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Jasmonate-responsive transcriptional regulation in Catharanthus roseus

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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.

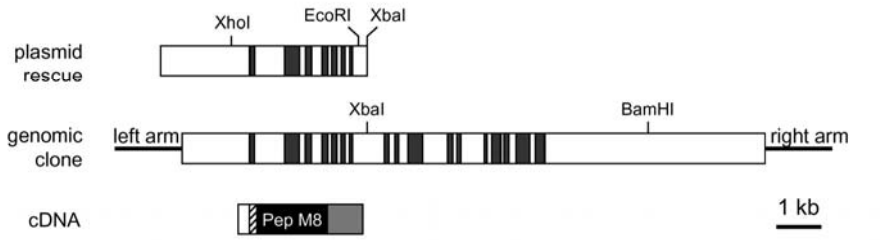

Figure 1. Schematic representation of *CrA42* DNA clones.
At the top is the DNA fragment rescued from T-DNA tagged *C. roseus* cell line A42 with XbaI. The left border flanked the T-DNA activation construct. A XhoI/EcoRI subfragment was used to screen a genomic library. The orientation of the genomic 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.

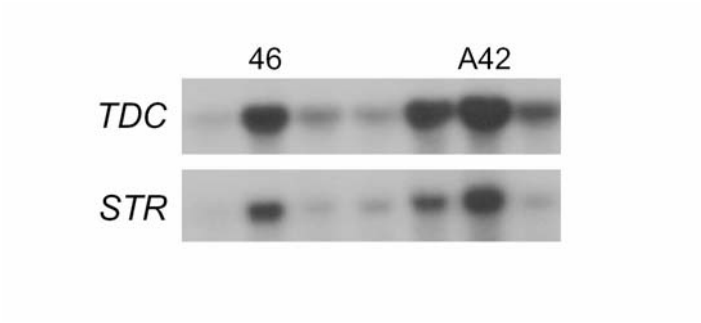

Figure 2. Expression analysis of independent T-DNA tagged *C. roseus* BIX lines. Northern blots containing identical amounts of total RNA from each line were hybridized with *TDC* or *STR* cDNA probes. Only relevant line numbers are indicated. In line 46 the *ORCA3* gene was T-DNA tagged (van der Fits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

