Jasmonate-responsive transcriptional regulation in Catharanthus roseus

Zhang, H.

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.

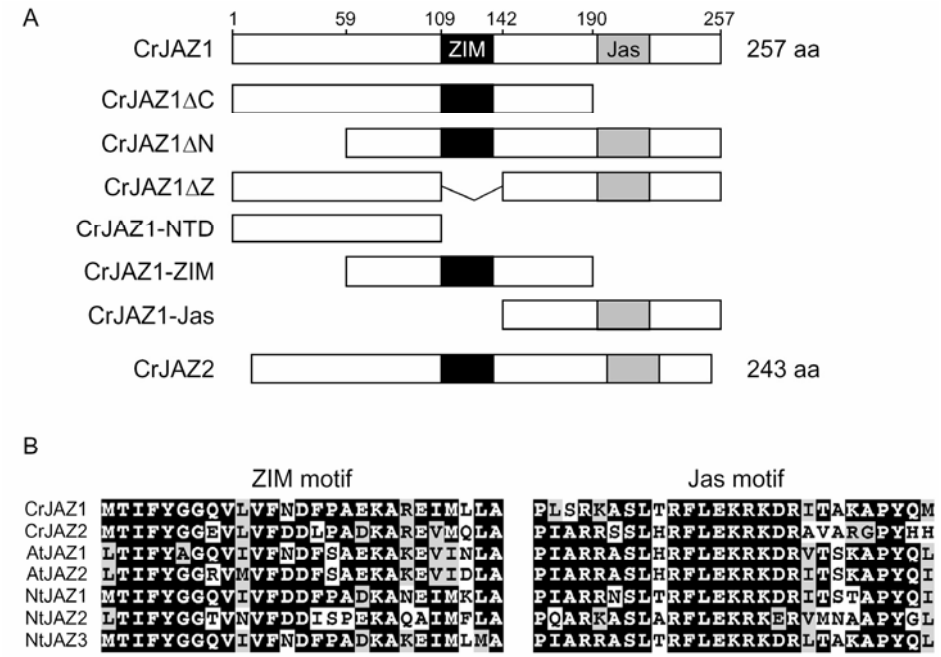

Figure 1. JAZ proteins from *C. roseus*. A, Schematic representation of CrJAZ1 and CrJAZ2 proteins and truncated versions. Numbers indicate amino acid (aa) positions. Relative sizes and positions of conserved ZIM and Jas motifs are shown. B, Alignment of deduced amino acid sequences of the ZIM and Jas 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).

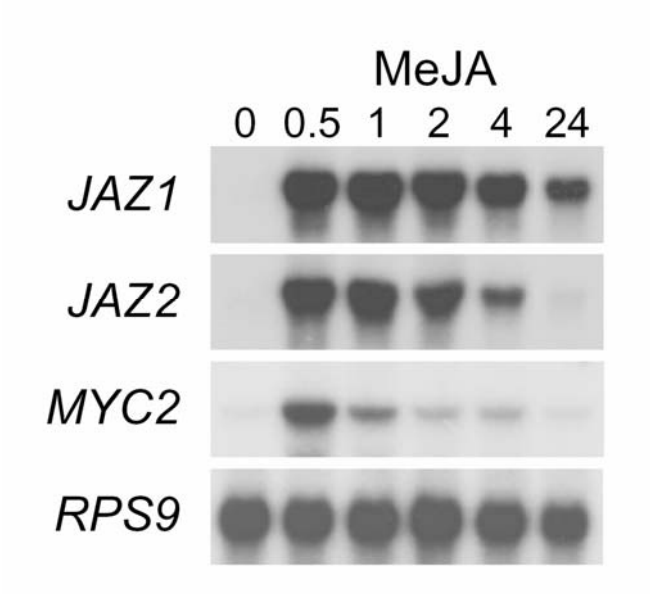

Figure 2. *CrJAZ* gene expression is induced by MeJA. *C. roseus* MP183L cells were treated with 10 µM MeJA for the number of hours indicated. Northern blots were hybridized with the probes indicated at the left.

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.

