were tightly correlated among independent control or RNAi lines. Among the lines transformed with the *CrMYC2* silencing construct, only line #15 showed a marked decrease in the *CrMYC2* and *ORCA3* mRNA levels. No differences were observed between control line #15 and the RNAi line #15 in the levels of *RPS9* mRNA (encoding ribosomal protein S9) or of *CrMYC1* or *CrMYC4* mRNA (Figure 9b). This showed that the RNAi construct did not affect mRNA levels in general and that it specifically knocked down *CrMYC2* mRNA levels.

RNAi-*MYC2* line #15 was analysed in more detail for the effect of knocking down *CrMYC2* expression on MeJA-responsive gene expression using a time course of MeJA treatment (Figure 9b). RNAi-*CrMYC2* line #15 showed a strong reduction in the levels of MeJA-responsive *ORCA3* and *ORCA2* mRNA accumulation compared to the vector control line #15. MeJA-induced *STR* and *TDC* mRNA levels were also negatively affected but to a lesser extent than *ORCA* mRNA levels.

These results show that CrMYC2 is essential for MeJA-responsive *ORCA* gene expression.

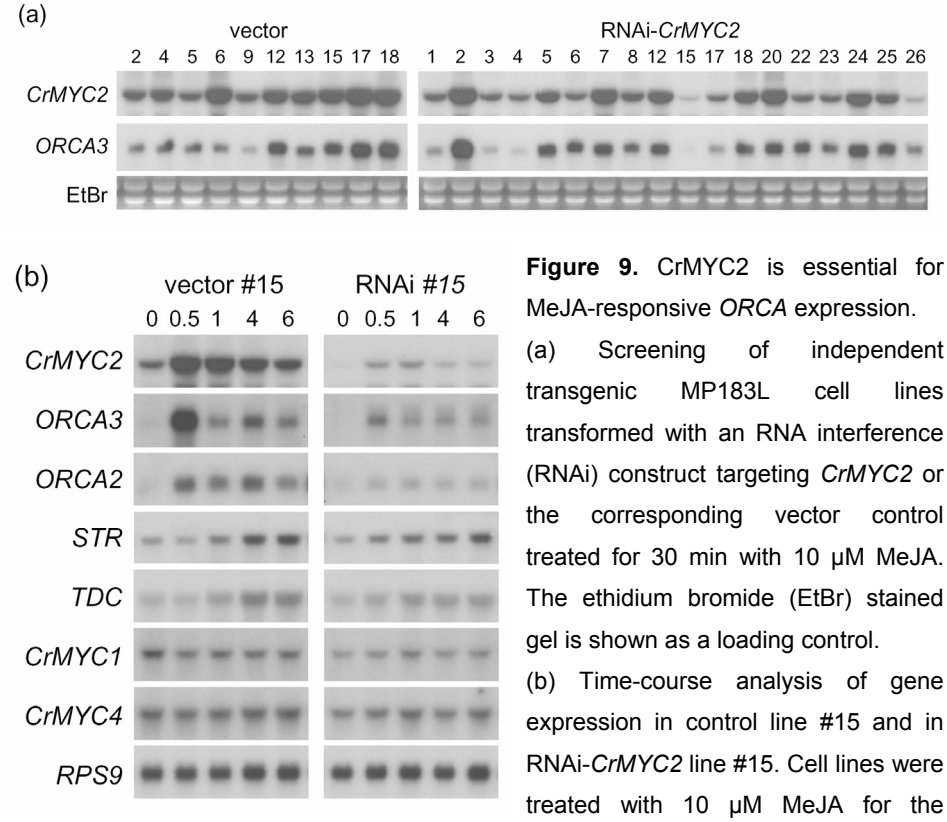

Figure 9. CrMYC2 is essential for MeJA-responsive *ORCA* expression.
(a) Screening of independent transgenic MP183L cell lines transformed with an RNA interference (RNAi) construct targeting *CrMYC2* or the corresponding vector control treated for 30 min with 10 µM MeJA. The ethidium bromide (EtBr) stained gel is shown as a loading control.
(b) Time-course analysis of gene expression in control line #15 and in RNAi-*CrMYC2* line #15. Cell lines were treated with 10 µM MeJA for the number of hours indicated. The *CrMYC2* probe corresponds to an N-

Overexpression of CrMYC2 induces ORCA gene expression

The RNAi experiment demonstrated that CrMYC2 is required for MeJA-responsive *ORCA* gene expression. To determine whether an elevated level of *CrMYC2* expression is sufficient for activation of *ORCA* gene expression, we made stable lines transformed with a construct carrying the *CrMYC2* open reading frame under the control of the CaMV 35S promoter. Screening of independent transgenic lines showed that several lines had moderately elevated *CrMYC2* mRNA levels compared to control lines (Figure 10a). No

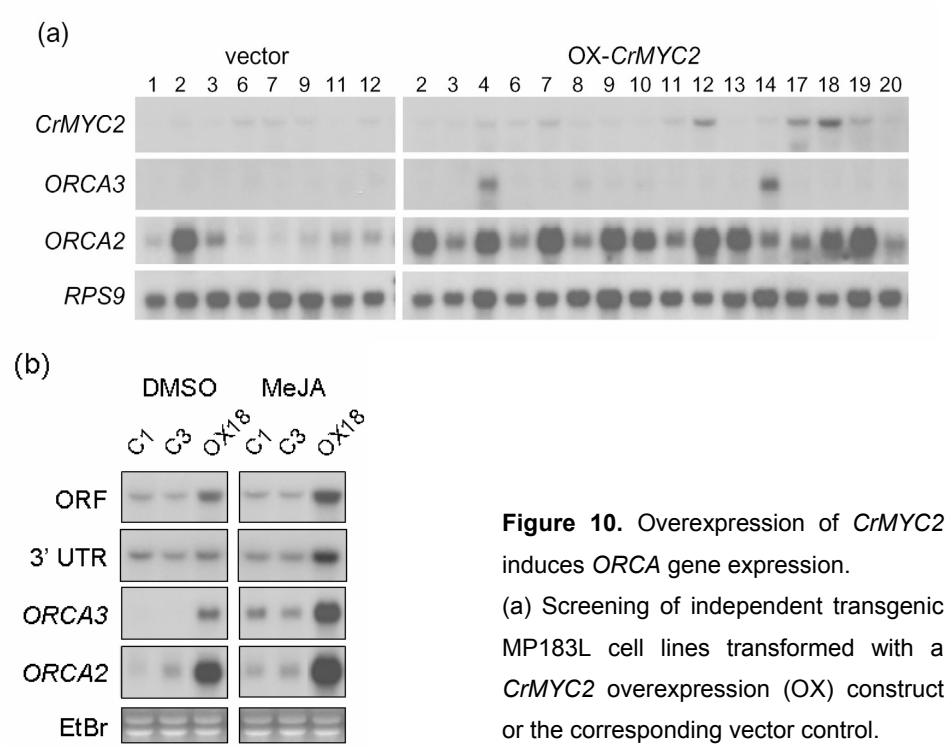

Figure 10. Overexpression of *CrMYC2* induces *ORCA* gene expression.
(a) Screening of independent transgenic MP183L cell lines transformed with a *CrMYC2* overexpression (OX) construct or the corresponding vector control.
(b) Control lines (C) #1 and #3 and overexpression line #18 were treated for 30 min with 0.1% (v/v) of the solvent DMSO or 50 nM MeJA. The ethidium bromide (EtBr) stained gel is shown as a loading control. The probe consisting of the 3' untranslated region (3' UTR) of the *CrMYC2* gene has no overlap with the *CrMYC2* open reading frame (ORF) used for the overexpression constructs.

correlation between the levels of *CrMYC2* mRNA and *ORCA3* mRNA was observed among independent transgenic lines. But it was clear that differences existed in *ORCA* mRNA levels between control lines and overexpression lines. Among 8 independent control lines none showed an elevated *ORCA3* mRNA level and only one an elevated *ORCA2* mRNA level, whereas among 16 independent overexpression lines 2 showed elevated *ORCA3* mRNA levels and 9 showed elevated *ORCA2* mRNA levels.

CrMYC2 overexpression line #18, showing the highest *CrMYC2* mRNA level and an elevated *ORCA2* mRNA level in the initial screening, was further analysed in comparison with control lines #1 and #3. In this second gene

expression analysis, line #18 showed elevated levels of *ORCA2* as well as of *ORCA3* mRNA. Apparently the effect of *CrMYC2* overexpression on downstream gene expression was not stable over time in this line. To determine whether an elevated *CrMYC2* expression level might make the cells more sensitive to MeJA, line #18 was treated for 30 min with 50 nM MeJA. Although this concentration had no effect on *CrMYC2* and *ORCA2* mRNA levels in the control lines, it did cause a slight but significant induction of *ORCA3* expression (Figure 10b). Cells overexpressing *CrMYC2* were more sensitive to MeJA, since they showed induction of *CrMYC2* and *ORCA2* mRNA levels. Induction of *CrMYC2* mRNA accumulation indicates that CrMYC2 auto-regulates its own MeJA-responsive gene expression.

Discussion

We show here that CrMYC2 is the regulator of MeJA-responsive *ORCA* gene expression. This crucial role for CrMYC2 is deduced from the observations that the *CrMYC2* gene is an immediate-early MeJA-responsive gene, that the CrMYC2 protein binds *in vitro* to the qualitative sequence within the *ORCA3* JRE, that it trans-activates gene expression via the same sequence in Catharanthus cells, and that knock-down of the *CrMYC2* mRNA level caused a significant reduction in the level of MeJA-responsive *ORCA* gene expression.

Among the 162 members of the bHLH family in Arabidopsis the closest homologue of CrMYC2 is AtMYC2 (Table S1). AtMYC2 regulates a subset of JA-responsive genes in Arabidopsis (Lorenzo *et al*., 2004). The activity of AtMYC2 has been suggested to be regulated by putative repressors belonging to the family of Jasmonate ZIM-domain (JAZ) proteins (Chini *et al*., 2007). Certain JAZ proteins were shown to bind *in vitro* and in yeast to AtMYC2 (Chini *et al*., 2007; Melotto *et al*., 2008). In addition certain JAZ proteins were shown to be rapidly degraded in response to jasmonate (Chini *et al*., 2007; Thines *et al*., 2007). In tobacco, members of the JAZ family were proposed to act as repressors of MeJA-responsive nicotine biosynthesis by repressing the expression of biosynthesis genes (Shoji *et al*., 2008). Therefore it seems likely

that the activity of CrMYC2 is also modulated by members of the Catharanthus JAZ family.

The levels of *CrMYC2* and *ORCA3* mRNAs were tightly correlated in all analyses of expression levels except for the *CrMYC2* overexpression lines. Induction of *ORCA3* gene expression was just as fast as *CrMYC2* induction, and *ORCA3* is also an immediate-early MeJA response gene since its induction was insensitive to CHX (Figure 1c; van der Fits and Memelink, 2001). Therefore, *ORCA3* induction is not dependent on *de novo* CrMYC2 protein synthesis, but instead is caused by activation of pre-existing CrMYC2 protein. As proposed above, this activation step probably consists of degradation of JAZ repressor proteins which are inhibitors of CrMYC2 activity.

In *CrMYC2* overexpression lines *ORCA3* mRNA levels did not correlate with *CrMYC2* mRNA levels. At first thought tight correlation would maybe be expected in a model where CrMYC2 directly controls *ORCA* gene expression. However, at second thought lack of correlation is not unexpected, when one takes into consideration that CrMYC2 activity is likely to be regulated by JAZ repressors. The expression of *JAZ* genes in Arabidopsis is induced by jasmonates (Thines *et al*., 2007; Chini *et al*., 2007), and is controlled by AtMYC2 (Chini *et al*., 2007). AtMYC2 and JAZ proteins therefore form an oscillator, where JAZ proteins negatively regulate AtMYC2 activity at the protein level and AtMYC2 switches on the expression of JAZ repressors at the gene level. The proposed existence of a similar oscillator in Catharanthus cells would cause unpredictable and unstable CrMYC2 activity upon overexpression, since its activity at any given time point would depend on the relative ratios of the activator CrMYC2 and of the JAZ repressors. The oscillator would also explain our observation that one cell line with relatively high *CrMYC2* mRNA levels had low *ORCA3* mRNA levels in a first screening, but elevated *ORCA3* mRNA levels in a later analysis.

In transient expression assays we found that CrMYC2 works synergistically with certain AT-hook proteins. Mutation of their binding site corresponding to the quantitative sequence in the context of the *ORCA3* D tetramer reduces the MeJA-responsive expression level conferred by this

promoter derivative (Vom Endt *et al*., 2007), corroborating the notion that AT-hook proteins act as co-activators of CrMYC2. The function of this type of AT-hook proteins containing a single AT-hook motif coupled to the domain of unknown function DUF296 in plants is in fact very poorly described. Our results suggest that they may act as enhancers of the activity of other signal-responsive transcription factors without reacting themselves to the signal (Vom Endt *et al*., 2007).

For several plant species it has been shown that bHLH transcription factors regulating genes encoding enzymes of the anthocyanin biosynthesis pathway function together with certain members of the MYB family of transcription factors (Mol *et al*., 1998). Our block mutation scanning of the D region from the *ORCA3* promoter did not uncover a MYB binding site important for the MeJA-responsive expression level (Vom Endt *et al*., 2007). In addition, CrMYC2 was able to trans-activate gene expression via a simple minimal promoter consisting of a multimerized G-box fused to a TATA box. These observations suggest that CrMYC2 does not require a MYB protein partner for its activity.

CrMYC2 was initially isolated in a yeast one-hybrid screening using a tetramer of a G-box from the *STR* promoter as bait (Pré *et al*., 2000). We found that in *C. roseus* cells it was also able to activate gene expression via this tetramer. Surprisingly, CrMYC2 was unable to activate gene expression via native *STR* promoter derivatives. A possible explanation may be that compared to the G-box-like sequence from the *ORCA3* promoter the *STR* G-box is a relatively low affinity binding site which only overcomes the threshold for productive binding upon multimerization. Whatever the explanation may be, it seems unlikely that CrMYC2 directly regulates *STR* expression via this G-box.

We have shown here 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 regulate in turn a subset of alkaloid biosynthesis genes including *TDC* and *STR*.

Transcription factors may form useful tools for engineering the production of valuable secondary metabolites (Gantet and Memelink, 2002).

CrMYC2 may form a very valuable tool, since it occupies the highest position in the regulatory hierarchy and it controls the expression of both the *ORCA2* and *ORCA3* genes and possibly of other genes encoding transcription factors involved in alkaloid biosynthesis. However the usefulness of CrMYC2 would be seriously limited by negative regulation by JAZ repressors. Therefore it is important to determine whether CrMYC2 activity is indeed modulated by JAZ repressors, and if so, whether it is possible to uncouple its activity from this negative regulation.

Experimental procedures

Cell cultures, stable transformation, and treatments

C. roseus cell suspension lines MP183L and BIX were grown in 50 ml of Linsmaier and Skoog (LS) medium containing 88 mM sucrose, 2.7 µM NAA and 0.23 µM kinetin at 28°C in a 16/8 hour light/dark regime. *C. roseus* cell line C20D was grown in 50 ml of Gamborg B5 medium containing 58 mM sucrose and 4.5 µM 2,4-D in the dark at 24°C. Cultures were grown on a rotary shaker at 100-120 rpm. Cultures were maintained by weekly transfer of 5 ml to 45-50 ml of fresh medium. Treatments were 4 d after transfer. MeJA was diluted in DMSO. Control cultures were treated with DMSO at a final concentration of 0.1% (v/v). For stable transformations of cell line MP183L, plasmid constructs of interest were co-transformed with the plasmid pGL2 (Bilang *et al*., 1991) carrying a hygromycin selection gene driven by the CaMV 35S promoter in a ratio of 4:1 by particle bombardment (van der Fits and Memelink, 1997). Transgenic cells were selected on solid medium containing 50 µg/ml hygromycin-B and individual transgenic calli were converted to cell suspensions.

Isolation of full-length cDNA clones

To isolate full-length clones, 5' sequences were isolated by PCR with a gene-specific primer and a vector primer using a pACTII cDNA library of YE-treated

MP183L cells (Menke *et al*., 1999) (*CrMYC2*, *CrMYC3 and CrMYC5*) or a pAD-GAL4-2.1 cDNA library of MeJA-treated MP183L cells (Vom Endt *et al*., 2007) (*CrMYC4*) as templates. The sequences were deposited in GenBank with the accession numbers indicated in parentheses: *CrMYC2* (AF283507), *CrMYC3* (FJ004233), *CrMYC4* (FJ004234) and *CrMYC5* (FJ004235).