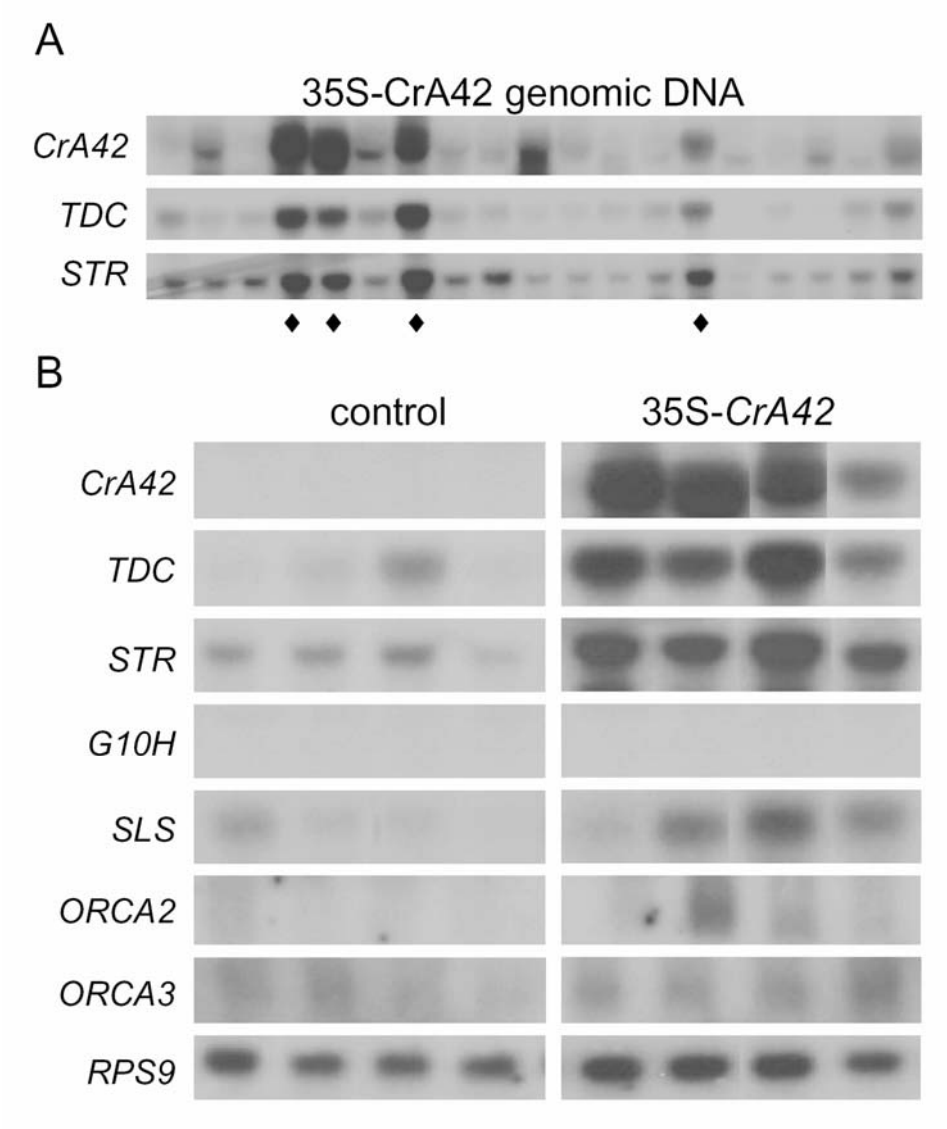

Figure 3. Gene expression analysis in *C. roseus* MP183L lines transformed with a genomic *CrA42* overexpression construct.

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).

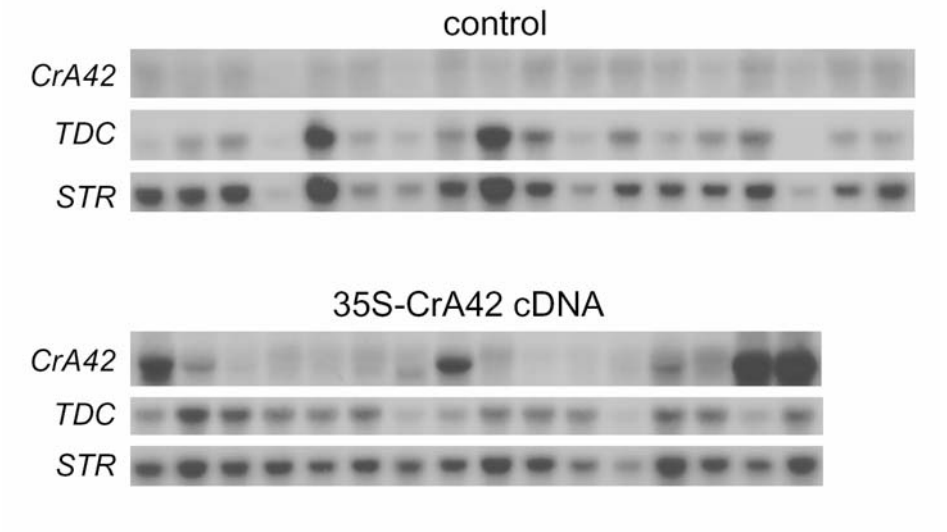

Figure 4. Gene expression analysis in *C. roseus* MP183L lines transformed with a *CrA42* cDNA overexpression construct.

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.

