the plant journal

SEB Society for Experimental Biology

The Plant Journal (2011) **67**, 61–71 doi: 10.1111/j.1365-313X.2011.04575.x

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

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Received 14 February 2011; revised 9 March 2011; accepted 10 March 2011; published online 26 April 2011.
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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 several 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 the 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 activator 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 transactivates reporter gene expression via this sequence in transient assays. Knock-down of the *CrMYC2* expression level via RNA interference caused a strong reduction in the level of MeJA-responsive *ORCA3* mRNA accumulation. In addition, MeJA-responsive expression of the related transcription factor gene *ORCA2* was significantly reduced. 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 in turn regulate a subset of alkaloid biosynthesis genes.

Keywords: AP2/ERF, bHLH, G-box, periwinkle, secondary metabolism, terpenoid indole alkaloids.

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 insect or 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 transactivate the *STR* promoter via sequence-specific binding to a jasmonate- and elicitor-responsive element (JERE), consisting of a GCC box (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 signalling (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 transactivate 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 activator 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; Dombrecht *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 five 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 *C. roseus* cDNA libraries as templates. Analysis of the encoded proteins (Table S1) showed that CrMYC2 is a close homologue of AtMYC2.

The deduced amino acid sequence of CrMYC2 (Figure S1) 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 (Archley and Fitch, 1997). A domain rich in acidic residues is present, and has been suggested to be a transcriptional activation domain in other bHLH transcription factors.

Several putative nuclear localization signals (NLS1–4) are indicated in Figure S1. It was previously reported that the two C-terminal NLS, together with a cryptic NLS, were responsible for targeting CrMYC2 to the nucleus in onion epidermal cells (Hedhili *et al.*, 2010). To confirm that CrMYC2 is a nuclear protein in *C. roseus* cells, we introduced fusions between CrMYC2 and GFP by particle bombardment. As shown in Figure S2, the CrMYC2-GFP fusion protein was exclusively localized in the nucleus of *C. roseus* cells in the absence of jasmonate treatment. The GFP-CrMYC2 fusion protein showed a similar nuclear localization (data not shown). GFP alone was localized both in the nucleus and in the cytoplasm (Figure S2).

CrMYC2 is an immediate-early jasmonate-responsive gene

To get a first indication of 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 with the re-establishment of basal levels after 24 hours (Figure 1a). The expression of the *ORCA3* gene was 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).

The early response of *CrMYC2* was analysed at shorter time points. As shown in Figure 1b, induction of *CrMYC2* by MeJA was already observed at the shortest time point of 5 min after treatment. The 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

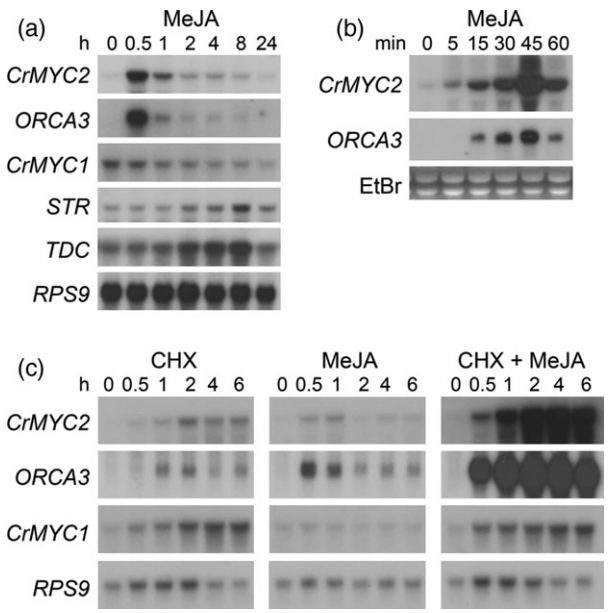

Figure 1. *CrMYC2* is an immediate-early jasmonate-responsive gene.
(a) *Catharanthus roseus* MP183L cells were exposed to 10 μM methyljasmonate (MeJA) for the number of hours indicated. Northern blots were hybridized with cDNAs as indicated.
(b) *Catharanthus roseus* MP183L cells were exposed to 10 μM MeJA for the number of minutes indicated. The ethidium bromide (EtBr) stained gel is shown as a loading control.
(c) *Catharanthus roseus* BIX cells were treated with 100 μM cycloheximide (CHX), 10 μM MeJA or both compounds for the number of hours indicated.

kinetics as observed in cell line MP183L (Figure S3). *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 1c). CHX alone had an inducing effect on *CrMYC2* expression. Together with MeJA, CHX caused superinduction of *CrMYC2* mRNA accumulation. Superinduction 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 superinducing 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 that 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 extending from positions −162 to −88 from the *ORCA3* promoter, which contains the JRE (Figure 3a; Vom Endt *et al.*, 2007). Mutant derivatives contain a block mutation changing five or six nucleotides in their complementary nucleotides (Figure 2a). Block mutations M2 and M3 define the quantitative sequence that 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 2a).

Analysis of recombinant CrMYC2 protein produced in *Escherichia coli* by SDS-PAGE and Coomassie Brilliant Blue staining or western blotting and immunoprobing 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 2b).