Plasmid constructs

The *CrMYC1* open reading frame (ORF) was PCR amplified with the primers 5'-CCT CGA GAT GGA TCA GCA AGC GT CGT AT-3' and 5'-GGA ATT CTC ATG TTG TTC TTT CAA AAC CAC CAC CA-3' using the pACTII cDNA library as template, digested with *Xho*I/*Eco*RI and cloned in pRT101 (Töpfer *et al*., 1987) digested with *Xho*I/*Eco*RI. The *CrMYC2* ORF was amplified with the primers 5'-CCT CGA GAT GAC GGA CTA TAG GCT ACA AC-3' and 5'-CCT CGA GTC TAG ATC ATA CCA AGA GCC TCA TCG AGT TT-3' using the pAD-GAL4-2.1 cDNA library as template, digested with *Xho*I and cloned in pRT101 digested with *Xho*I. This construct was used in most of the transient assays. For the experiment in Figure 5c, *CrMYC2* was amplified with the primers 5'-CCG GTC GAC CCA CTT TTC TCT CCC C-3' and 5'-CCG GTC GAC CCT CCA ATC TAA CAG C-3', digested with *Sal*I and cloned in the expression vector pLBR19 containing a double-enhanced CaMV 35S promoter. The *CrMYC3* ORF was amplified with the primers 5'-GGA ATT CAT GCT TTC TTC ATA TCT CTT TC-3' and 5'-GGA ATT CGG ATC CTC ATA ATA GGT CAC CAT GCA TC-3' using the pACTII cDNA library as template, digested with *Bam*HI/*Eco*RI and cloned in pRT101 digested with *Bam*HI/*Eco*RI. The *CrMYC4* ORF was amplified with the primers 5'-CCT CGA GCA TAT GCA AGC TGG AGG TGG AGG AGG AAA C-3' and 5'-GGA TCC TTA CTC TTT GGC AGA GCA TTT ACA TTT C-3' using the pAD-GAL4-2.1 cDNA library as template, digested with *Bam*HI/*Xho*I and cloned in pRT101 digested with *Bam*HI/*Xho*I. The *CrMYC5* ORF was amplified with the primers 5'-CCT CGA GAA AAT GGC GAA TAT GCA TGG CGG AC-3' and 5'-GGG ATC CTA GAC TCT TTC TCT ACT TGA TAT TAT G-3' using the pACTII cDNA library as template, digested with

*Bam*HI/*Xho*I and cloned in pRT101 digested with *Bam*HI/*Xho*I. The *CrMYC2* RNAi construct consisted of an inverted repeat of the central part of *CrMYC2* in pHannibal (Wesley *et al*., 2001). A *Spe*I/*Bam*HI (positions 1127 to 1669 in GenBank acc.no. AF283507) fragment was cloned in pHannibal digested with *Xba*I/*Bam*HI. An *Spe*I/*Avr*II (positions 1127 to 1685) fragment was first cloned in pIC-20R (Marsh *et al*., 1984) digested with *Xba*I such that the *Avr*II site flanked the *Kpn*I site, re-excised with *Xho*I/*Kpn*I and cloned into the pHannibal silencing construct digested with *Xho*I/*Kpn*I creating the inverted repeat. The *CrBPF1* overexpression construct was made by transferring the *CrBPF1* ORF as a *Bam*HI/*Bgl*II fragment to pMOG184 digested with *Bam*HI. pMOG184 is a pUC18 derivative carrying a double-enhanced CaMV 35S promoter. The NR tetramer construct was made by cloning the *Nsi*I/*Rsa*I (NR) fragment from the *STR* promoter in pIC-19R digested with *Pst*I/*Nru*I. A head-to-tail tetramer was constructed using the flanking compatible *Sal*I/*Xho*I sites from pIC-19R. The NR tetramer was transferred as a *Bam*HI/*Xho*I fragment to GusSH-47 (Pasquali *et al*., 1994) digested with *Bam*HI/*Sal*I. *ORCA3* promoter-*GUS* constructs and *AT-hook* overexpression constructs in pRT101 were described by Vom Endt *et al*. (2007). Wild-type and mutant *STR* G-box tetramer constructs (4SW-GusSH-47 and 4SM-GusSH-47, respectively) were described by Ouwerkerk and Memelink (1999). *GUS* reporter plasmids carrying the *STR* promoter derivatives BH and NH were described by Pasquali *et al*. (1999). The *ORCA2* and *ORCA3* overexpression constructs in pMOG184 (Menke *et al*., 1999) and pRT101 (van der Fits and Memelink, 2001), respectively, were previously described.

Transient expression assays

Cell line MP183L was transformed by particle bombardment as described (van der Fits and Memelink, 1997) using a home-made helium gun and 1.8 µm tungsten particles (Pioneer Hi-Bred, http://www.pioneer.com). C20D cells were transformed similarly using a PDS-1000 Biorad helium gun with 1800 Psi rupture disks (Biorad, http://biorad.com) and tungsten M-25 particles (Biorad). Cells were co-bombarded in triplicate with 2-3 µg of a *GUS* reporter construct

and effector plasmids as indicated in the figure legends. Co-transformations with the empty overexpression vectors served as controls. In transformations of C20D cells 1 µg of 35S-*CAT* reference plasmid was included. GUS and chloramphenicol acetyl transferase (CAT) activity assays were performed as described (van der Fits and Memelink, 1997) and protein concentrations were measured using Bradford protein assay reagent (BioRad). GUS activities in MP183L protein extracts were expressed as nmoles 4-methyl umbelliferone (MU) formed per min per mg protein, whereas GUS activities in C20D protein extracts were related to the corresponding CAT activities. In experimental setups with a single reporter gene GUS activities were depicted as fold induction compared with the vector control.

RNA extraction and Northern blot analyses

Total RNA was extracted from frozen cells by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% ethanol, and resuspended in water. Ten µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose/1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences, http://www.perkinelmer.com). Probes were ^{32}P-labeled by random priming. (Pre-) hybridization and subsequent washings of blots were performed as described (Memelink *et al*., 1994) with minor modifications. cDNAs used as probes were: *ORCA2* (GenBank acc. No. AJ238740), *ORCA3* (AJ251250), *STR* (X61932), *TDC* (M25151), *RPS9* (AJ749993), *CrMYC1* (AF283506), *CrMYC2* (AF283507), and *CrMYC4* (FJ004234). The 3' untranslated region of the *CrMYC2* gene was amplified with the primers 5'-AGG GTA GAT TAA ACT CAA GAA AAG TTG-3' and 5'-AGC AAA CCT CCA ATC TAA CAG CAC AAC-3' using as the partial pACT2-CrMYC2 which was isolated by yeast one hybrid screening and starts at position 787 in Genbank acc. No. AF283507 a template.

Isolation of recombinant CrMYC2 protein and EMSA

The *CrMYC2* ORF was PCR-amplified with the primers 5'-GGT ACC ACA TAT GAC GGA CTA TAG GCT ACA AC-3' and 5'-CTC GAG GGT ACC AAG AGC CTC ATC GAG TTT C-3', digested with *Kpn*I and cloned in pASK-IBA45plus (IBA, http://www.iba-go.com) cut with *Kpn*I. Double Strep/His-tagged protein was expressed in *E. coli* strain BL21 (DE3) pLysS and purified by sequential Ni-NTA agarose (Qiagen, http://www1.qiagen.com) and Strep-Tactin sepharose (IBA) chromatography. For quality analysis the recombinant protein was run on a 10% (w/v) SDS-PAA gel, transferred to Protran nitrocellulose (Whatman, http://www.whatman.com) by semidry electroblotting, and the Western blot was probed with mouse monoclonal RGS-His antibodies (Qiagen) using goat anti-mouse Immunoglobulins-HRP (Dako, http://www.dako.com) as second antibodies. Antibody binding was detected by incubation in 250 µM sodium luminol, 0.1 M Tris-HCl pH 8.6, 3 mM H_2O_2, 67 µM p-coumaric acid and exposure to X-ray film. Monomeric D wild-type and mutant fragments (Vom Endt *et al*., 2007) were isolated from pIC-20H with *Xba*I/*Xho*I and labeled by filling in the overhangs with the Klenow fragment of DNA polymerase I and α-^{32}P-dCTP. DNA-binding reactions contained 0.1 ng of end-labeled DNA fragment, 500 ng of poly(dAdT)-poly(dAdT), binding buffer (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10% v/v glycerol) and protein extract in a 10 µl volume. Following addition of protein extract, reactions were incubated for 30 min at room temperature before loading on 5% w/v acrylamide/bisacrylamide (37:1)-0.5x Tris-Borate-EDTA gel under tension. After electrophoresis at 125 V for 1 hr, the gel was dried on Whatman DE81 paper and autoradiographed.

Acknowledgements

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References

Adhikary, S. and Eilers, M. (2005) Transcriptional regulation and transformation by Myc proteins. *Nat. Rev. Mol. Cell Biol.* **6**, 635-645.

Altschul, S. F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped Blast and PSI-Blast: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.

Alvarez, J. and Smyth, D.R. (1999) *CRABS CLAW* and *SPATULA*, two Arabidopsis genes that control carpel development in parallel with *AGAMOUS*. *Development* **126**, 2377–2386.

Balbi, V. and Devoto, A. (2008) Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. *New Phytol.* **177**, 301-318.

Bilang, R., Iida, S., Peterhans, A., Potrykus, I. and Paszkowski, J. (1991) The 3'-terminal region of the hygromycin-B-resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*. *Gene* **100**, 247-250.

Chatel, G., Montiel, G., Pré, M., Memelink, J., Thiersault, M., Saint-Pierre, B., Doireau, P. and Gantet, P. (2003) CrMYC1, a *Catharanthus roseus* elicitor- and jasmonate-responsive bHLH transcription factor that binds the G-box element of the *strictosidine synthase* gene promoter. *J. Exp. Bot.* **54**, 2587-2588.

Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J.M., Lorenzo, O., García-Casado, G., López-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L. and Solano, R. (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**, 666-671.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.-H., Hong, X., Agarwal, M. and Zhu, J.-K. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* **17**, 1043-1054.

de Pater, S., Pham, K., Memelink, J. and Kijne, J. (1997) RAP-1 is an Arabidopsis MYC-like R protein homologue, that binds to G-box sequence motifs. *Plant Mol. Biol.* **34**, 169-174.

Edwards, D.R. and Mahadevan, L.C. (1992) Protein synthesis inhibitors differentially superinduce c-fos and c-jun by three distinct mechanisms: lack of evidence for labile repressors. *EMBO J.* **11**, 2415–2424.

Gantet, P., Imbault, N., Thiersault, M. and Doireau, P. (1998) Necessity of a functional octadecanoid pathway for indole alkaloid synthesis by *Catharanthus roseus* cell suspensions cultured in an auxin starved medium. *Plant Cell Physiol.* **39**, 220-225.

Gantet, P. and Memelink, J. (2002) Transcription factors: tools to engineer the production of pharmacologically active plant metabolites. *Trends Pharmacol. Sci.* **23**, 563-569.

Gundlach, H., Müller, M.J., Kutchan, T.M. and Zenk, M.H. (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci. USA* **89**, 2389-2393.

Heim, M.A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B. and Bailey, P.C. (2003) The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. *Mol. Biol. Evol.* **20**, 735-747.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J. and Solano, R. (2004) *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell* **16**, 1938-1950.

Marsh, J.L., Erfle, M. and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene* **32**, 481-485.

Melotto, M., Mecey, C., Niu, Y., Chung, H.S., Katsir, L., Yao, J., Zeng, W., Thines, B., Staswick, P., Browse, J., Howe, G.A. and He, S.Y. (2008) A

critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. *Plant J.*, doi: 10.1111/j.1365-313X.2008.03566.x.

Memelink, J., Swords, K.M.M., Staehelin, L.A. and Hoge, J.H.C. (1994) Southern, Northern and Western blot analysis. In *Plant Molecular Biology Manual* (Gelvin, S.B., Schilperoort, R.A. and Verma, D.P.S., eds), Dordrecht: Kluwer Academic Publishers, pp. F1-F23.

Memelink, J., Verpoorte, R. and Kijne, J.W. (2001) ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. *Trends Plant Sci.* **6**, 212-219.

Menke, F.L.H., Champion, A., Kijne, J.W. and Memelink, J. (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *EMBO J.* **18**, 4455-4463.

Mol, J., Grotewold, E. and Koes, R. (1998) How genes paint flowers and seeds. *Trends Plant Sci.* **3**, 212-217.

Ouwerkerk, P.B.F. and Memelink, J. (1999) A G-box element from the *Catharanthus roseus* strictosidine synthase (*Str*) gene promoter confers seed-specific expression in transgenic tobacco plants. *Mol. Gen. Genet.* **261**, 635-643.

Pasquali, G., Ouwerkerk, P.B.F. and Memelink, J. (1994) Versatile transformation vectors to assay the promoter activity of DNA elements in plants. *Gene* **149**, 373-374.

Pasquali, G., Erven, A.S.W., Ouwerkerk, P.B.F., Menke, F.L.H. and Memelink, J. (1999) The promoter of the *strictosidine synthase* gene from periwinkle confers elicitor-inducible expression in transgenic tobacco and binds nuclear factors GT-1 and GBF. *Plant Mol. Biol.* **39**, 1299-1310.

Pauw, B., Hilliou, F.A.O., Sandonis Martin, V., Chatel, G., de Wolf, C.J.F., Champion, A., Pre, M., van Duijn, B., Kijne, J.W., van der Fits, L. and Memelink, J. (2004) Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in *Catharanthus roseus*. *J. Biol. Chem.* **279**, 52940-52948.

Pré, M., Sibéril, Y., Memelink , J., Champion, A., Doireau, P. and Gantet, P. (2000) Isolation by the yeast one-hybrid system of cDNAs encoding transcription factors that bind to the G-box element of the *strictosidine synthase* gene promoter from *Catharanthus roseus*. *Int. J. Bio-chrom.* **5**, 229-244.

Szécsi, J., Joly, C., Bordji, K., Varaud, E., Cock, J.M., Dumas, C. and Bendahmane, M. (2006) BIGPETALp, a bHLH transcription factor is involved in the control of Arabidopsis petal size. *EMBO J.* **25**, 3912-3920.

Shoji, T., Ogawa, T. and Hashimoto, T. (2008) Jasmonate-induced nicotine formation in tobacco is mediated by tobacco *COI1* and *JAZ* genes. *Plant Cell Physiol.* **49**, 1003-1012.

Solano, R., Stepanova, A., Chao, Q. and Ecker, J.R. (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE RESPONSE-FACTOR1. *Genes Dev.* **12**, 3703-3714.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A. and Browse, J. (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. *Nature* **448**, 661-665.

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H.-H. (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Res.* **15**, 5890.

Turner, J.G., Ellis, C. and Devoto, A. (2002) The jasmonate signal pathway. *Plant Cell* **14**, S153-S164.

van der Fits, L. and Memelink, J. (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. *Plant Mol. Biol.* **33**, 943-946.

van der Fits, L. and Memelink, J. (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science* **289**, 295-297.

van der Fits, L. and Memelink, J. (2001) The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. *Plant J.* **25**, 43-53.

van der Fits, L., Zhang, H., Menke, F.L.H., Deneka, M. and Memelink, J. (2000) A *Catharanthus roseus* BPF-1 homologue interacts with an elicitor-responsive region of the secondary metabolite biosynthetic gene *Str* and is induced by elicitor via a JA-independent signal transduction pathway. *Plant Mol. Biol.* **44**, 675-685.

Vom Endt, D., Soares e Silva, M., Kijne, J.W., Pasquali, G. and Memelink, J. (2007) Identification of a bipartite jasmonate-responsive promoter element in the *Catharanthus roseus ORCA3* transcription factor gene that interacts specifically with AT-Hook DNA-binding proteins. *Plant Physiol.* **144**, 1680-1689.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**, 581-590.

Chapter 4

CrJAZ proteins repress CrMYC2 activity and jasmonate-responsive gene expression in *Catharanthus roseus*

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ABSTRACT

Jasmonates are plant signalling molecules that play key roles in defence against insects and certain pathogens, among others by controlling the biosynthesis of protective secondary metabolites. In *Catharanthus roseus*, jasmonate-responsive expression of terpenoid indole alkaloid biosynthesis genes is controlled by a transcription factor cascade consisting of the basic Helix-Loop-Helix (bHLH) protein CrMYC2 regulating *ORCA* gene expression, and the Octadecanoid-derivative Responsive Catharanthus APETALA2-domain transcription factors ORCA2 and ORCA3, which regulate in turn a subset of alkaloid biosynthesis genes. Here we show that the activity of CrMYC2 is repressed by members of the Jasmonate ZIM (JAZ) family of proteins. CrJAZ1 and CrJAZ2 interacted with CrMYC2 in yeast, and repressed CrMYC2 activity in transient trans-activation assays. The N-terminal domain of CrJAZ1 was necessary and sufficient for CrMYC2 repression. CrJAZ1 was shown to be degraded via the 26S proteasome in response to jasmonate, whereas a deletion derivative lacking the C-terminal Jas domain was stable. Overexpression of this CrJAZ1ΔC derivative negatively affected the jasmonate-responsive expression of the genes *CrJAZ1*, *CrJAZ2*, *CrMYC2*, *ORCA2*, *ORCA3* and the genes encoding the alkaloid biosynthetic enzymes tryptophan decarboxylase (TDC) and strictosidine synthase (STR). Jasmonate-responsive *CrJAZ* gene expression was controlled by CrMYC2. Our current model is that CrMYC2 is inhibited by CrJAZs. Upon jasmonate-responsive CrJAZ degradation, de-repressed CrMYC2 induces the expression of the *ORCA* genes, which in turn regulate a subset of alkaloid biosynthesis genes. CrMYC2 also activates the expression of the *CrJAZ* genes, leading to de novo synthesis of CrJAZ proteins and re-repression of CrMYC2.

INTRODUCTION

Jasmonates constitute a family of bioactive oxylipins that are involved in the regulation of a number of processes in plants, including certain developmental processes, senescence, and responses to wounding and pathogen attack (Turner *et al*., 2002; Balbi and Devoto, 2008). Bioactive jasmonates include jasmonic acid (JA), methyl-JA (MeJA), 12-oxo-phytodienoic acid (OPDA) and the isoleucine conjugate JA-Ile (Wasternack, 2007). In addition, a bacterial toxin called coronatine which is a structural and functional mimic of JA-Ile is commonly used in jasmonate-related research.

An important defence response that depends on jasmonates as regulatory signals is the induction of secondary metabolite accumulation (Gundlach *et al*., 1992; Memelink *et al*., 2001). Jasmonates induce secondary metabolism at the transcriptional level by switching on the coordinate expression of a set of biosynthesis genes (Memelink *et al*., 2001).