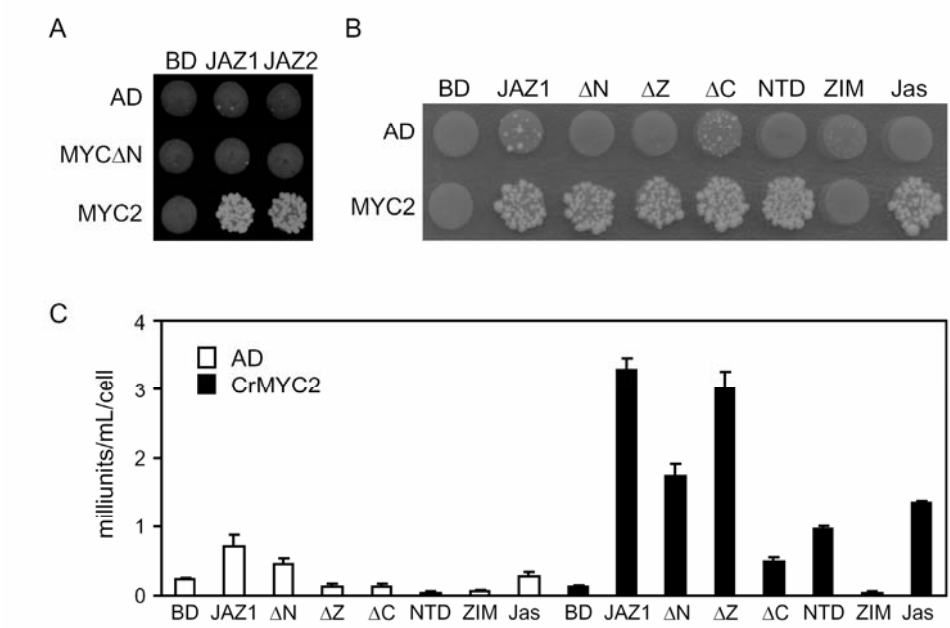

Figure 3. CrMYC2 interacts with the N-terminal and C-terminal domains of CrJAZ1 in yeast. A, CrJAZs interact with the N-terminal part of CrMYC2. Yeast cells expressing the GAL4AD fused to CrMYC2 and the GAL4DBD fused to the indicated CrJAZ were spotted on SD medium selecting for expression of the *ADE* 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

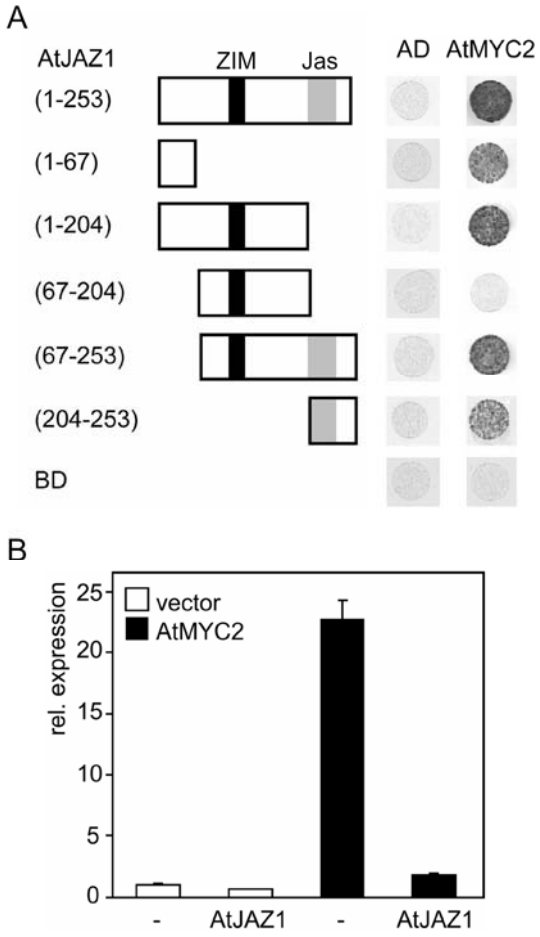

Figure 4. AtJAZ1 interacts with AtMYC2 in yeast and represses AtMYC2 activity in tobacco protoplasts. A, AtMYC2 interacts with the N-terminal and C-terminal domains of AtJAZ1 in yeast. Schematic drawings represent AtJAZ1 and truncated versions. Numbers indicate amino acid positions. Relative sizes and 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).