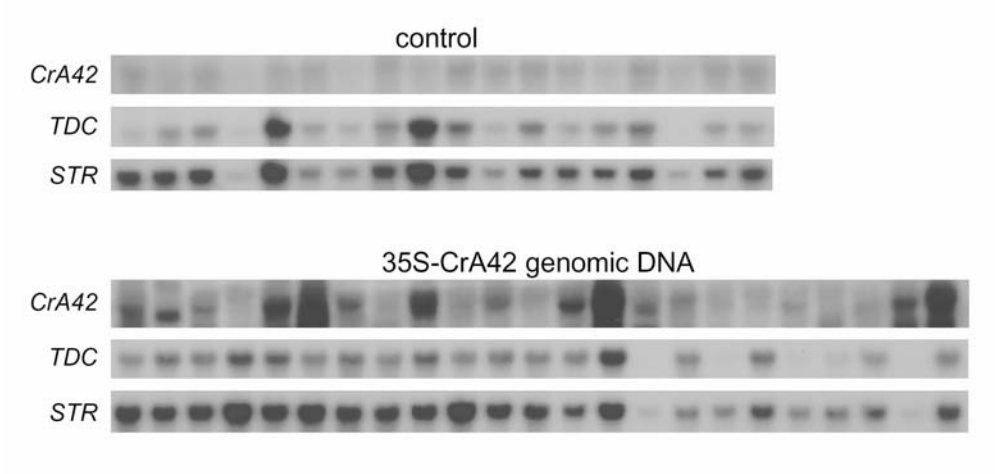

Figure 5. Gene expression analysis in *C. roseus* MP183L lines transformed with a genomic *CrA42* overexpression construct.
The results shown are from a repetition of the experiment shown in Fig. 3. Northern blots containing identical amounts of total RNA from independent control lines or lines transformed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

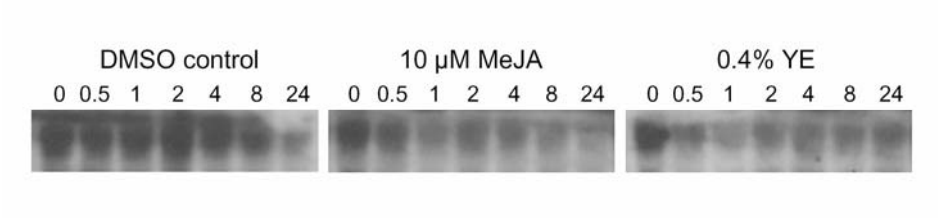

Figure 6. The *CrA42* gene is not induced by MeJA or yeast extract.
Cells from *C. roseus* cell line MP183L were treated with 0.1% (v/v) DMSO, 10 µM MeJA or 0.4% (w/v) yeast extract (YE) for the indicated number of hours. The Northern blot was hybridized with the *CrA42* cDNA.

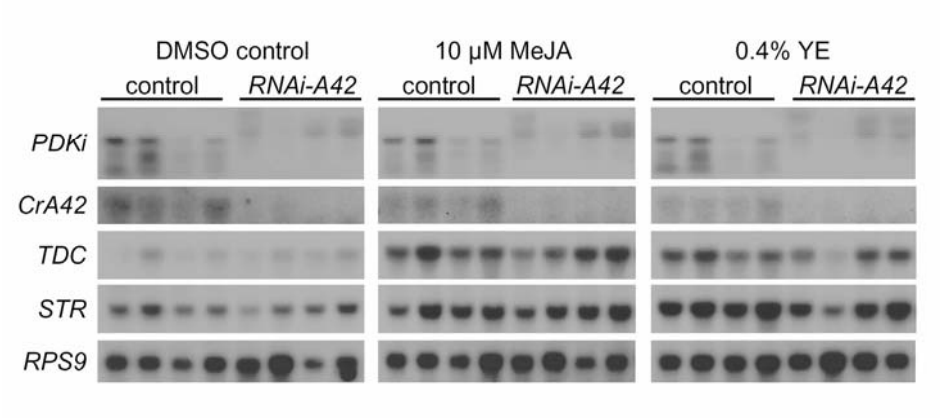

Figure 7. Silencing of *CrA42* does not affect MeJA- or YE-responsive *TDC* and *STR* expression.
Four independent control cell lines and four independent cell lines transformed with an RNAi construct targeting *CrA42* mRNA were treated for 6 hours with 0.1% (v/v) DMSO, 10 µM MeJA or 0.4% (w/v) yeast 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.