MeJA stimulates terpenoid indole alkaloid (TIA) metabolism in cell suspensions of *Catharanthus roseus* (Gantet *et al*., 1998) and induces the expression of all of the TIA biosynthesis genes tested (van der Fits and Memelink, 2000). The MeJA-responsive expression of a number of these biosynthesis genes, including the strictosidine synthase (*STR*) and the tryptophan decarboxylase (*TDC*) genes, is controlled by the transcription factor Octadecanoid-derivative Responsive Catharanthus AP2-domain protein 3 (ORCA3) (van der Fits and Memelink, 2000). ORCA3 and the related transcription factor ORCA2 (Menke *et al*., 1999) contain a DNA-binding domain of the APETALA2/Ethylene Response Factor (AP2/ERF) type.

The expression of the *ORCA* genes themselves is rapidly induced by MeJA (Menke *et al*., 1999; van der Fits and Memelink, 2001), which is controlled by the basic Helix-Loop-Helix (bHLH) transcription factor CrMYC2. The *ORCA3* promoter contains an autonomous jasmonate-responsive element (JRE), which is composed of 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 (Vom Endt *et al*., 2007). CrMYC2 binds to the qualitative

sequence in the *ORCA3* JRE *in vitro*, and trans-activates reporter gene expression via this sequence in transient assays.

CrMYC2 is the Catharanthus orthologue of AtMYC2, which regulates a subset of jasmonate-responsive genes in *Arabidopsis thaliana* (Lorenzo et al., 2004). The activity of AtMYC2 has been proposed to be regulated by putative repressors belonging to the Jasmonate ZIM-domain (JAZ) family of proteins (Chini et al., 2007; Chico et al., 2008), a subfamily of the tify protein family (Vanholme et al., 2007). Several AtJAZ proteins were shown to bind to AtMYC2 in yeast (*Saccharomyces cereviseae*; Chini et al., 2007; Melotto et al, 2008) and in vitro (Chini et al., 2007). In addition these JAZ proteins were shown to bind to the F-box protein CORONATINE-INSENSITIVE1 (COI1) in yeast (Thines et al., 2007; Melotto et al., 2008), but only when JA-Ile or coronatine were included in the growth medium. COI1 is the receptor for JA-Ile (Thines et al., 2007; Katsir et al., 2008). In response to jasmonates several JAZ proteins were shown to be rapidly degraded via the 26S proteasome in a COI1-dependent manner (Chini et al., 2007; Thines et al., 2007). The expression of the *AtJAZ* genes themselves is induced by jasmonates (Chini et al., 2007; Thines et al., 2007), which is controlled by AtMYC2 (Chini et al., 2007). The current model therefore is that AtMYC2 is inhibited by JAZs and becomes active upon COI1-mediated degradation in response to JA-Ile and induces the expression of the *JAZ* genes, leading to de novo synthesis of JAZ proteins and re-repression of AtMYC2 (Chico et al., 2008).

The biosynthesis of nicotine and related alkaloids in tobacco (*Nicotiana tabacum*) is induced by jasmonates (Imanishi et al., 1998; Memelink et al., 2001; Goossens et al., 2003; Katoh et al., 2005; Shoji et al., 2008). MeJA induces a number of alkaloid biosynthesis genes in tobacco plants (Shoji et al., 2000; Memelink et al., 2001; Katoh et al., 2005) and in suspension-cultured cells (Imanishi et al., 1998; Goossens et al., 2003). MeJA-responsive nicotine biosynthesis is controlled by NtCOI1 and depends on degradation of members of the NtJAZ repressor family (Shoji et al., 2008), showing that JAZ proteins are conserved components of jasmonate signalling in plants.

Here we show that members of the JAZ family in *C. roseus* repress the activity of CrMYC2 and the MeJA-responsive expression of downstream genes including the *ORCA*, *TDC* and *STR* genes.

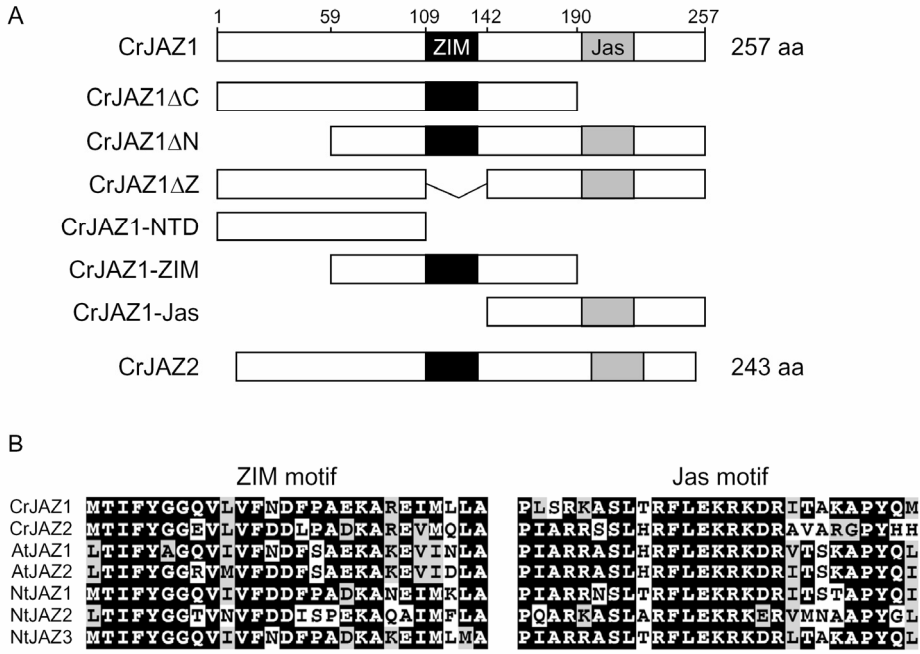

Figure 1. JAZ proteins from *C. roseus*. A, Schematic representation of CrJAZ1 and CrJAZ2 proteins and truncated versions. Numbers indicate amino acid (aa) positions. Relative sizes and positions of conserved ZIM and Jas motifs are shown. B, Alignment of deduced amino acid sequences of the ZIM and Jas motifs from CrJAZs, tobacco JAZs (NtJAZ) and the most similar JAZ proteins from Arabidopsis (AtJAZ). Black, identical residues; grey, conservative substitutions.

RESULTS

JAZ genes from *Catharanthus roseus* are MeJA-responsive

Two cDNA- amplified fragment length polymorphism (AFLP) fragments (CRG294 and CRG331) corresponding to MeJA-responsive *C. roseus* genes encoding proteins with ZIM motifs have been reported previously (Rischer et al., 2006). We isolated full-length cDNA clones via PCR using a Catharanthus

cDNA library as a template. Analysis of the encoded proteins corresponding to tags CRG331 and CRG294 showed that they were most similar to AtJAZ1 and AtJAZ2, respectively, with overall amino acid identities of 38% and 35%. The proteins were named CrJAZ1 and CrJAZ2. Both proteins contain ZIM and Jas motifs (Fig.1A, B), which are highly similar to corresponding motifs from the AtJAZ proteins (Chini et al., 2007; Thines et al., 2007) and from NtJAZ proteins from *Nicotiana tabacum* (Shoji et al., 2008).

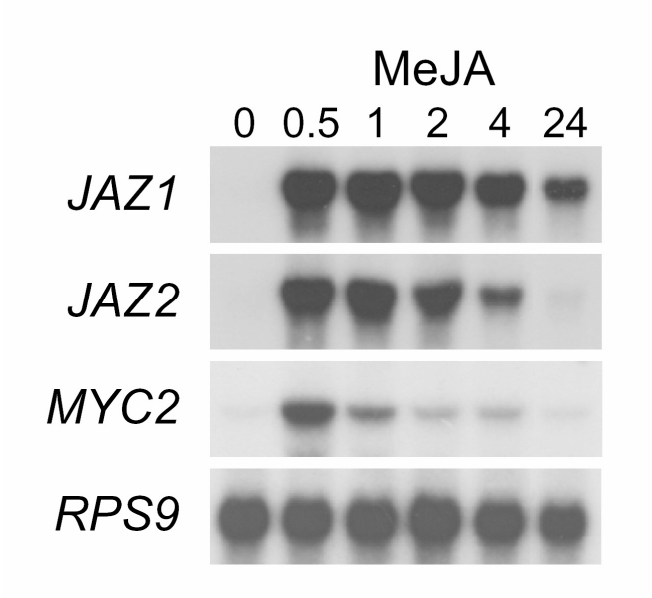

Figure 2. *CrJAZ* gene expression is induced by MeJA. *C. roseus* MP183L cells were treated with 10 µM MeJA for the number of hours indicated. Northern blots were hybridized with the probes indicated at the left.

First we confirmed by Northern blot analysis that *CrJAZ* gene expression is induced by MeJA as suggested by the amplification of MeJA-inducible cDNA-AFLP tags (Rischer et al., 2006). *CrJAZ* mRNAs had low basal levels and accumulated rapidly and transiently after MeJA addition (Fig. 2). The expression of the *JAZ* genes was similar to *CrMYC2* (Fig.2) and *ORCA3* (van der Fits and Memelink, 2001) with the same kinetics**.**

MYC2 interacts with the N-terminal and C-terminal domains of JAZ1 in yeast

Several AtJAZ proteins including AtJAZ1 were shown to interact with the bHLH transcription factor AtMYC2 in yeast (Chini et al., 2007; Melotto et al., 2008).

Therefore we tested whether similar interactions occur between the CrJAZ proteins and CrMYC2, the Catharanthus orthologue of AtMYC2.

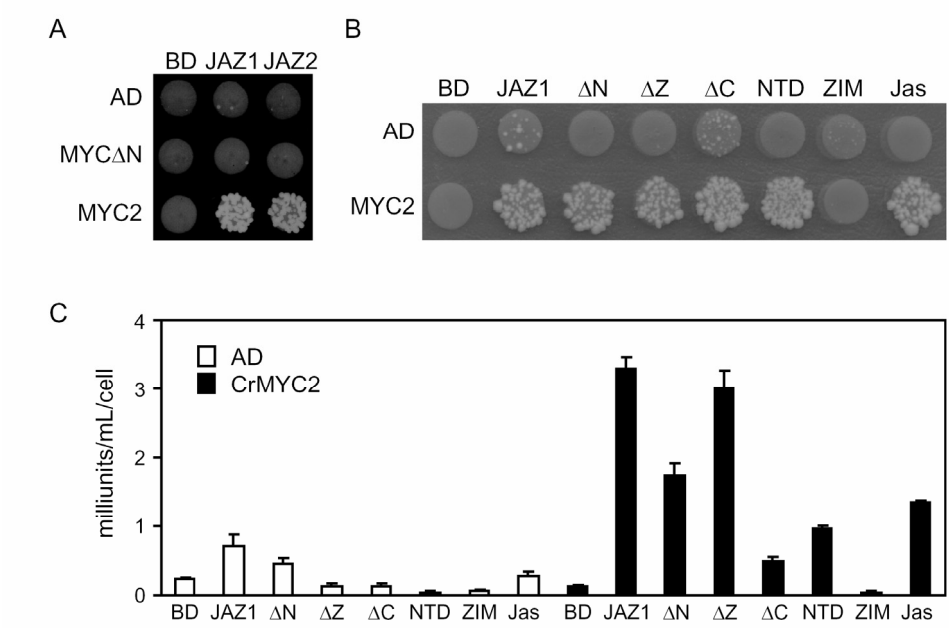

Figure 3. CrMYC2 interacts with the N-terminal and C-terminal domains of CrJAZ1 in yeast. A, CrJAZs interact with the N-terminal part of CrMYC2. Yeast cells expressing the GAL4AD fused to CrMYC2 and the GAL4DBD fused to the indicated CrJAZ were spotted on SD medium selecting for expression of the *ADE* reporter gene. B, Yeast two-hybrid assay of CrMYC2 and CrJAZ1 interaction. Schematic drawings of CrJAZ1 derivatives are shown in Figure 1A. Yeast transformations with the indicated plasmid combinations were spotted on SD medium selecting for expression of the *ADE* reporter gene. Pictures were taken after 5 days of growth. C, α-Galactosidase activity encoded by the GAL4-controlled *MEL1* gene excreted by yeast cells containing the indicated plasmid combinations. Bars represent means + SE (n = 3). The empty pACT2 and pGBT9 vectors were used as activation domain (AD) and binding domain (BD) controls, respectively.

Full-length CrJAZ1 had a low auto-activation activity (Fig. 3A), which was probably due to the ZIM domain since the ZIM domain alone also slightly auto-activated *ADE* reporter gene expression (Fig. 3B). As shown in Fig. 3A,

both CrJAZ proteins interacted with CrMYC2 in yeast. Deletion of the N-terminal domain of CrMYC2 abolished the interaction, indicating that similar to its Arabidopsis counterpart (Chini et al., 2007) CrMYC2 interacts with JAZ proteins via its N-terminal domain. Next we performed a detailed interaction analysis of deletion derivatives of CrJAZ1 with structures shown schematically in Fig. 1A to determine which domain(s) interacted with CrMYC2. As shown in Figs. 3B and Fig. 3C, deletions of either the N-terminal domain or the ZIM or Jas motifs did not abolish interaction in yeast, indicating that at least two distinct domains in CrJAZ1 interacted with CrMYC2. Indeed, when the domains were directly tested for interaction, both the N-terminal domain and the non-overlapping Jas domain interacted with CrMYC2, whereas the ZIM domain showed no interaction. Quantitative measurements of *MEL1* reporter gene activity suggested that the interaction with the C-terminal Jas domain was the strongest in yeast, since deletion of this domain had a stronger negative effect on reporter gene activity than deletion of the N-terminal domain, and since it stimulated reporter gene activity more strongly than the N-terminal domain when tested directly for interaction with CrMYC2 (Fig. 3C).

AtJAZ3 has been reported to interact with AtMYC2 in vitro and in yeast through its C-terminal domain only (Chini et al., 2007). We wondered whether the difference in interaction domains reflected differences between CrJAZs and AtJAZs, or instead differences between JAZ1 and JAZ3. Therefore we tested the interaction between AtMYC2 and different deletion derivatives of AtJAZ1. As shown in Fig. 4A, both the N-terminal and C-terminal parts of AtJAZ1 interacted with AtMYC2, indicating that this dual interaction is a conserved property of JAZ1 proteins and that AtJAZ1 and AtJAZ3 have different modes of interaction with AtMYC2.

JAZ proteins repress MYC2 activity

The AtJAZ proteins have been suggested to act as repressors based on indirect evidence (Chini et al., 2007; Thines et al., 2007), but this has never been directly shown. This prompted us to test the effect of CrJAZ proteins on

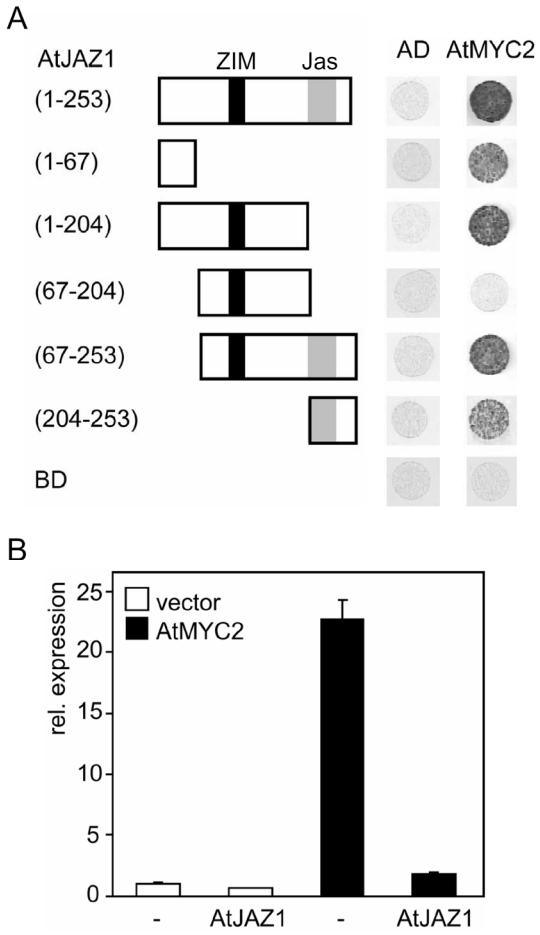

Figure 4. AtJAZ1 interacts with AtMYC2 in yeast and represses AtMYC2 activity in tobacco protoplasts. A, AtMYC2 interacts with the N-terminal and C-terminal domains of AtJAZ1 in yeast. Schematic drawings represent AtJAZ1 and truncated versions. Numbers indicate amino acid positions. Relative sizes and positions of conserved ZIM and Jas motifs are shown. Yeast cells expressing GAL4AD fused to AtMYC2 and the GAL4DBD fused to the indicated AtJAZ1 derivative were spotted on SD medium selecting for the expression of the *HIS* reporter gene. The empty pGAD424 and pGBT9 vectors were used as activation domain (AD) and binding domain (BD) controls, respectively. B, AtJAZ1 represses AtMYC2 activity. Tobacco BY-2 protoplasts were co-transfected with a *fLUC* reporter construct carrying an *AtJAZ1* promoter fragment, an *rLUC* normalization construct, *AtMYC2* effector plasmid and *AtJAZ1* effector plasmid as indicated. Bars represent means + SE (n=8). Normalized fLUC activities are shown as relative expression compared with the control effector plasmid.

CrMYC2 activity. As a read-out for CrMYC2 activity we used the expression of a β-glucuronidase (*GUS*) reporter gene driven by 4 copies of the D fragment from the *ORCA3* promoter (Vom Endt et al., 2007). CrMYC2 was shown to interact in vitro with a qualitative regulatory sequence containing a T/G-box in the D fragment, and to trans-activate reporter gene expression via this sequence in vivo (Chapter 3).