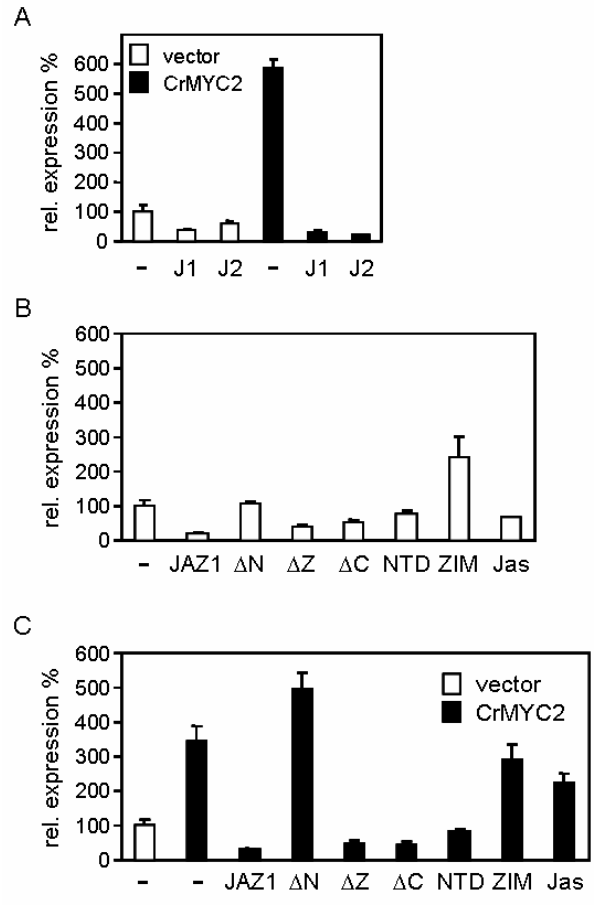

Figure 5. CrJAZ proteins repress CrMYC2 activity. A, CrJAZ proteins repress CrMYC2 activity. *C. roseus* cells were transiently co-transformed with a *GUS* reporter construct carrying the D tetramer, an effector plasmid carrying *CrMYC2* and *CrJAZ1* (J1) or *CrJAZ2* (J2) effectors as indicated. B, The 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.

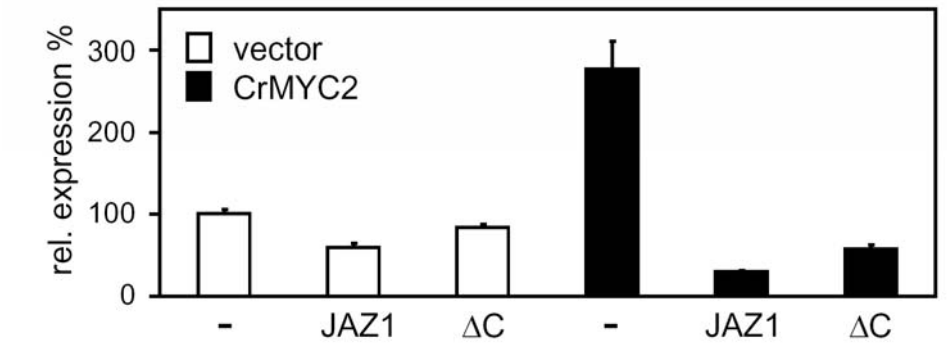

Figure 6. CrJAZ1-GFP fusion proteins repress CrMYC2 activity. A, *C. roseus* MP183L cells were transiently co-transformed with a *GUS* reporter construct carrying the *ORCA3* D tetramer, *CrMYC2* effector plasmid and *CrJAZ1-GFP* or *CrJAZ1ΔC-GFP* effectors. Bars represent means + SE (n = 3). Normalized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