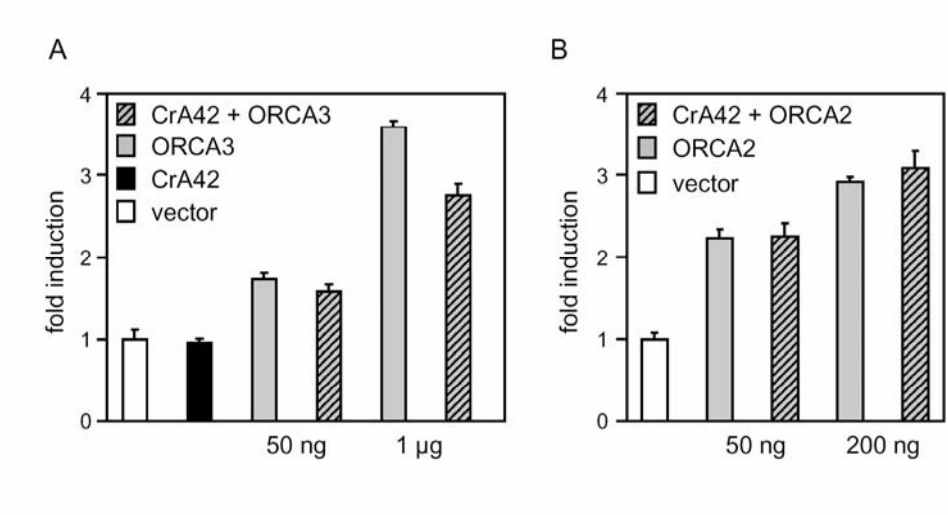

Figure 8. CrA42 does not trans-activate *STR* promoter activity alone or with the ORCAs. A. *C. roseus* MP183L cells were transiently co-transformed with an *STR-GUS* reporter construct and 6 µg of *CrA42* effector plasmid or the indicated amount of *ORCA3* effector plasmid alone or combined as indicated. 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.

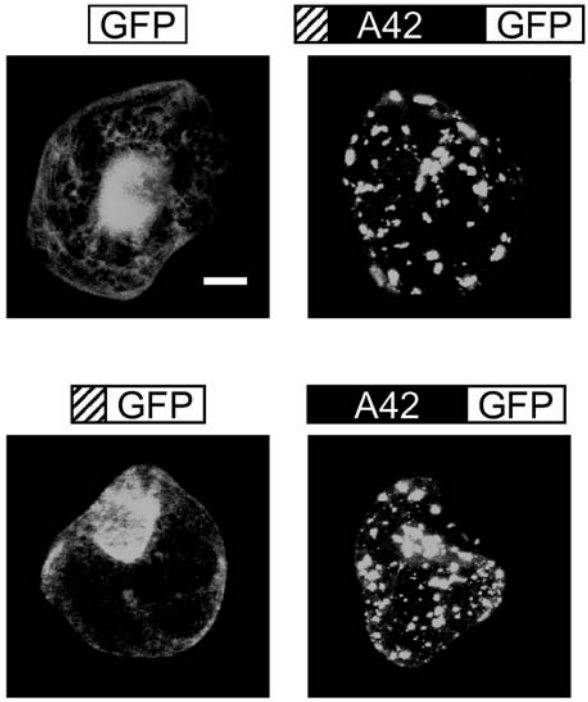

Figure 9. CrA42 protein is localized in cytoplasmic speckles in *C. roseus* cells.
Confocal laser scanning microscopy at 63x magnification of *C. roseus* MP183L cells transiently transformed with plasmids carrying *GFP* or *GFP* fusions with *CrA42* as indicated. The hatched box indicates the N-terminal 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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