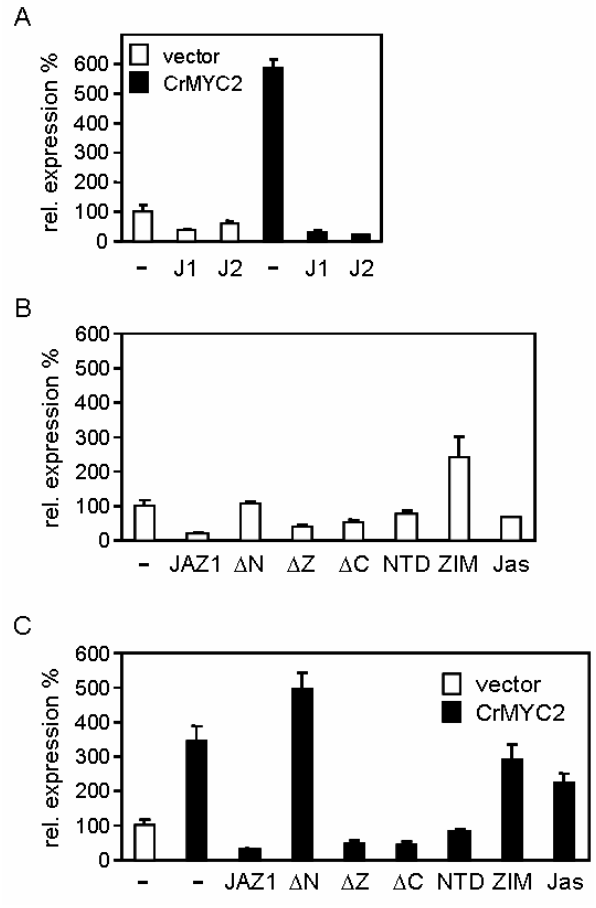

Figure 5. CrJAZ proteins repress CrMYC2 activity. A, CrJAZ proteins repress CrMYC2 activity. *C. roseus* cells were transiently co-transformed with a *GUS* reporter construct carrying the D tetramer, an effector plasmid carrying *CrMYC2* and *CrJAZ1* (J1) or *CrJAZ2* (J2) effectors as indicated. B, The ZIM domain is an activator of reporter gene expression. *C. roseus* cells were transiently co-transformed with the 4D-*GUS* reporter construct and effectors carrying *CrJAZ1* derivatives with structures as shown in Figure 1A. C, The N-terminal domain of CrJAZ1 is important for repression of CrMYC2 activity. *C. roseus* cells were transiently co-transformed with the 4D-*GUS* reporter construct, *CrMYC2* effector plasmid and effectors carrying *CrJAZ1* derivatives with structures as shown in Figure 1A. Bars represent means + SE (n = 3). Normalized GUS activities are shown as relative expression compared with the vector control. Panels B and C are from the same experiment.

First we tested whether CrJAZs functioned as repressors of CrMYC2 activity. As shown in Fig. 5A, addition of *CrJAZ* effectors resulted in a strong repression of CrMYC2 activity. *CrJAZ* effectors had also an inhibiting effect on basal reporter gene activity, presumably by inhibition of endogenous CrMYC2 activity. We then proceeded to determine which domains in CrJAZ1 are necessary for repression of CrMYC2 activity. When various *CrJAZ1* derivatives were tested as effectors in the absence of CrMYC2, we made the surprising discovery that the ZIM domain alone acted as an activator of *GUS* reporter gene expression (Fig. 5B). As discussed below we hypothesise that the overexpression of the ZIM domain titrates out a negative regulatory protein via a process coined “squelching” (Gill and Ptashne, 1988). When the *CrJAZ1* derivatives were combined with *CrMYC2* (Fig. 5C), the full-length protein was effective as a repressor as shown in Fig. 5A. Deletion of the N-terminal domain abolished repressor activity, whereas deletion of the ZIM domain did not affect repressor activity. When the individual domains were tested as effectors, the N-terminal domain worked as a repressor, whereas the ZIM domain and the Jas domain had little effect on CrMYC2 activity. Although the ZIM domain by itself worked as an activator, it did not enhance the activity of co-expressed CrMYC2.

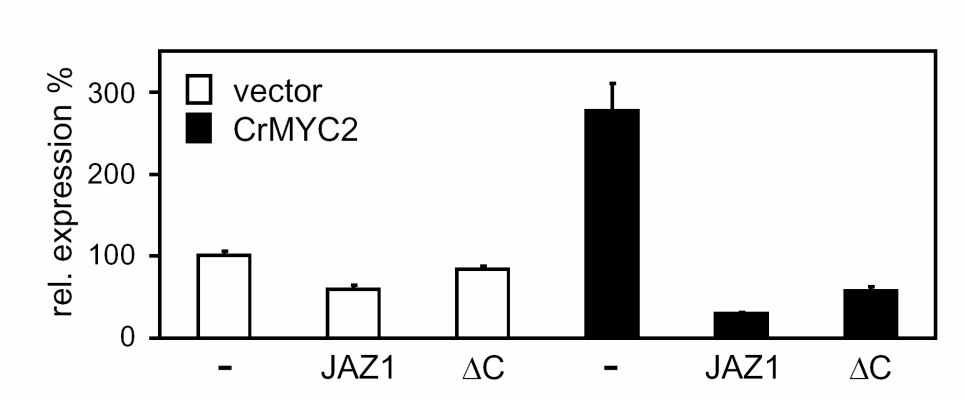

Figure 6. CrJAZ1-GFP fusion proteins repress CrMYC2 activity. A, *C. roseus* MP183L cells were transiently co-transformed with a *GUS* reporter construct carrying the *ORCA3* D tetramer, *CrMYC2* effector plasmid and *CrJAZ1-GFP* or *CrJAZ1ΔC-GFP* effectors. Bars represent means + SE (n = 3). Normalized GUS activities are shown as relative expression compared with the vector control.

The results show that the full-length CrJAZ proteins worked as repressors of CrMYC2 activity, and that the N-terminal but not the C-terminal domain of CrJAZ1 was necessary and sufficient for repressor activity.

To establish whether full-length AtJAZ1 similarly repressed AtMYC2 activity, we tested the activity of a firefly luciferase (*fLUC*) reporter gene fused to part of the *AtJAZ1* promoter in transient assays in tobacco protoplasts. The results in Fig. 4B show that AtMYC2 strongly activated the *AtJAZ1* promoter consistent with its proposed role as a regulator of *AtJAZ* gene expression. Addition of *AtJAZ1* effector together with *AtMYC2* effector caused a strong repression of *AtJAZ1* promoter activity down to the basal level. These results show that analogous to its *C. roseus* counterparts full-length AtJAZ1 was a potent repressor of AtMYC2 activity.

Full-length CrJAZ1 but not CrJAZ1ΔC is rapidly degraded by the 26S proteasome in response to JA

For AtJAZ (Chini et al., 2007; Thines et al., 2007) and NtJAZ (Shoji et al., 2008) proteins it was shown that they are rapidly degraded via the 26S proteasome in response to jasmonate. Deletion of the C-terminal domain resulted in stable proteins. We wanted to determine whether similar mechanisms apply to the CrJAZ proteins. Cells of *C. roseus* cell line MP183L were stably transformed with a control *GFP* construct or with constructs carrying *GFP* fused to the C-terminus of *JAZ1* or *JAZ1ΔC*. To verify that the JAZ1 derivatives were active as GFP fusions, we tested their activities as repressors of CrMYC2 activity and found that they were active in these transient assays (Fig. 6). Stably transformed transgenic cell lines with high expression levels of *GFP* constructs were selected (Fig. 9). Analysis of the cell lines via confocal laser scanning microscopy showed that the JAZ1-GFP fusion was localized in the nuclei (Fig. 7) but excluded from the nucleoli (Fig. 8 and data not shown). GFP in contrast was found everywhere in the cells including in the nuclei except for the nucleoli. The localization of the JAZ1ΔC-GFP fusion was different from that of the JAZ1-

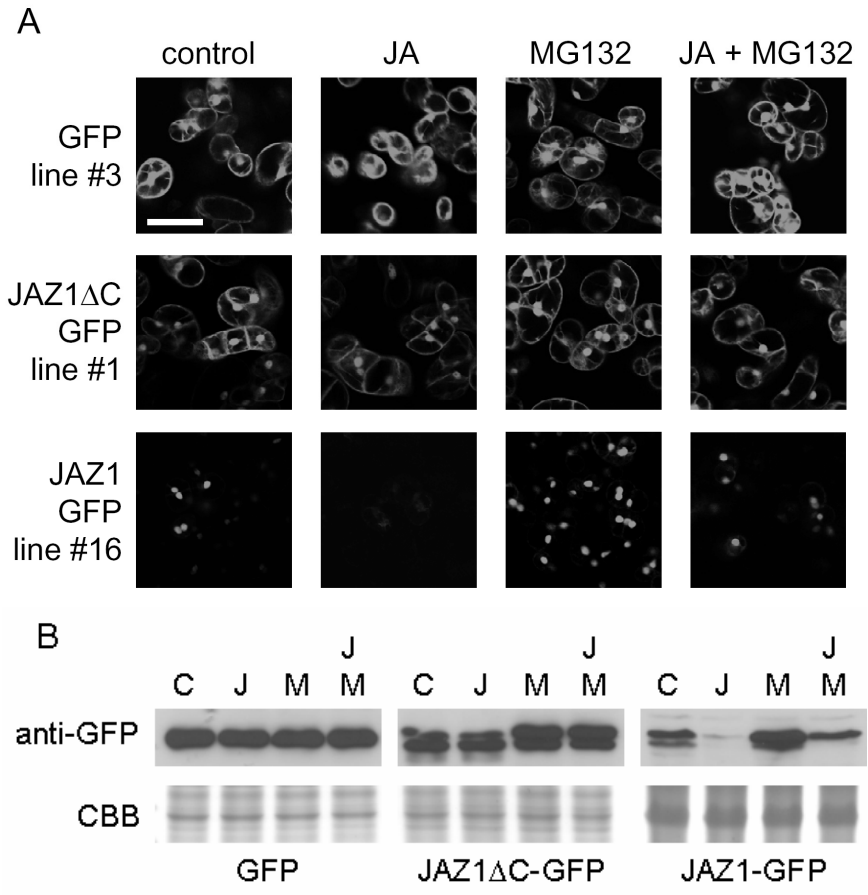

Figure 7. Full-length CrJAZ1 but not CrJAZ1ΔC is rapidly degraded by the 26S proteasome in response to JA. A, *C. roseus* MP183L cell lines stably transformed with *GFP*, *CrJAZ1ΔC-GFP* or *CrJAZ1-GFP* were treated with 50 µM MG132 without or with 50 µM JA for 3 hours. MG132 was added 1 hour before JA addition. DMSO concentrations in all treatments were adjusted to 0.1% (v/v). Cells were viewed by confocal laser scanning microscopy at 20x magnification with identical settings for each cell line. Each picture shows a representative independent cell sample. The size bar in the upper left picture corresponds to 100 µm. B, Western blot of proteins extracted from the cell lines and treatments as in panel A. Protein samples of 10 µg (GFP and JAZ1ΔC-GFP) or 50 µg (JAZ1-GFP) were separated by SDS-PAGE and either stained with coomassie brilliant blue (CBB) or Western blotted with anti-GFP antibodies. The most abundant protein band from the CBB-stained gels with an estimated molecular mass of 38 kD is shown as a loading control.

GFP fusion. It was present both in the nucleus (except the nucleoli) and the cytoplasm with a similar distribution as GFP although it appeared to be more preferentially localized in the nucleus than GFP.

We then analysed the effects of JA and MG132 on localization and abundance of the proteins. Both compounds had no effect on GFP. The JAZ1-GFP protein was stabilized by MG132 alone, indicating that it underwent continuous turnover via the 26S proteasome. Treatment with JA resulted in degradation of JAZ1-GFP. This occurred via the 26S proteasome since simultaneous application of MG132 inhibited JA-induced degradation. The JAZ1ΔC-GFP fusion was not degraded upon JA treatment.

Experiments with transiently transformed *C. roseus* cells showed similar results (Fig. 8). These experiments also showed predominant nuclear localization of the full-length JAZ1-GFP fusion, whereas the localization of the JAZ1ΔC-GFP fusion was similar to GFP. Whereas the full-length JAZ1-GFP fusion was almost totally degraded within 2 hours after addition of MeJA, the JAZ1ΔC-GFP fusion was relatively stable and similar to GFP.

To obtain a more quantitative picture reflecting the total cellular protein amounts after different treatments we performed a Western blot analysis with anti-GFP antibodies of the cell lines treated as in Fig. 7A. As shown in Fig. 7B, GFP amounts were not affected by the treatments. The JAZ1ΔC-GFP derivative was stable after JA treatment, and became somewhat more abundant after MG132 treatment. Two bands differing about 5 kD in size were visible. Without MG132 the lower band was more abundant, whereas after MG132 treatment the upper band was more abundant. The occurrence of two bands might either be the result of proteolytic cleavage at the N-terminus (lower band) or post-translational modification (upper band) by for example ubiquitin. The JAZ1-GFP protein was more difficult to detect, indicating that its concentration in total cell extract was relatively low. After MG132 treatment the JAZ1-GFP fusion protein became more abundant, indicating that without treatment it underwent degradation via the 26S proteasome. Upon JA treatment the amount of fusion protein became significantly lower, whereas upon simultaneous treatment with JA and MG132 it was present at a similar amount as without treatment.

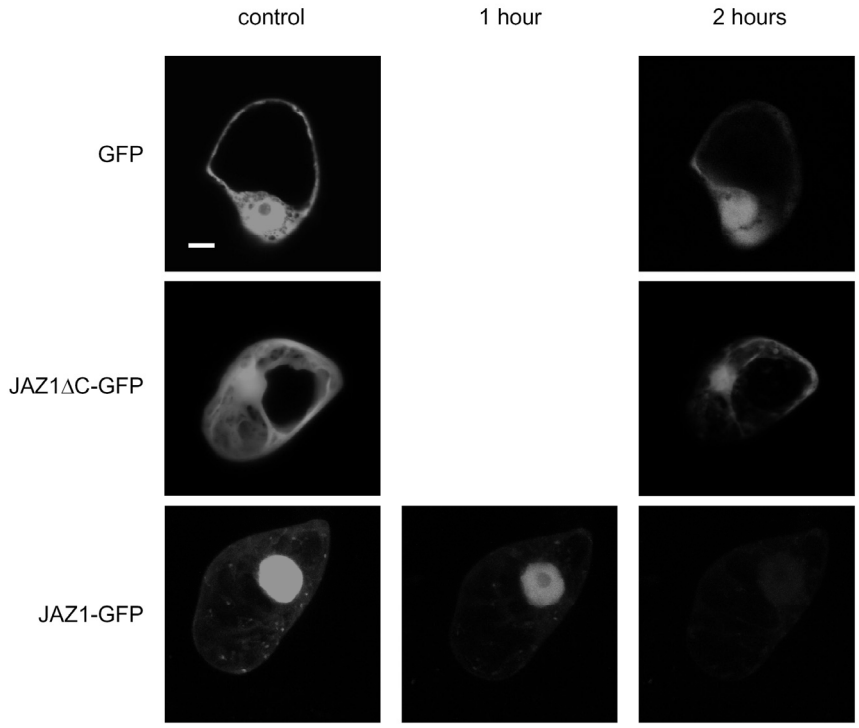

Figure 8. Full-length CrJAZ1 but not CrJAZ1ΔC is rapidly degraded in response to MeJA. Confocal laser scanning microscopy at 63x magnification of *C. roseus* MP183L cells transiently transformed with plasmids carrying *GFP*, *CrJAZ1ΔC-GFP* or *CrJAZ1-GFP* and treated with 50 µM MeJA for the number of hours indicated. For each plasmid construct the same cell was followed in time. The size bar corresponds to 10 µm.