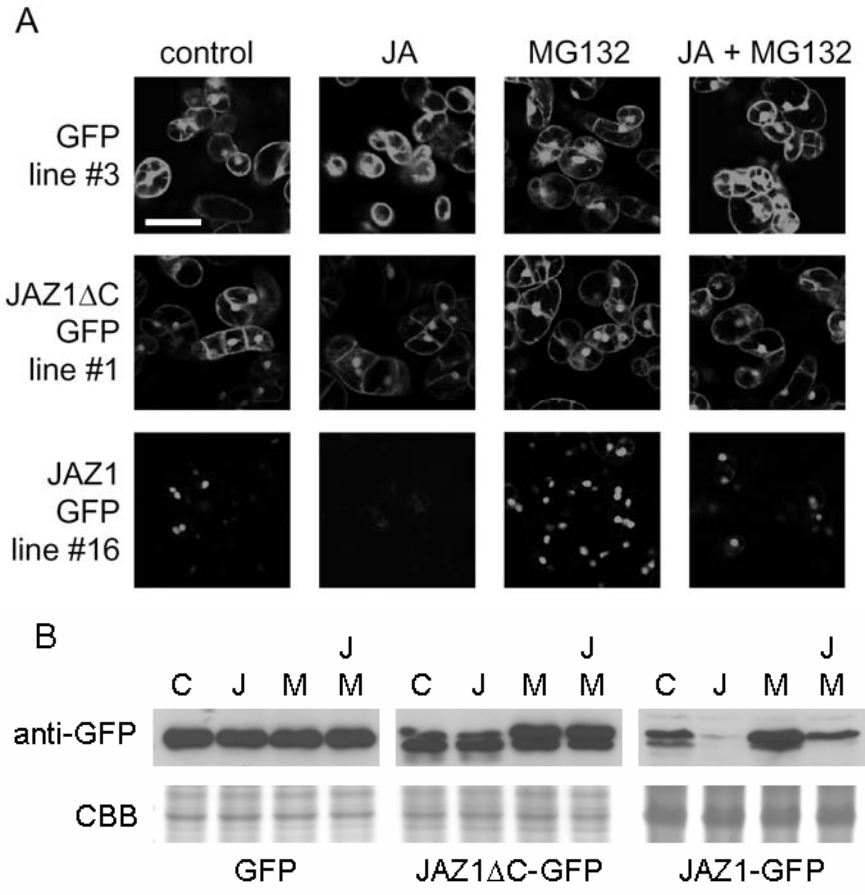

Figure 7. Full-length CrJAZ1 but not CrJAZ1ΔC is rapidly degraded by the 26S proteasome in response to JA. A, *C. roseus* MP183L cell lines stably transformed with *GFP*, *CrJAZ1ΔC-GFP* or *CrJAZ1-GFP* were treated with 50 µM MG132 without or with 50 µM JA for 3 hours. MG132 was added 1 hour before JA 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.

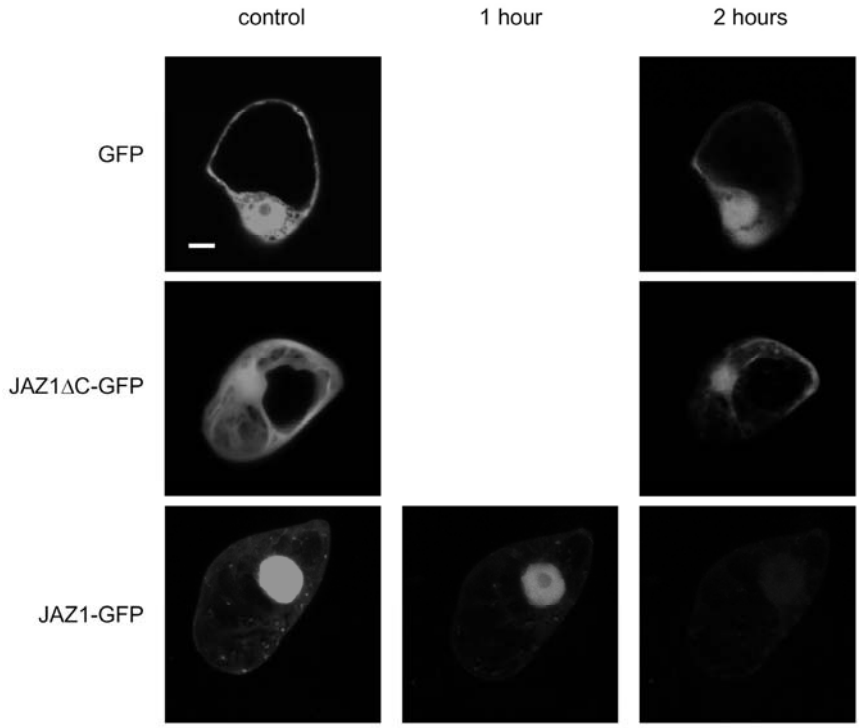

Figure 8. Full-length CrJAZ1 but not CrJAZ1ΔC is rapidly degraded in response to MeJA. Confocal laser scanning microscopy at 63x magnification of *C. roseus* MP183L cells transiently transformed with plasmids carrying *GFP*, *CrJAZ1ΔC-GFP* or *CrJAZ1-GFP* and treated with 50 µM MeJA for the number of 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

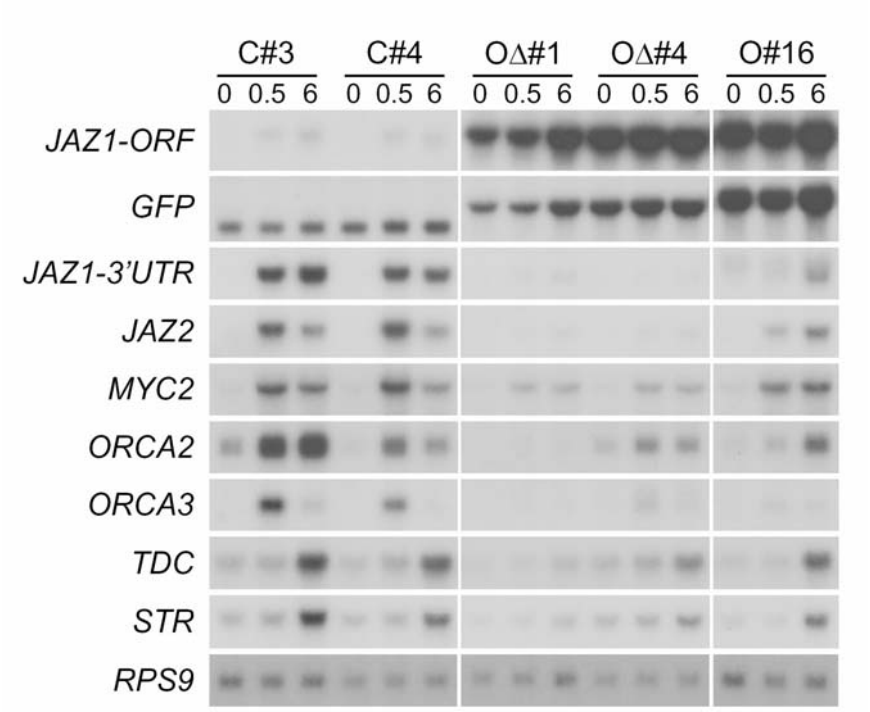

Figure 9. Overexpression of *CrJAZ1-GFP* fusions represses MeJA-responsive gene expression. Transgenic control lines (C) and overexpression lines expressing *CrJAZ1ΔC-GFP* (OΔ) or *CrJAZ1-GFP* (O) were treated with 10 µM MeJA for the indicated number of hours. Northern blots were hybridized with the 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.

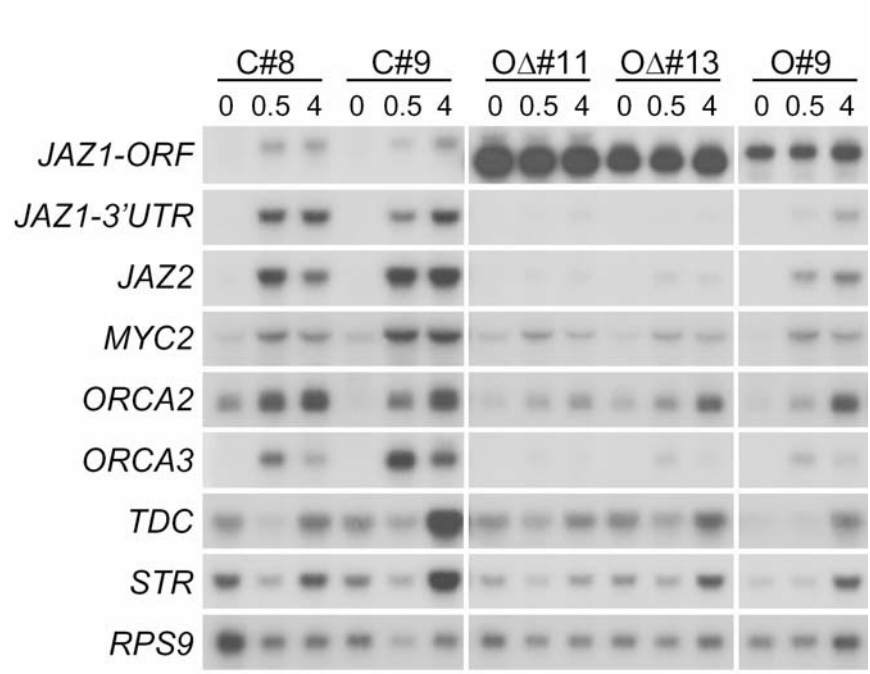

Figure 10. Overexpression of CrJAZ1 represses MeJA-responsive gene expression. Control lines (C) and overexpression lines expressing *CrJAZ1ΔC* (OΔ) or *CrJAZ1* (O) were treated with 10 µM MeJA for the indicated number of hours. Northern blots were hybridized with the probes indicated at the left. The 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.