Overexpression of *CrJAZ1-GFP* fusions represses MeJA-responsive gene expression

For AtJAZ proteins it has been shown that overexpression of a stable C-terminal deletion derivative inhibited JA responses including JA-responsive gene expression (Chini et al., 2007; Thines et al., 2007). In tobacco overexpression of C-terminal deletion derivatives had similar effects and was shown to inhibit MeJA-responsive expression of the gene encoding putrescine N-methyltransferase (PMT) and MeJA-responsive nicotine biosynthesis (Shoji et al., 2008). We wanted to determine whether overexpression of a C-terminal

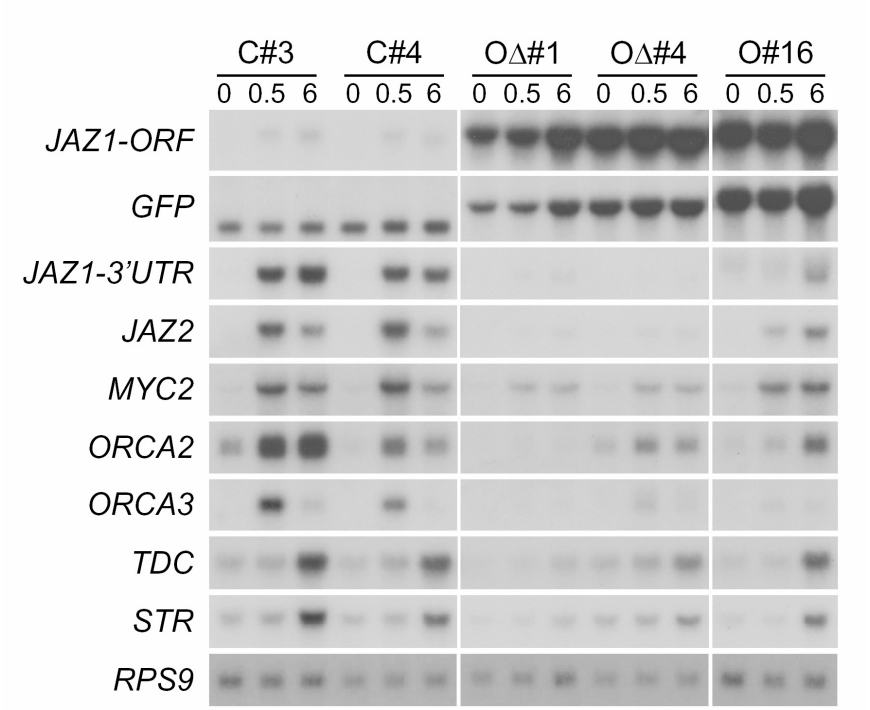

Figure 9. Overexpression of *CrJAZ1-GFP* fusions represses MeJA-responsive gene expression. Transgenic control lines (C) and overexpression lines expressing *CrJAZ1ΔC-GFP* (OΔ) or *CrJAZ1-GFP* (O) were treated with 10 µM MeJA for the indicated number of hours. Northern blots were hybridized with the probes indicated at the left. The probe consisting of the 3' untranslated region (3' UTR) of the *CrJAZ1* gene has no overlap with the *CrJAZ1* open reading frame (ORF) used for the overexpression constructs.

deletion derivative of Catharanthus JAZ1 would have similar effects, as would be expected from the observations that it inhibited CrMYC2 activity (Fig. 5) and that it was relatively stable upon jasmonate treatment (Fig. 7). We also wanted to determine whether overexpression of full-length JAZ1 would have effects on MeJA responses. CrJAZ1 derivatives were overexpressed as GFP fusion proteins (Fig. 9) or in their native form (Fig. 10). As shown in Fig.9, the GFP fusion constructs had high expression levels that far exceeded the MeJA-induced expression levels of the endogenous *JAZ* genes. Overexpression of CrJAZ1ΔC had a strong negative effect on MeJA-responsive expression of the

JAZ genes and of the *CrMYC2* gene. Also the expression of the *ORCA* genes, which are target genes of CrMYC2, was strongly reduced. In addition the expression of the TIA biosynthesis genes *TDC* and *STR*, which are target genes of ORCA2 and ORCA3 (Menke et al., 1999; van der Fits and Memelink, 2000), was reduced. Overexpression of full-length JAZ1-GFP also had negative effects on MeJA-responsive *JAZ* and *ORCA3* gene expression, but the effects on *CrMYC2*, *ORCA2*, *TDC* and *STR* gene expression were less pronounced.

Analysis of transgenic cell lines overexpressing the native CrJAZ1 derivatives led to similar conclusions (Fig. 10). Overexpression of JAZ1ΔC had a negative effect on the MeJA-responsive expression of all genes tested. Overexpression of full-length CrJAZ1 had weaker but clear effects on *JAZ* gene expression, whereas downstream genes were less affected.

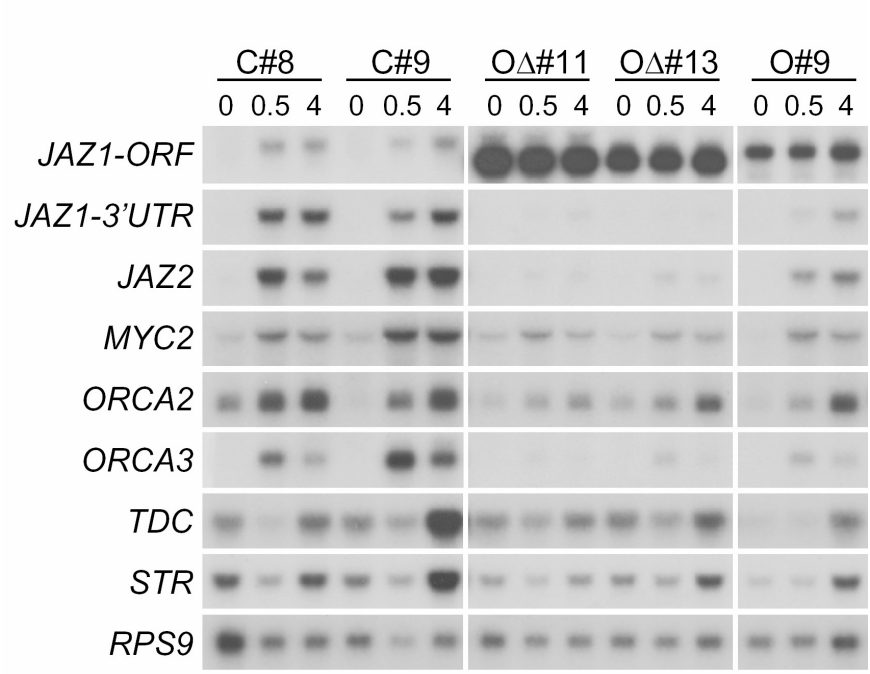

Figure 10. Overexpression of CrJAZ1 represses MeJA-responsive gene expression. Control lines (C) and overexpression lines expressing *CrJAZ1ΔC* (OΔ) or *CrJAZ1* (O) were treated with 10 µM MeJA for the indicated number of hours. Northern blots were hybridized with the probes indicated at the left. The probe consisting of the 3' untranslated region (3' UTR) of the *CrJAZ1* gene has no overlap with the *CrJAZ1* open reading frame (ORF) used for the overexpression constructs.

CrMYC2 controls MeJA-responsive *CrJAZ* gene expression

AtMYC2 was shown to control the MeJA-responsive expression of *AtJAZ* genes (Chini et al., 2007). To determine whether similarly CrMYC2 controls the expression of the *CrJAZ* genes, we analysed *JAZ* gene expression in a transgenic cell line which contained reduced levels of *CrMYC2* mRNA due to the presence of an RNA interference (RNAi) construct targeting *CrMYC2*. As shown in Fig.11A, knock-down of the *CrMYC2* mRNA level caused a strong reduction in the levels of *CrJAZ* mRNA accumulation in response to treatment with 10 µM MeJA, indicating that CrMYC2 is required for MeJA-responsive *CrJAZ* expression.

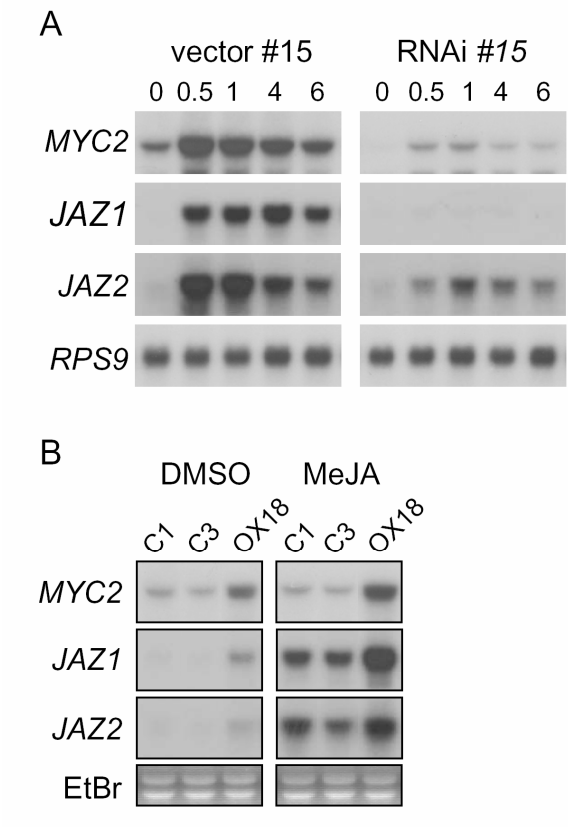

Figure 11. CrMYC2 controls MeJA-responsive *CrJAZ* expression. A, CrMYC2 is required for MeJA-responsive *CrJAZ* expression. Time-course analysis of gene expression in control line #15 and in RNAi-*CrMYC2* line #15. Cell lines were treated with 10 µM MeJA for the number of hours indicated. The *CrMYC2* probe corresponds to an N-terminal fragment that does not contain sequences present in the RNAi construct. B, Overexpression of *CrMYC2* induces *CrJAZ* gene expression. Control lines (C) #1 and #3 and overexpression line (OX) #18 were treated for 30 min with 0.1% (v/v) of the solvent DMSO or 50 nM MeJA. The ethidium bromide (EtBr) stained gel is shown as a loading control. Northern blots were hybridized with the probes indicated at the left.

To determine whether an elevated level of *CrMYC2* expression is sufficient for activation of *JAZ* gene expression, we analysed gene expression in stable lines transformed with a construct carrying the *CrMYC2* open reading frame under the control of the CaMV 35S promoter. As shown in Fig. 11B, overexpression of CrMYC2 caused elevated levels of *JAZ* mRNA accumulation. Overexpression of CrMYC2 also caused increased sensitivity to very low levels of MeJA. Upon treatment with a limiting concentration of 50 nM MeJA, the overexpression line showed higher expression levels of the JAZ genes compared to the control lines.

These experiments demonstrate that CrMYC2 controls MeJA-responsive *CrJAZ* gene expression.

DISCUSSION

Here we show that two members of the JAZ family in *C. roseus* are repressors of the activity of the bHLH transcription factor CrMYC2. Our findings are integrated in the model in Fig. 12. We previously showed that CrMYC2 controls MeJA-responsive expression of the *ORCA* genes, and that certain members of the AT-hook family of transcription factors are co-activators of *ORCA3* promoter activity. In response to a jasmonate, CrJAZ proteins are degraded which presumably leads to activation of CrMYC2. CrMYC2 then activates *ORCA* gene expression as well as *CrJAZ* gene expression. De novo synthesis of CrJAZ proteins presumably restores the uninduced situation. We hypothesize that CrJAZ degradation is mediated by the *C. roseus* COI1 orthologue analogous to the situation in Arabidopsis (Chini et al., 2007; Thines et al., 2007) and tobacco (Shoji et al., 2008).

Full-length CrJAZ proteins were potent repressors of CrMYC2 activity in transient trans-activation assays. A repressor role has been suggested previously for the AtJAZ proteins (Chini et al., 2007; Thines et al., 2007). One of the reasons for this assumption was that overexpression of C-terminal deletion derivatives repressed jasmonate responses including jasmonate-responsive gene expression. However, for transcription factors it is not uncommon that

deletion derivatives work as repressors when overexpressed whereas the full-length proteins work as activators (e.g. Gill and Ptashne, 1988; Miao and Lam, 1995; Mizukami et al., 1996; Fan and Dong, 2002). Conversely we show here that the ZIM domain alone worked as an activator when overexpressed, whereas the full-length CrJAZ proteins were repressors. Our results demonstrate that the assumption that JAZ proteins are repressors was correct and thus confirm this aspect of the proposed model for jasmonate signalling (Chico et al., 2008).

The observation that the ZIM domain alone worked as an activator was surprising. A similar observation was made when the transcriptional activation domain of the yeast transcription factor GAL4 was overexpressed, except that in this case repression was observed (Gill and Ptashne, 1988). The phenomenon was called “squelching”, and was explained by assuming that a co-activator interacting with the activation domain was titrated out and therefore unavailable for interaction with full-length GAL4. Later on, the GAL4 activation domain was shown to interact in vivo with the co-activator complexes SAGA and Mediator (Traven et al., 2006). In analogy to GAL4, we hypothesize that the ZIM domain titrates out a co-repressor which normally keeps the reporter gene silent, implying that the normal function of the ZIM domain is to attract this co-repressor to a promoter bound by CrMYC2.

Our yeast two-hybrid assays showed that both the C-terminal and the N-terminal regions of CrJAZ1 interacted with CrMYC2. In contrast, it has been reported for AtJAZ3 that only the C-terminal domain interacted with AtMYC2 in vitro and in yeast (Chini et al., 2007). The dual interaction with MYC2 in yeast is a conserved property of JAZ1 proteins, since AtJAZ1 also had two distinct AtMYC2 interaction domains. Therefore it appears that there are differences between different JAZ proteins in the modes of interaction with MYC2, at least in yeast.

In the trans-activation assays we found that a deletion derivative lacking the N-terminal domain did not repress CrMYC2 activity. A C-terminal deletion derivative on the other hand was an efficient repressor. When tested separately, only the N-terminal domain was an efficient repressor, whereas the ZIM and Jas domains had little or no effect. These observations can be explained by

assuming that in plant cells the N-terminal domain is the main interaction interface with CrMYC2. If the assumption that the ZIM domain binds a co-repressor is correct, it is surprising that the N-terminal domain alone was an efficient repressor. It indicates that binding of the N-terminal domain is sufficient to block CrMYC2 activity, maybe by interfering with CrMYC2 DNA-binding capacity or with the binding of a co-activator.

In localization studies of JAZ derivatives we found that CrJAZ1 is a nuclear protein, consistent with the reported localization of AtJAZ1 in Arabidopsis (Thines et al., 2007). The localization of the AtJAZ1ΔC derivative has not been reported, but for AtJAZ3 both the full-length protein as well as the C-terminal deletion derivative were reported to be nuclear-localized in Arabidopsis roots (Chini et al., 2007). The CrJAZ1ΔC derivative however was not exclusively nuclear-localized and had in fact a similar localization as GFP. Careful inspection of Fig.2 in the paper of Chini et al. (2007) shows that the AtJAZ3ΔC-GFP fusion stains the nuclei in a much more diffuse pattern than AtJAZ3-GFP and stains also other parts of the root cells, and its localization is thus in fact not that different from CrJAZ1ΔC localization.

JAZ proteins are crucial regulators of jasmonate-responsive expression of genes involved in alkaloid biosynthesis both in tobacco (Shoji et al., 2008) as well as in *C. roseus*. In tobacco, the transcription factor that is the target for JAZ repression is not known. Based on the conserved interaction between JAZs and MYC2 in Arabidopsis (Chini et al., 2007; Melotto et al., 2008) and *C. roseus*, we speculate that a tobacco MYC2 orthologue is the target for JAZ repression. In *C. roseus*, CrMYC2 is part of a small transcription factor cascade including the ORCA transcription factors. We further speculate that in tobacco a similar transcription factor cascade is operative. Two tobacco members of the AP2/ERF-domain transcription factor family called NtORC1 and NtJAP1 were shown to upregulate the activity of the *PMT* promoter in transient assays in tobacco protoplasts (De Sutter et al., 2005). Together the transcription factors caused a strong synergistic activation of the *PMT* promoter. NtORC1 is a close homologue of the *C. roseus* transcription factor ORCA3. Both *NtORC1* and

NtJAP1 gene expression is induced by MeJA (Goossens et al., 2003). Therefore we speculate that *NtORC1* and *NtJAP1* genes are regulated by the tobacco MYC2 orthologue.

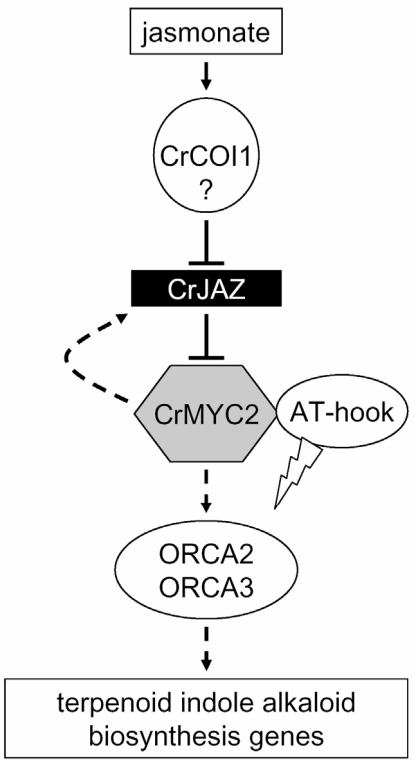

Figure 12. Model for jasmonate signal transduction leading to the expression of terpenoid indole alkaloid biosynthesis genes in *C. roseus*. A jasmonate forms the molecular glue between CrCOI1 and CrJAZ, leading to degradation of the latter proteins. CrMYC2 then activates transcription of the genes encoding the AP2/ERF transcription factors ORCA2 and ORCA3, which in turn activate the expression of terpenoid indole alkaloid biosynthesis genes. CrMYC2 also activates transcription of *CrJAZ* genes as part of a negative feedback loop. Certain members of the AT-hook transcription factor family co-stimulate the expression of the *ORCA* genes. The position of CrCOI1 in this signal transduction pathway is hypothetical as indicated by the question mark. Solid lines indicate interactions between proteins and broken lines indicate interactions between proteins and genes.

Jasmonate signalling appears to be highly conserved between plant species. The recent discoveries concerning mechanisms of jasmonate signalling in Arabidopsis (Chini et al., 2007; Thines et al., 2007) are perfectly translatable to the crop species tobacco (Shoji et al., 2008) and to the pharmaceutical plant species *C. roseus*. The commonalities between the components involved in regulation of jasmonate-responsive alkaloid metabolism in tobacco and in *C. roseus* suggest that the overall regulatory circuits are highly similar. It will be interesting to see to whether other jasmonate-responsive secondary metabolic pathways in other plant species (Memelink et al., 2001) are regulated in a similar manner.