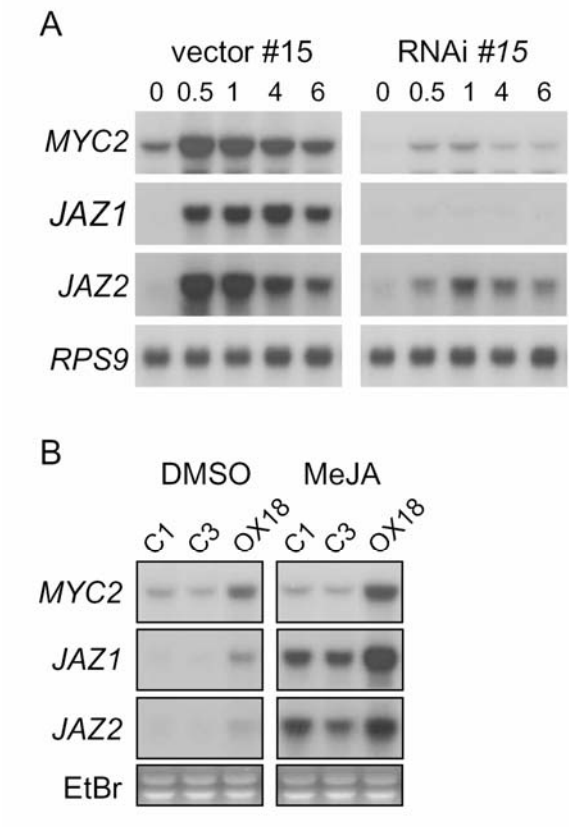

Figure 11. CrMYC2 controls MeJA-responsive *CrJAZ* expression. A, CrMYC2 is required for MeJA-responsive *CrJAZ* expression. Time-course analysis of gene expression in control line #15 and in RNAi-*CrMYC2* line #15. Cell lines were treated with 10 µM MeJA for the number of hours indicated. The *CrMYC2* 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.

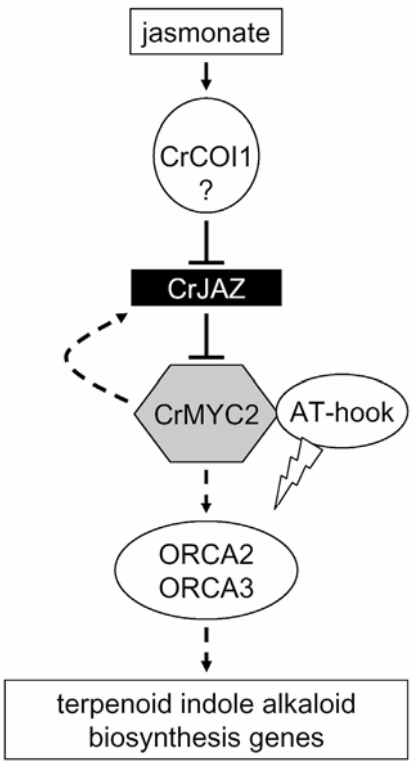

Figure 12. Model for jasmonate signal transduction leading to the expression of terpenoid indole alkaloid biosynthesis genes in *C. roseus*. A jasmonate forms the molecular glue between CrCOI1 and CrJAZ, leading to degradation of the latter proteins. CrMYC2 then activates transcription of the genes encoding 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*).

ACKNOWLEDGMENTS

H.Z. was supported by the Dutch Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO; grant # 812.06.002). J.M. was supported by a van der Leeuw grant from NWO. The authors thank Wim Grunewald for providing the AtJAZ1 deletion constructs, Bernd Weisshaar for providing the pENTRY-AtMYC2 construct, and Pasqua Vernile for cloning the N-terminal domain of CrJAZ1.

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