MATERIALS AND METHODS

Cell Cultures, Stable Transformation, Treatments, and GFP analysis

Catharanthus roseus cell suspension line MP183L was maintained by weekly 10-fold dilution in 50 mL of Linsmaier and Skoog (LS) medium containing 88 mM sucrose, 2.7 µM 1-NAA and 0.23 µM kinetin and was grown at 28°C in a 16/8 hour light/dark regime at 200 µE m^{-2} s^{-1} at 70% relative humidity on a rotary shaker at 120 rpm. Treatments were 4 d after transfer. MeJA and MG132 were diluted in dimethylsulfoxide (DMSO). Control cultures were treated with DMSO at a final concentration of 0.1% (v/v). For stable transformations of cell line MP183L, plasmid constructs of interest were co-transformed with the plasmid pGL2 (Bilang et al., 1991) carrying a hygromycin selection gene driven by the CaMV 35S promoter in a ratio of 4 to 1 by particle bombardment (van der Fits and Memelink, 1997). Transgenic cells were selected on solid medium containing 50 µg/mL hygromycin-B and individual transgenic calli were converted to cell suspensions. GFP fluorescence was examined with a Leica inverted microscope (DM IRBE) equipped with a Leica SP1 confocal scanhead with an argon laser at an excitation wavelength of 488 nm and collection of emitted fluorescence after passage through a broad band pass filter (500-550 nm). The resulting signal was amplified, digitalized and the consistent picture reconstituted by Leica software.

Isolation of CrJAZ cDNA Clones

To isolate full-length clones, 5' and 3' sequences were isolated by PCR with gene-specific primers and corresponding vector primer using a pAD-GAL4-2.1 cDNA library of MeJA-treated MP183L cells (Vom Endt et al., 2007) as template.

Plasmid Constructs

The *CrJAZ1* ORF was amplified with Gateway adaptor sites with the primers 5'-AAA AAG CAG GCT CAA TGG CTT CAT CGG AGA T-3' and 5'-AGA AAG CTG GGT TTT AAA AAG GAA AGC CAA T-3' using the pAD-GAL4-2.1 cDNA library as template. The amplicon was transferred via BP clonase (Invitrogen) in pDONR221 (Invitrogen), the resulting entry clone sequence was verified, and the *CrJAZ1* ORF was then recombined via LR clonase in the destination vector p2GW7 (Karimi et al., 2005) to yield p2GW7-CrJAZ1. An EcoRV fragment from p2GW7-CrJAZ1, carrying the full-length open reading frame (ORF) of *CrJAZ1*, was cloned in pIC-20H (Marsh et al., 1984). The *CrJAZ1* ORF was PCR amplified with the primers 5'-GAA TTC ATG GCT TCA TCG GAG ATG ATT ATG-3' and 5'-GGA TCC TTA AAA AGG AAA GCC AAT TTC TAT ACT-3' using pIC20H-CrJAZ1 as a template, digested with EcoRI and BamHI and cloned in pRT101 (Töpfer et al., 1987) digested with EcoRI and BamHI. The *CrJAZ2* ORF was amplified with the primers 5'-CGG GAT CCG GAA TTC ATG TCA AGT TCT AAG AAA GGT TTT AG-3' and 5'-CCG CTC GAG GGA TTC TTA TAA TTT CAA TTC AAG TTG TTC TTG-3' using the pAD-GAL4-2.1 cDNA library as template, cloned into pGEM-T Easy vector (Promega) such that the stop codon flanked the SpeI site, excised with EcoRI and SpeI and cloned in pRT101 digested with EcoRI /XbaI. The *CrMYC2* ORF was amplified with the primers 5'-CCT CGA GAT GAC GGA CTA TAG GCT ACA AC-3' and 5'-CCT CGA GTC TAG ATC ATA CCA AGA GCC TCA TCG AGT TT-3' using the pAD-GAL4-2.1 cDNA library as template, digested with XhoI and cloned in pRT101 digested with XhoI. The *CrMYC2* RNAi construct consisted of an inverted repeat of the central part of *CrMYC2* in pHannibal (Wesley et al., 2001). A

SpeI/BamHI (positions 1127 to 1669 in GenBank acc.no. AF283507) fragment was cloned in pHannibal digested with XbaI/BamHI. An SpeI/AvrII (positions 1127 to 1685) fragment was first cloned in pIC-20R digested with XbaI such that the AvrII site flanked the KpnI site, re-excised with XhoI/KpnI and cloned into the pHannibal silencing construct digested with XhoI/KpnI creating the inverted repeat. The N-terminal domain of *CrJAZ1* was PCR amplified with the primers 5'-GAA TTC ATG GCT TCA TCG GAG ATG ATT ATG-3' and 5'-CGG GAT CCT TAT TGT GCT GTG TCT GGT TCA GAT TTT GC-3', the ZIM domain was amplified with the primers 5'-CGG GAT CCG GAA TTC GTC GAC ATG AAT TTG CTA TCA ACG ATG GAT A -3' and 5'-GGA TCC TTA ATC ATT GAT GCG TGG ATA AAG AG-3', the Jas domain was amplified with the primers 5'-CGG GAT CCG GAA TTC GTC GAC ATG CTT AAT TTC ACC CCT AAA CCA GCT G-3' and 5'-GGA TCC TTA AAA AGG AAA GCC AAT TTC TAT ACT-3' , CrJAZ1ΔN was amplified with the primers 5'-CGG GAT CCG GAA TTC GTC GAC ATG AAT TTG CTA TCA ACG ATG GAT A -3' and 5'-GGA TCC TTA AAA AGG AAA GCC AAT TTC TAT ACT-3' , CrJAZ1ΔC was amplified with the primers 5'-GAA TTC ATG GCT TCA TCG GAG ATG ATT ATG-3' and 5'-GGA TCC TTA ATC ATT GAT GCG TGG ATA AAG AG-3'. EcoRI/BamHI fragments were then cloned in pRT101and pGBT9 (GenBank acc.no. U07646) digested with EcoRI/BamHI. To generate pRT101-CrJAZ1ΔZ the N-terminal domain was amplified with the primers 5'-GAA TTC ATG GCT TCA TCG GAG ATG ATT ATG-3'and 5'-ACG CGT CGA CTT GTG CTG TGT CTG GTT CAG ATT TTG C, digested with EcoRI/SalI and cloned in pRT101-Jas digested with EcoRI/SalI. To generate pGBT9-CrJAZ1ΔZ, an EcoRI/BamHI fragment was excised from pRT101-CrJAZΔZ and cloned in pGBT9 digested with EcoRI/BamHI. The *CrJAZ1* ORF without stop codon was amplified with the primers 5'-GGA ATT CGG TCG ACA TGG CTT CAT CGG AGA TGA TTA TG-3' and 5'-CAT GCC ATG GGA AAA GGA AAG CCA ATT TCT ATA CTT GG-3' and *CrJAZ1*ΔC lacking the stop codon was amplified with the primers 5'-GGA ATT CGG TCG ACA TGG CTT CAT CGG AGA TGA TTA TG-3'and 5'-CAT GCC ATG GGT AAA TCA TTG ATG CGT GGA TAA AG-3'. PCR fragments were then digested with SalI/NcoI and cloned into pTH2 (Chiu et al., 1996; Niwa

et al., 1999) digested with SalI/NcoI. To generate pACT-CrMYC2, the *CrMYC2* ORF was amplified with the primers 5'-GGC CAT GGC CAT GAC GGA CTA TAG GCT ACA ACC-3' and 5'-CCT CGA GTC TAG ATC ATA CCA AGA GCC TCA TCG AGT TT-3' using pRT101-CrMYC2 as a template, digested with NcoI/XhoI and cloned in pACT2 (acc. No. U29899) digested with NcoI/XhoI. pACT2-CrMYC2ΔN was isolated by yeast one hybrid screening and starts at position 787 in Genbank acc. No. AF283507. *AtJAZ1* fragments were amplified with Gateway adaptor sites and recombined with pDONR221 or pDONR207. N-terminal fragments were amplified with the primers 5'-AAA AAG CAG GCT CGA TGT CGA GTT CTA TGG AAT G-3' and 5'-AGA AAG CTG GGT CTC AGG TTG TTG TCG GCT GAC GTG-3' (1-67) or 5'-AGA AAG CTG GGT GAG CAA TAG GAA GTT CTG-3' (1-204). C-terminal fragments were amplified with 5'-AAA AAG CAG GCT TCA TGA GTT TAT TCC CTT-3' (67-253) or 5'-AAA AAG CAG GCT TCA CCA TGA GAA GAG CTT-3' (204-253) and 5'-AGA AAG CTG GGT GTA TTT CAG CTG CTA AAC CGA G-3'. For derivative 67-204, the third and fourth primers were combined. For the *AtJAZ1-fLUC* (firefly luciferase) reporter construct, a 1356 bp fragment of the *AtJAZ1* promoter was amplified with the primers 5'-GGG GAC AAC TTT GTA TAG AAA AGT TGG ACT GCA CAC TTG CCA ACC TTC TTT CC-3' and 5'-GGG GAC TGC TTT TTT GTA CAA ACT TGT CTT TAA CAA TTA AAA CTT TC-3' and Gateway recombined with pDONRP4P1R (Invitrogen) to yield pENTRY-ProAtJAZ1. Subsequently, the latter vector was recombined by Gateway MultiSite LR cloning with pENTRY-fLUC and pm42GW7,3 (Karimi et al., 2007) to yield ProAtJAZ1:fLUC. The p2GW7-AtJAZ1 and p2GW7-AtMYC2 effector plasmids were previously described (Pauwels et al., 2008).

Yeast Two-Hybrid Assays

CrJAZ derivatives cloned in pGBT9 (GenBank acc. no. U07646) and CrMYC2 derivatives cloned in pACT2 (acc. No. U29899) were transformed to *Saccharomyces cereviseae* strain pJ69-4A (James et al., 1996) and plated on

solidified minimal synthetic defined (SD)-glucose medium (BD Biosciences) supplemented with Met/Ura/His. *MEL1* reporter gene activity was measured and calculated according to the Clontech Yeast Protocols Handbook (Protocol No. PT3024-1 version No. PR742227). pENTRY vectors holding *AtJAZ1* or derivatives and pENTRY-MYC2 were recombined with pGBT9gate and pGADgate respectively. These Gateway destination vectors were derived from pGBT9 and pGAD424 (acc. No. U07647) by inserting a Gateway cassette. The yeast strain MaV203 (Invitrogen) was co-transformed with a bait and a prey plasmid and selected on solidified SD-glucose medium supplemented with all amino acids except Leu and Trp. As controls, empty pGAD424 and pGBT9 vectors were used. Three individual transformants were grown for 2 days in the same medium. Subsequently, 10-fold dilutions were dropped on solidified SD-glucose medium supplemented with 20 mM 3-amino-1,2,4-triazole and all amino acids except, Leu, Trp and His, and allowed to grow for several days at 30°C.

Transient Expression Assays

Cell line MP183L was transformed by particle bombardment as described (van der Fits and Memelink, 1997) using a home-made helium gun and 1.8 µm tungsten particles (Pioneer Hi-Bred). Cells were co-bombarded in triplicate with 2 µg of a 4D-*GusSH-47* reporter construct, 200 ng of *CrMYC2* effector plasmid and 6 µg of *CrJAZ* effector plasmid as indicated in the figures. Total effector amount was adjusted to 6 µg in all transformations using empty vector plasmid pRT101. GUS activities measured as described (van der Fits and Memelink, 1997) were corrected for protein concentrations measured using Bio-Rad protein assay reagent and expressed as relative activities compared with the vector control. Tobacco BY-2 protoplasts were transfected as described (De Sutter et al., 2005) with 2 µg *fLUC* reporter construct carrying an *AtJAZ1* promoter fragment, 2 µg of a *rLUC* (Renilla luciferase) normalization construct, 2 µg *AtMYC2* effector plasmid and 1 µg *AtJAZ1* effector plasmid as indicated in

the figure. Total effector amount was adjusted to 3 µg in all transfections using a control *GUS* effector plasmid.

RNA Extraction and Northern Blot Analyses

Total RNA was extracted from frozen cells by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% (v/v) ethanol, and resuspended in water. Ten µg RNA samples were subjected to electrophoresis on 1.5% w/v agarose/1% v/v formaldehyde gels and blotted onto Genescreen nylon membranes (Perkin-Elmer Life Sciences). Probes were ^{32}P-labeled by random priming. (Pre-) hybridization and subsequent washings of blots were performed as described (Memelink et al., 1994) with minor modifications. cDNAs used as probes were: *ORCA2* (GenBank acc. No. AJ238740), *ORCA3* (AJ251250), *STR* (X61932), *TDC* (M25151), *RPS9* (AJ749993), *CrMYC2* (AF283507), *CrJAZ1* (FJ040204) and *CrJAZ2* (FJ040205). The 3' untranslated region of the *CrJAZ1* gene was amplified with the primers 5'- CTC AAG AAC TTC TTG GGG TTG GTT GA-3' and T7 primer using a pAD-GAL4-2.1 cDNA library of MeJA-treated MP183L cells (Vom Endt et al., 2007) as template.

Western blotting

C. roseus cells from 10 mL samples of 4-d-old suspension cultures were ground in liquid nitrogen and thawed in 1 mL extraction buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 50 mM β-glycerophosphate, 10% v/v glycerol, 1% v/v IGEPAL, 0.5% w/v deoxycholate, 0.1% w/v SDS, 1 mM phenylmethylsulfonylfluoride (PMSF), 1x proteinase inhibitor cocktail (Roche)). Protein concentrations in the supernatant after centrifugation for 5 min at 13000 rpm were determined using Bio-Rad protein assay reagent. Protein samples were separated by 10% (w/v) SDS-PAGE and

transferred to Protran nitrocellulose (Whatman). Western blots were probed with rabbit anti-GFP antibodies (Invitrogen) using goat anti-rabbit IgG-HRP conjugate (Sigma) as second antibodies. Antibody binding was detected by incubation in 250 µM sodium luminol, 0.1 M Tris-HCl pH 8.6, 3 mM H_2O_2, 67 µM p-coumaric acid and exposure to X-ray film.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers FJ040204 (*CrJAZ1*) and FJ040205 (*CrJAZ2*).

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References

Balbi V, Devoto A (2008) Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. New Phytol 177: 301-318

Bilang R, Iida S, Peterhans A, Potrykus I, Paszkowski J (1991) The 3'-terminal region of the hygromycin-B-resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*. Gene 100: 247-250

Chico JM, Chini A, Fonseca S, Solano R (2008) JAZ repressors set the rhythm in jasmonate signaling. Curr Opin Plant Biol 11: 486-494

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano

R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448: 666-671

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6: 325-330

De Sutter V, Vanderhaeghen R, Tilleman S, Lammertyn F, Vanhoutte I, Karimi M, Inzé D, Goossens A, Hilson P (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. Plant J 44: 1065-1076

Fan W, Dong X (2002) In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell 14: 1377-1389

Gantet P, Imbault N, Thiersault M, Doireau P (1998) Necessity of a functional octadecanoid pathway for indole alkaloid synthesis by *Catharanthus roseus* cell suspensions cultured in an auxin starved medium. Plant Cell Physiol 39: 220-225

Gill G, Ptashne M (1988) Negative effect of the transcriptional activator GAL4. Nature 334: 721-724.

Goossens A, Häkkinen ST, Laakso I, Seppänen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila, AM, Söderlund H, Zabeau M, Inzé D, Oksman-Caldentey KM (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl Acad Sci USA 100: 8595-8600

Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA 89: 2389–2393

Imanishi S, Hashizume K, Nakakita M, Kojima H, Matsubayashi Y, Hashimoto T, Sakagami Y, Yamada Y, Nakamura K (1998) Differential induction by methyl jasmonate of genes encoding ornithine decarboxylase and other enzymes involved in nicotine biosynthesis in tobacco cell cultures. Plant Mol Biol 38: 1101-1111

James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144: 1425-1436

Katoh A, Ohki H, Inai K, Hashimoto T (2005) Molecular regulation of nicotine biosynthesis. Plant Biotechnology 22: 389-392

Karimi M, Bleys A, Vanderhaeghen R, Hilson P (2007) Building blocks for plant gene assembly. Plant Physiol 145: 1183-1191

Karimi M, De Meyer B, Hilson P (2005) Modular cloning in plant cells. Trends Plant Sci 10: 103-105

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci USA 105: 7100-7105

Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R (2004) *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell 16: 1938-1950

Marsh JL, Erfle M, Wykes EJ (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32: 481-485

Melotto M, Mecey C, Niu Y, Chung HS, Katsir L, Yao J, Zeng W, Thines B, Staswick P, Browse J, Howe GA, He SY (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. Plant J 55: 979-988

Memelink J, Swords KMM, Staehelin LA, Hoge JHC (1994) Southern, Northern and Western blot analysis. In Plant Molecular Biology Manual, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds (Dordrecht: Kluwer Academic Publishers), pp. F1-F23

Memelink J, Verpoorte R, Kijne, JW (2001) ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 6: 212-219

Menke FLH, Champion A, Kijne JW, Memelink, J (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18: 4455-4463

Miao ZH, Lam E (1995) Construction of a trans-dominant inhibitor for members of the TGA family of transcription factors conserved in higher plants. Plant J 7: 887-896

Mizukami Y, Huang H, Tudor M, Hu Y, Ma H (1996) Functional domains of the floral regulator AGAMOUS: characterization of the DNA binding domain and analysis of dominant negative mutations. Plant Cell 8: 831-845

Niwa Y, Hirano T, Yoshimoto K, Shimizu M, Kobayashi H (1999) Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. Plant J 18: 455-463

Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inzé D, Goossens A (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. Proc Natl Acad Sci USA 105: 1380-1385

Pré M, Sibéril Y, Memelink J, Champion A, Doireau P, Gantet P (2000) Isolation by the yeast one-hybrid system of cDNAs encoding transcription factors that bind to the G-box element of the *strictosidine synthase* gene promoter from *Catharanthus roseus*. Int J Bio-chrom 5: 229-244

Rischer H, Oresic M, Seppänen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MC, Inzé D, Oksman-Caldentey KM, Goossens A (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in Catharanthus roseus cells. Proc Natl Acad Sci USA 103: 5614-5619

Shoji T, Yamada Y, Hashimoto T (2000) Jasmonate induction of putrescine N-methyltransferase genes in the root of *Nicotiana sylvestris*. Plant Cell Physiol 41: 831-839

Shoji T, Ogawa T, Hashimoto T (2008) Jasmonate-induced nicotine formation in tobacco is mediated by tobacco *COI1* and *JAZ* genes. Plant Cell Physiol 49: 1003-1012

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature 448: 661-665

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss H-H (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res 15: 5890

Traven A, Jelicic B, Sopta M (2006) Yeast Gal4: a transcriptional paradigm revisited. EMBO Rep 7: 496-499

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell 14: S153-S164

van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. Plant Mol Biol 33: 943-946

van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295-297

van der Fits L, Memelink, J (2001) The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J 25: 43-53

Vanholme B, Grunewald W, Bateman A, Kohchi T, Gheysen G (2007) The tify family previously known as ZIM. Trends Plant Sci 12: 239-244

Vom Endt D, Soares e Silva M, Kijne JW, Pasquali G, Memelink, J (2007) Identification of a bipartite jasmonate-responsive promoter element in the *Catharanthus roseus ORCA3* transcription factor gene that interacts specifically with AT-Hook DNA-binding proteins. Plant Physiol 144: 1680-1689

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot 100: 681-697

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27: 581-590

Chapter 5

General discussion

Hongtao Zhang and Johan Memelink

Introduction

The biosynthesis of many different types of secondary metabolites that serve defensive functions in different plant species is regulated by hormones belonging to the group of jasmonate compounds. Regulation acts at the level of transcription of genes encoding biosynthetic enzymes. Chapters 3 and 4 describe mechanisms of signal transduction initiated by jasmonates leading to the activation of transcription factors. Here unifying models for jasmonate signal transduction regulating tobacco alkaloid biosynthesis and terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* are presented.

How do JAZ proteins, MYC2 and COI1 interact?

One of the new findings in Chapter 4 was that the N-terminal domain of CrJAZ1 was able to interact with CrMYC2 in yeast. The C-terminal domain also interacted with CrMYC2 in yeast, in line with the report of Chini et al. (2007) of interaction between the C-terminal domain of AtJAZ3 and AtMYC2. These authors did not find an interaction between the N-terminal domain and AtMYC2. Chapter 4 also reports that the N-terminal and C-terminal domains of AtJAZ1 interacted with AtMYC2. This dual interaction therefore is a conserved property of JAZ1 proteins, which apparently differ from AtJAZ3.

The C-terminal domains of tomato JAZ1 (Katsir et al., 2008) and Arabidopsis JAZ1, JAZ3 and JAZ9 (Melotto et al, 2008) were shown to be necessary and sufficient for binding to COI1 in a JA-Ile or coronatine dependent manner. Importantly, no interaction between COI1 and JAZ proteins was detected in the absence of JAs (Thines et al., 2007; Katsir et al., 2008; Melotto et al., 2008).

This contrasts with the reported interaction between AtCOI1 and AtJAZ1 or AtJAZ3 in in vitro pull-down assays in the absence of JAs (Chini et al., 2007). In addition, Chini et al. (2007) showed that in the absence of JAs the N-terminal but not the C-terminal domain of AtJAZ3 interacts with AtCOI1 (Figure 1A).

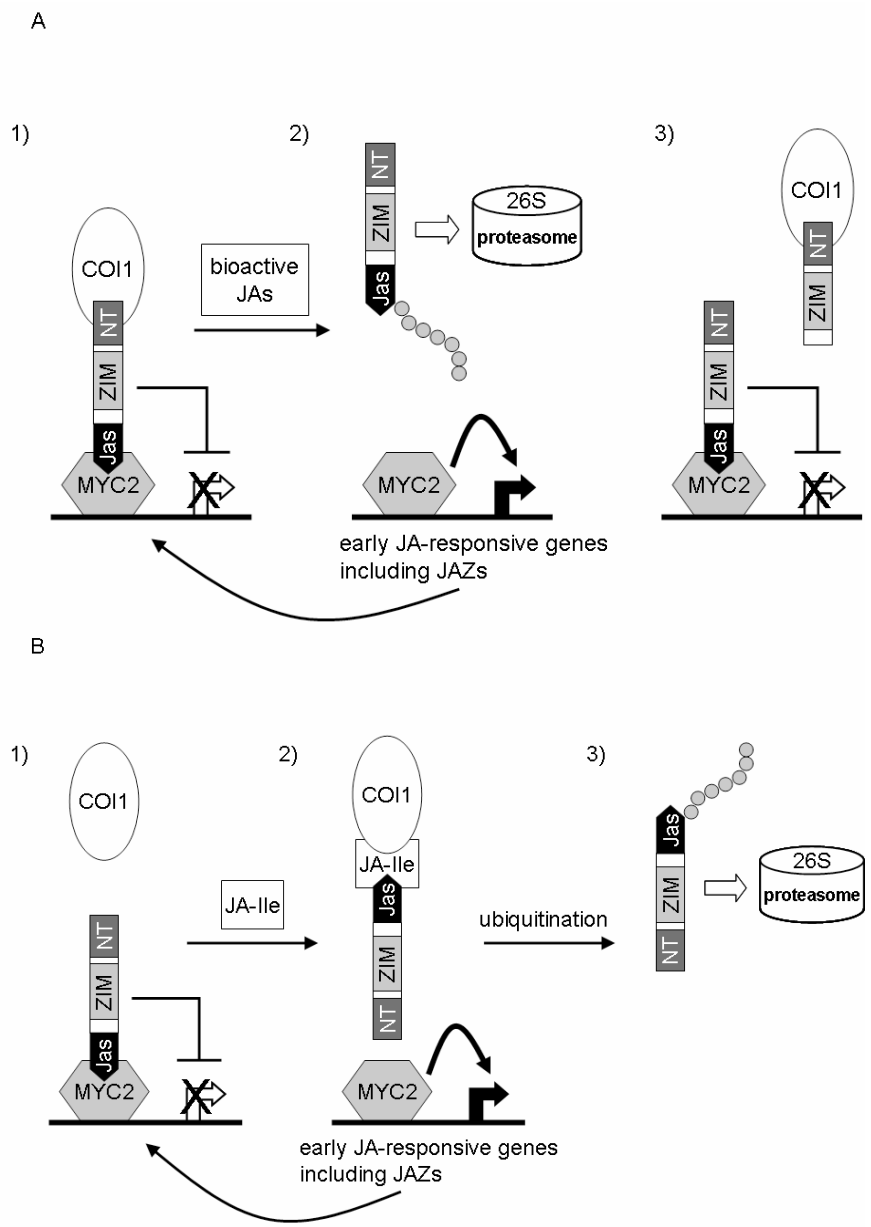
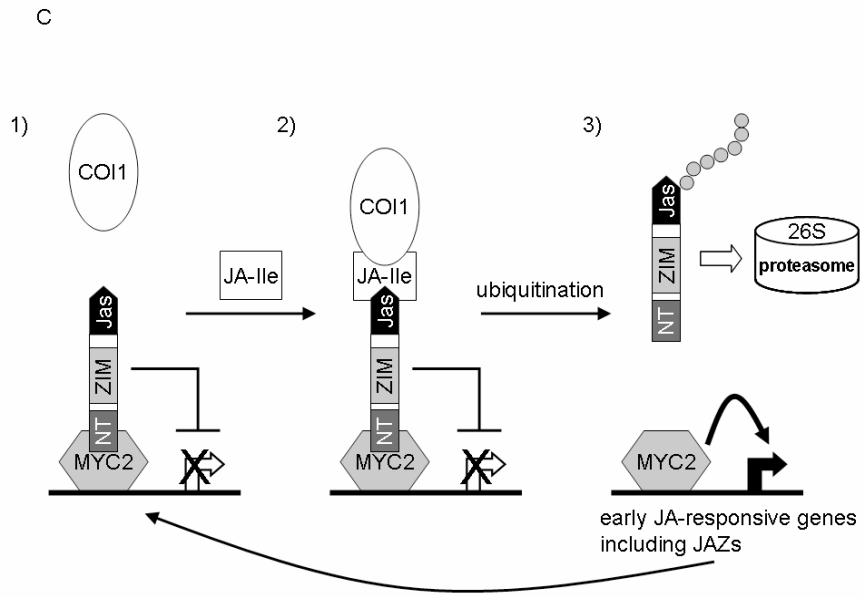


Figure 1. Models for jasmonate signal transduction leading to expression of AtMYC2-regulated genes. Although depicted as a single protein, COI1 forms part of the E3 ubiquitin ligase SCF^{COI1}. A) Model proposed by Chini et al. (2007). 1) In the absence of bioactive JAs, JAZ3 interacts through its Jas motif with AtMYC2 maintaining this transcription factor inactive. With its N-terminal domain JAZ3 interacts with COI1 in the absence of bioactive JAs. (To be continued on the next page)



(Continued from the previous page) 2) In the presence of bioactive JAs, JAZ3 is ubiquitinated by the SCF^{COI1} complex and degraded via the 26S proteasome. AtMYC2 is liberated and activates transcription of target genes, including genes encoding JAZ proteins, resulting in a negative feedback loop.3) Situation in the *jai3-1* mutant. The C-terminal deletion derivative of JAZ3 can still bind to COI1 and blocks its activity. As a result, other members of the JAZ family binding to AMYC2 cannot be ubiquitinated in the presence of bioactive JAs.

B) Model proposed by Melotto et al. (2008). 1) In the absence of JA-Ile, certain members of the JAZ family interact through a subdomain of the Jas motif with AtMYC2 maintaining this transcription factor inactive. 2) JA-Ile forms the molecular glue between another subdomain of the Jas motif 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.

C) Model proposed here. 1) In the absence of JA-Ile, certain members of the JAZ family interact through their N-terminal domain with AtMYC2 maintaining this transcription factor inactive, probably through the repressive action of the ZIM domain via interaction with a co-repressor. 2) JA-Ile forms the molecular glue between the Jas domain of certain members of the JAZ family 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.

The original model proposed by Chini et al. (2007) (Figure 1A), although consistent with all their reported data, has several elements that are difficult to understand. For example, what is the function of biologically active JAs in inducing ubiquitination of JAZ proteins if it is not by forming the molecular glue between COI1 and JAZ proteins? More importantly, why is a C-terminal deletion derivative of AtJAZ3 stable whereas according to the model it can still interact with AtCOI1? And why is this same deletion derivative, which still according to the model cannot interact anymore with AtMYC2, a strong repressor? The explanation presented by these authors is that the C-terminal deletion derivative of AtJAZ3 still binds to AtCOI1 and blocks its activity, thereby inhibiting degradation of other members of the AtJAZ family that can still bind and inactivate AtMYC2.

However, this “poison complex” model seems no longer tenable based on the convincing evidence that in the presence of JA-Ile the C-terminal domain of AtJAZ proteins including AtJAZ3 interacts with AtCOI1 (Melotto et al., 2008). Melotto et al. (2008) presented an alternative model (Figure 1B) which proposes that distinct subdomains of the Jas motif interact with AtMYC2 in the absence of JA-Ile and with AtCOI1 in the presence of JA-Ile. One major problem with that model is that it does not explain why a C-terminal deletion derivative of AtJAZ3 lacking the entire Jas motif which according to the model cannot interact with either AtMYC2 or AtCOI1 acts as a strong repressor.

Another problem with the model in Figure 1B is that it does not explain the observation of Chini et al. (2007) that the C-terminal deletion derivative of AtJAZ3 stabilizes other AtJAZ proteins in trans in the presence of JA-Ile. The model in Figure 1A explains this observation by proposing that the deletion derivative binds to AtCOI1 and blocks its activity. Apart from the fact that this interaction is being questioned (Melotto et al., 2008), it also does not appear to be fully consistent with other observations made by Chini et al. (2007). They report that in a genome-wide micro-array analysis only 31 genes showed a lower expression in the *jai3-1* mutant after JA treatment compared to JA-treated wild-type plants. If the C-terminal deletion derivative of AtJAZ3 expressed in the *jai3-1* mutant blocks AtCOI1, this should affect a much larger number of JA-

responsive COI1-dependent genes, which are estimated to comprise over 500 genes (Devoto et al., 2005).

A third model proposed here based on the data from Chapter 4 that the N-terminal domain of a JAZ protein from *C. roseus* interacts with the Catharanthus orthologue of MYC2 is shown in Figure 1C. It assumes that in the presence of JA-Ile the C-terminal JAZ domain interacts with COI1 in accordance with the data presented for Arabidopsis by Melotto et al. (2008). The model implies that a C-terminal deletion derivative of a JAZ protein that cannot interact anymore with COI1 is stable, and since it can still interact with MYC2 it is an active repressor. This model is in agreement with the results from our studies of CrJAZ1 and CrMYC2, but is not supported by the reported interaction between the C-terminal domain of AtJAZ3 and AtMYC2. The latter interaction was confirmed in a yeast two-hybrid assay (unpublished results) in accordance with the data reported by Chini et al. (2007). One possible explanation for these apparently contradictory results is that in planta the highest affinity interaction domains in AtJAZ3 are different from those in yeast, for example due to plant-specific protein modifications. A major problem with the model in Figure 1C is that it provides no explanation for the observation that a C-terminal deletion derivative of a JAZ protein stabilizes other JAZ proteins in trans. Maybe JAs-independent interaction between the N-terminal domain of a JAZ lacking the C-terminus and COI1 blocks the activity of the latter and thereby causes the dominant-negative effect as proposed by Chini et al. (2007).

It is clear that identification of the mechanisms of interaction between JAZ proteins, MYC2 and COI1 in planta requires more detailed studies, including crystal structure analyses of COI1-JAZ complexes in the presence and absence of JA-Ile or coronatine and of MYC2-JAZ complexes.

Jasmonate signalling in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*

The results in Chapter 3 demonstrate that MeJA-responsive *ORCA* expression is controlled by CrMYC2, and that at least for the *ORCA3* gene members of the

AT-hook family of transcription factors contribute to the level of expression (Figure 2).

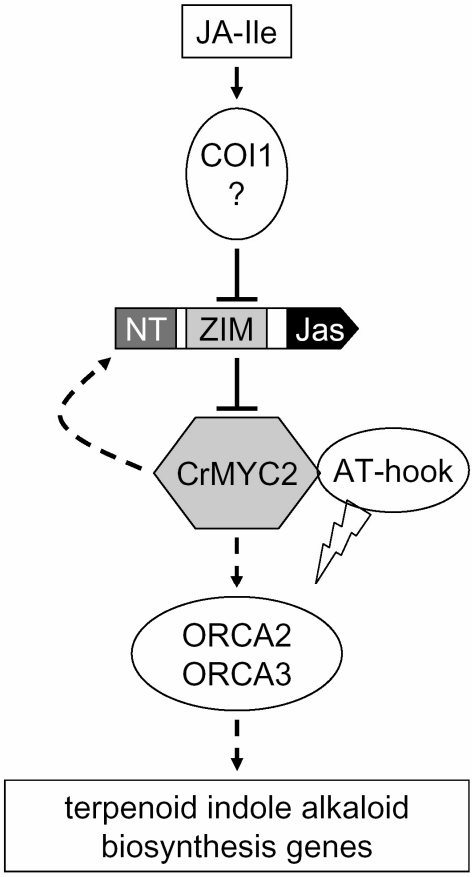


Figure 2. Model for jasmonate signal transduction leading to the expression of terpenoid indole alkaloid biosynthesis genes in *Catharanthus roseus*.

As depicted in Figure 1B, JA-Ile forms the molecular glue between CrCOI1 and CrJAZs, leading to degradation of the latter proteins. CrMYC2 then activates transcription of the genes encoding the ERF transcription factors ORCA2 and ORCA3, which in turn activate the expression of terpenoid indole alkaloid biosynthesis genes. CrMYC2 also activates transcription of *CrJAZ* genes as part of a negative feedback loop. Certain members of the AT-hook transcription factor family co-stimulate the expression of the *ORCA* genes. The position of CrCOI1 in this signal transduction pathway is hypothetical as indicated by the question mark. Solid lines indicate interactions between proteins and broken lines indicate interactions between proteins and genes.

Chapter 4 shows that the full-length CrJAZ proteins are repressors that negatively regulate CrMYC2 activity. For CrJAZ1 evidence was obtained that it is degraded in response to jasmonate via the 26S proteasome.

Based on the results in Chapters 3 and 4 the following model is proposed for signalling by JAs in *C. roseus* leading to alkaloid biosynthesis (Figure 2). Although MeJA was used in the studies, this compound is probably de-methylated in *C. roseus* cells, and then converted to the bioactive jasmonate JA-Ile. But this is clearly an issue that needs to be resolved. Perception of JA-Ile by CrCOI1 results in the degradation of CrJAZ proteins. CrMYC2 then activates the expression of the *ORCA* genes, which in turn activate the expression of a subset of TIA biosynthesis genes including *TDC* and *STR*. Simultaneous activation of *JAZ* genes by CrMYC2 restores the un-induced situation by inhibition of CrMYC2 activity. The involvement of CrCOI1 in this sequence of events still needs to be experimentally confirmed.

Jasmonate signalling in tobacco alkaloid biosynthesis

For jasmonate signalling in tobacco leading to the biosynthesis of nicotine and related alkaloids, a similar model is proposed. In transient assays in tobacco protoplasts two members of the tobacco ERF transcription factor family called NtORC1 and NtJAP1 were shown to upregulate the activity of the promoter of the tobacco gene encoding putrescine N-methyltransferase (PMT), which catalyzes the first committed step in nicotine biosynthesis (De Sutter et al., 2005). Together the transcription factors caused a strong synergistic activation of the *PMT* promoter. NtORC1 is a close homologue of the Catharanthus ERF transcription factor ORCA3. Both *NtORC1* and *NtJAP1* gene expression is induced by MeJA (Goossens et al., 2003).

Genetic studies using low-nicotine tobacco varieties demonstrated that the low-nicotine phenotype is caused by synergistic effects of two non-linked loci, called *nic1* and *nic2* (Katoh et al., 2005). Tobacco plants with the *nic1nic2* genotype have highly reduced nicotine contents (about 5% of wild type) and strongly decreased expression levels of nicotine biosynthesis genes. The genes corresponding to the *nic* loci have not been cloned yet.

MeJA-responsive nicotine biosynthesis is controlled by the jasmonate receptor COI1 and depends on degradation of members of the JAZ repressor family (Shoji et al., 2008). There are no published data yet about the nature of the transcription factor(s) repressed by the JAZ proteins in tobacco, but in the model in Figure 3 it is speculated that it is the tobacco homologue of AtMYC2 and CrMYC2. It is also hypothesized that this NtMYC2 transcription factor controls the MeJA-responsive expression of the *NtORC1* and *NtJAP1* genes, which in turn are hypothesized to control the MeJA-responsive expression of the nicotine biosynthesis genes.

Conclusion

Unifying models for jasmonate signalling regulating alkaloid biosynthesis in tobacco and in *Catharanthus roseus* are presented here. The models propose that perception of the jasmonate hormone JA-Ile by the receptor COI1 results in the degradation of JAZ proteins. Since these JAZ proteins repress the activity of the bHLH transcription factor MYC2, MYC2 then activates the expression of genes encoding certain members of the ERF family of transcription factors, which in turn activate the expression of alkaloid biosynthesis genes.

For both species, certain elements in the model have not yet been experimentally confirmed. For the Catharanthus model, the involvement of the jasmonate receptor COI1 has not been experimentally confirmed. Given the conservation of COI1 as a jasmonate receptor in Arabidopsis (Xie et al., 1998), tomato (Li et al., 2004; Katsir et al., 2008), tobacco (Shoji et al., 2008) and *Nicotiana attenuata* (Paschold et al., 2007), the position of COI1 in the jasmonate signal transduction pathway in Catharanthus seems highly probable. In tobacco, especially the identities and roles of transcription factors need more solid experimental confirmation. With the speed at which this research field currently progresses, it is anticipated that it will not take long to confirm or disprove the model.

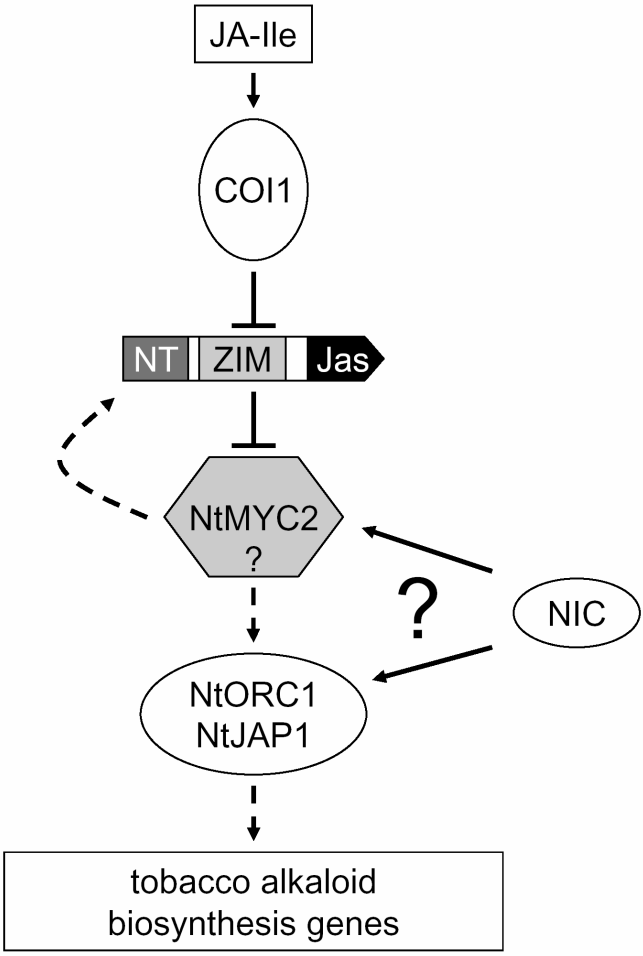


Figure 3. Model for jasmonate signal transduction leading to the expression of tobacco alkaloid biosynthesis genes.

As depicted in Figure 1B, JA-Ile forms the molecular glue between NtCOI1 and NtJAZs, leading to degradation of the latter proteins. NtMYC2 then activates transcription of the genes encoding the ERF transcription factors NtORC1 and NtJAP1, which in turn activate the expression of tobacco alkaloid biosynthesis genes. NtMYC2 also activates transcription of *NtJAZ* genes as part of a negative feedback loop. The elusive *NIC1* and *NIC2* genes may encode NtMYC2 and/or NtORC1 and NtJAP1 as indicated by the question mark. The position of NtMYC2 in this signal transduction pathway is hypothetical as indicated by the question mark. Solid lines indicate interactions between proteins and broken lines indicate interactions between proteins and genes.

It will be interesting to see to which degree the model will turn out to accurately reflect the mechanisms of jasmonate signal transduction in alkaloid biosynthesis, and to which degree this model applies to other secondary pathways regulated by JAs in different plant species.

References

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448: 666-671

De Sutter V, Vanderhaeghen R, Tilleman S, Lammertyn F, Vanhoutte I, Karimi M, Inzé D, Goossens A, Hilson P (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. Plant J 44:1065-1076

Devoto A, Ellis C, Magusin A, Chang HS, Chilcott C, Zhu T, Turner JG (2005) Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Mol Biol 58: 497-513

Goossens A, Häkkinen ST, Laakso I, Seppänen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila AM, Söderlund H, Zabeau M, Inzé D, Oksman-Caldentey KM (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl Acad Sci USA 100: 8595-8600

Katoh A, Ohki H, Inai K, Hashimoto T (2005) Molecular regulation of nicotine biosynthesis. Plant Biotechnology 22: 389-392

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci USA105: 7100-7105

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA (2004) The tomato homolog of *CORONATINE-INSENSITIVE1* is required for the maternal control of seed maturation, jasmonate-signaled

defense responses, and glandular trichome development. Plant Cell 16: 126-143

Melotto M, Mecey C, Niu Y, Chung HS, Katsir L, Yao J, Zeng W, Thines B, Staswick P, Browse J, Howe G, He SY (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interaction with the COI1 F-box protein. Plant J 55: 979-88

Paschold A, Halitschke R, Baldwin IT (2007) Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. Plant J 51: 79-91

Shoji T, Ogawa T, Hashimoto T (2008) Jasmonate-induced nicotine formation in tobacco is mediated by tobacco *COI1* and *JAZ* genes. Plant Cell Physiol 49: 1003-1012

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature 448: 661-665

Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) *COI1*: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280: 1091-1094

Samenvatting bij het proefschrift:

Jasmonzuur-responsieve transcriptionele regulatie in *Catharanthus roseus*

Onder bedreigende omstandigheden zoals infecties of vraat produceren planten laagmoleculaire beschermende stoffen, die secundaire metabolieten worden genoemd. Meestal worden secundaire metabolieten pas gemaakt wanneer ze nodig zijn, en is hun productie nauwkeurig gereguleerd. Wanneer de bedreigende omstandigheid wordt gesignaleerd, zorgt een keten van reacties in de cel voor verhoogde expressie van genen die coderen voor eiwitten (enzymen), die betrokken zijn bij de aanmaak van deze stoffen. Een centrale rol hierbij spelen DNA-bindende eiwitten die het expressieniveau van een gen kunnen reguleren, zogenaamde transcriptiefactoren. Bij de productie van secundaire metabolieten is vaak een groot aantal verschillende enzymen betrokken die achtereenvolgend actief zijn in een productieketen. Voor een gecoördineerde aanmaak van deze enzymen zijn dan ook vooral transcriptiefactoren van belang die de expressie van een aantal genen uit de keten tegelijk kunnen reguleren (zogenaamde hoofdschakelaars). Kennis van de regulatie van hoofdschakelaars in secundair metabolisme van planten kan worden toegepast voor een verhoogde productie van waardevolle plantenstoffen. Voorbeelden van hoofdschakelaars zijn de eiwitten ORCA2 en ORCA3 van *Catharanthus roseus*.

De subtropische plant *C. roseus*, de roze maagdenpalm, produceert onder bepaalde stresscondities terpenoïde indoolalkaloïden (TIA). Een aantal van deze TIA wordt door de mens gebruikt als geneesmiddel. Onderdeel van de reactieketen, die leidt tot de productie van TIA is de aanmaak van het plantenstresshormoon jasmonzuur (JA). Planten maken ook andere actieve varianten van dit hormoon, zoals methyl-jasmonzuur (MeJA) en een conjugaat met het aminozuur isoleucine (JA-Ile). MeJA induceert o.a. de expressie van de genen coderend voor de AP2-domein transcriptiefactoren ORCA2 en ORCA3, die beide de expressie stimuleren van een aantal genen betrokken bij TIA productie. Hiertoe behoren de *TDC* en *STR* genen, die coderen voor respectievelijk tryptofaan decarboxylase en strictosidine synthase, vroege enzymen in de TIA productieketen. De expressie van zowel *TDC* als *STR* wordt door ORCA2 en ORCA3 gestimuleerd. Het in dit proefschrift beschreven onderzoek betreft studies naar de manier waarop MeJA de expressie van de *ORCA* genen activeert.

Hoofdstuk 1 geeft een samenvatting van recente inzichten in de manier waarop JA genexpressie activeert gebaseerd op studies in de modelplant *Arabidopsis thaliana* (de zandraket). JA-Ile vormt de moleculaire lijm tussen JAZ (Jasmonate ZIM domein) repressoreiwitten en het F-box eiwit COI1 (CORONATINE INSENSITIVE1). In afwezigheid van JA-Ile houden de JAZ repressoren de transcriptiefactor AtMYC2 in een inactieve toestand. AtMYC2 behoort tot een andere klasse van transcriptiefactoren dan de ORCAs, nl. de basische Helix-Lus-Helix (bHLH) klasse. COI1 is onderdeel van een ubiquitine-eiwit ligase complex, dat na binding aan JAZ eiwitten in aanwezigheid van JA-Ile het kleine eiwitje ubiquitine aan de JAZ repressoren koppelt. Deze geubiquitineerde JAZ eiwitten worden vervolgens herkend door een eiwitafbraakmachine die het 26S proteasoom wordt genoemd. Door afbraak van de JAZ repressoren wordt AtMYC2 actief en schakelt een set van genen aan die betrokken is bij bescherming tegen verwonding en insectenvraat. AtMYC2 schakelt ook de *JAZ* genen aan, wat leidt tot synthese van nieuwe JAZ eiwitten die vervolgens AtMYC2 weer inactief maken en daarmee de door JA-Ile in gang gezette processen stoppen. In deze serie van gebeurtenissen functioneert COI1 (of het complex tussen COI1 en JAZ) als receptor voor JA-Ile.

Hoofdstuk 2 beschrijft experimenten die tot doel hebben om te onderzoeken of het eiwit CrA42 een bepalende rol speelt bij de expressie van de alkaloïdbiosynthesegenen *TDC* en *STR*. Het *CrA42* gen was oorspronkelijk geïsoleerd uit een *C. roseus* cellijn die een integratie van een T-DNA activatieconstruct in het genoom bevatte en ook verhoogde expressie vertoonde van deze genen. Het T-DNA was geïntegreerd vlak voor het *CrA42* gen. De hypothese was dat verhoogde expressie van het *CrA42* gen door de sterke promoter gelegen op het T-DNA construct de oorzaak was van de verhoogde *TDC* en *STR* expressie. In het onderzoek werd het volledige *CrA42* gen geïsoleerd en na koppeling aan een sterke promoter werd het geïntroduceerd in *C. roseus* cellen. Er kon echter hiermee niet op reproduceerbare wijze worden aangetoond dat verhoogde expressie van *CrA42* leidt tot een hogere expressie van de *TDC* en *STR* genen. Ook andere experimenten gericht op het ophelderen van de functie van CrA42 gaven geen resultaten die een functie van CrA42 als activator van genexpressie

ondersteunden. De aandacht werd daarom verlegd naar andere regulatoren die mogelijk de expressie van de *ORCA* genen zouden kunnen controleren.

In **Hoofdstuk 3** wordt de sleutelrol van CrMYC2 in de regulatie van de *ORCA* genen beschreven. CrMYC2 is het *C. roseus* equivalent van AtMYC2 uit Arabidopsis. De *ORCA3* promoter bevat een DNA sequentie die functioneert als een aan/uit schakelaar in respons op MeJA. CrMYC2 bindt in vitro aan deze sequentie. In *C. roseus* cellen activeert CrMYC2 de *ORCA3* promoter via binding aan de aan/uit schakelaar. Verlaging van het expressieniveau van het *CrMYC2* gen via de RNA interferentie (RNAi) techniek leidde tot sterk verlaagde expressie van de *ORCA* genen in reactie op MeJA toediening.

Hoofdstuk 4 beschrijft dat twee leden van de JAZ eiwitfamilie in *C. roseus* de activiteit van CrMYC2 stil houden. Beide JAZ eiwitten binden aan CrMYC2 in bakkersgist, en dus hoogst waarschijnlijk ook in plantencellen. Analyse van domeinen in CrJAZ1 betrokken bij inactivatie van CrMYC2 gaf aan dat het N-terminale domein een belangrijke rol speelt. Een fusie-eiwit bestaande uit CrJAZ1 en het fluorescente eiwit GFP (Green Fluorescent Protein) was gelocaliseerd in de kern van *C. roseus* cellen. Na toediening van JA verdween het eiwit. Dit kwam door afbraak door het 26S proteasoom, aangezien toediening van de specifieke proteasoominhibitor MG132 de door MeJA veroorzaakte verdwijning teniet deed. De expressie van de *JAZ* genen wordt aangeschakeld door MeJA. In *C. roseus* cellen met verlaagde *CrMYC2* expressie door RNAi is de *JAZ* genexpressie in respons op MeJA sterk verlaagd. Verhoogde expressie van *CrMYC2* door koppeling aan een sterke constitutieve promoter leidde tot verhoogde expressie van de *JAZ* genen. Deze resultaten geven aan dat, net als voor AtMYC2 en *AtJAZ* genen in Arabidopsis, de expressie van de *CrJAZ* genen in *C. roseus* wordt gecontroleerd door CrMYC2.

Hoofdstuk 5 vat de gevonden resultaten samen in een model, en vergelijkt de regulatie van TIA biosynthese in *C. roseus* met de regulatie van de biosynthese van nicotine en verwante alkaloïden in tabak. In *C. roseus* worden een aantal TIA biosynthesegenen gereguleerd door de ORCA transcriptiefactoren. De genen coderend voor de ORCA transcriptiefactoren worden weer gereguleerd door CrMYC2. CrMYC2 wordt normaal stil gehouden

door binding aan CrJAZ repressoren. Na aanmaak van JA in respons op stress worden de CrJAZ repressoren afgebroken door het 26S proteasoom en wordt CrMYC2 actief. Hoogstwaarschijnlijk worden de CrJAZ repressoren geubiquitineerd door een ubiquitine-eiwit ligasecomplex dat het COI1 equivalent van *C. roseus* bevat, maar dat dient nog aangetoond te worden. Actief CrMYC2 schakelt ook de *CrJAZ* genen aan, hetgeen door synthese van nieuwe CrJAZ eiwitten leidt tot het stilleggen van CrMYC2 en het herstellen van de basistoestand in de *C. roseus* cellen. Alhoewel in de experimenten MeJA of JA werd toegediend aan de *C. roseus* cellen, wordt dit hoogstwaarschijnlijk in de plantencellen omgezet in JA-Ile, maar ook dat dient nog aangetoond te worden.

In tabak (*Nicotiana tabacum*) is aangetoond dat de expressie van nicotine biosynthesegenen en de biosynthese van nicotine in respons op MeJA wordt gecontroleerd door NtCOI1 en NtJAZ eiwitten. Over transcriptiefactoren is minder bekend. Voor twee AP2-domein transcriptiefactoren, NtORC1 en NtJAP1, is aangetoond dat ze de activiteit van de promoter van een sleutelgen in de nicotinebiosynthese in tabakscellen kunnen verhogen. NtORC1 is het tabaksequivalent van ORCA3. In **Hoofdstuk 5** wordt de hypothese opgesteld dat NtORC1 en NtJAP1 de expressie van nicotine biosynthesegenen reguleren, en dat de expressie van de genen coderend voor deze transcriptiefactoren wordt gecontroleerd door NtMYC2, het tabaksequivalent van CrMYC2 en AtMYC2. Verder onderzoek moet uitwijzen of deze hypothese klopt.

In veel plantensoorten wordt de biosynthese van secundaire metabolieten gestimuleerd door (Me)JA. Hoogstwaarschijnlijk hebben COI1, JAZ, MYC2 en ORCA-achtige eiwitten een geconserveerde rol in planten in de regulatie van de biosynthese van secundaire metabolieten.

Curriculum Vitae

Hongtao Zhang was born on the 14th of September 1975 in Huadian, China. In 1994, he entered the College of Life Sciences at Nankai University in Tianjin, China. He received his Bachelor degree from Nankai University in June 1998. From September 1998 until August 2002, he worked as a research assistant in plant molecular biology at the Institute for Molecular Biology, Nankai University. From September 2002 until August 2004, he followed the master program of Molecular and Cellular Biology at Leiden University, the Netherlands. He received his degree of Master of Science in Biology in August 2004. From September 2004 until August 2008, he worked on his PhD research project under supervision of Prof. Dr. Johan Memelink at the Institute of Biology, Leiden University.