The EMSA experiments using the recombinant protein showed that it bound the wild-type D fragment as well as all mutants, except DM6 and DM7 (Figure 2c). In a competition experiment using the labelled wild-type D fragment as a probe in the absence or presence of a 20-fold excess of unlabeled wild-type fragment or mutant derivatives, all fragments competed for binding to the probe except mutant derivatives DM6 and DM7 (Figure S4). These results show

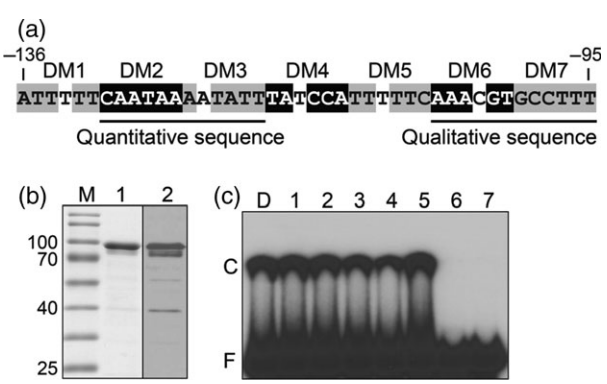

Figure 2. CrMYC2 binds *in vitro* to the qualitative sequence within the *ORCA3* jasmonate-responsive element (JRE).
(a) The wild-type sequence of part of the D fragment from the *ORCA3* promoter is shown. The numbering of mutations is given above the sequence. In each mutant, boxed nucleotides were changed into their complementary nucleotides. Mutations affecting the quantitative and the qualitative sequences are underlined. Nucleotide numbering is relative to the start codon of translation of the *ORCA3* gene.
(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 kD.
(c) *In vitro* binding of recombinant CrMYC2 to wild-type fragment D or mutated derivatives 1–7. Fragments indicated at the top were used as probes in *in vitro* binding. F indicates free probes, whereas C indicates DNA-CrMYC2 complexes.

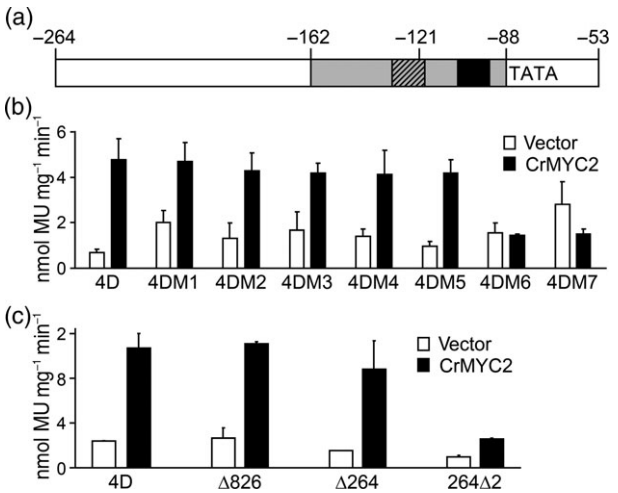

Figure 3. CrMYC2 activates gene expression via interaction with the qualitative sequence within the *ORCA3* jasmonate-responsive element (JRE).
(a) Schematic representation of the *ORCA3* promoter derivative Δ264. Numbering is relative to the ATG start codon. The D fragment is shaded, and the quantitative 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 transactivation. *Catharanthus 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 described in Figure 2a.
(c) *Catharanthus 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 + SEs (n = 3).

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 4DM6 and 4DM7 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 quantities of a plasmid carrying the *CrMYC2* open reading frame fused to the CaMV *35S* promoter showed that CrMYC2 was indeed able to activate gene expression via the D tetramer with the highest gene expression levels at 500 and 1000 ng of plasmid DNA (Figure S5). 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 3b, CrMYC2 transactivated all reporter genes, except those containing the DM6 and DM7 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 Δ826 (from positions –826 to –53, relative to the start codon) or Δ264 (from positions –264 to –53; Figure 3a) promoter derivatives to similar levels as the D tetramer construct (Figure 3c). Deletion of 33 nucleotides including the qualitative sequence (from positions –121 to –88) within the Δ264 promoter context (264Δ2) caused a significant reduction in the level of transactivation by CrMYC2.

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

CrMYC2 does not function synergistically with other CrMYCs

The bHLH proteins can homo- and heterodimerize 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. To be able to observe possible synergistic transactivation effects, 100-ng quantities of *CrMYC* effector plasmids were co-bombarded with the 4D-*GUS* reporter gene, with or without a suboptimal quantity of 100 ng of *CrMYC2* effector plasmid. Figure 4 shows that CrMYC2 was the only CrMYC protein that transactivated the reporter gene at an intermediate level at these effector plasmid levels. 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 transactivation by CrMYC2, whereas doubling the quantity of *CrMYC2* effector doubled the expression level of the reporter gene.

These experiments failed to demonstrate significant positive or negative effects of other CrMYC family members

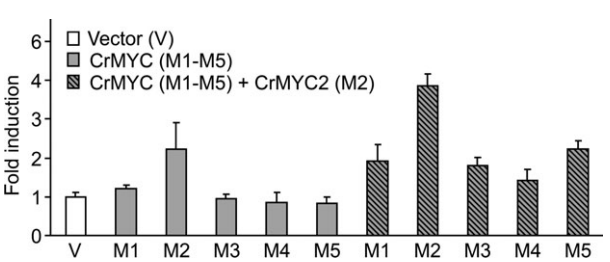

Figure 4. CrMYC2 does not function synergistically with other CrMYCs.
Catharanthus roseus MP183L cells were transiently co-transformed with a *GUS* reporter construct carrying the D tetramer and 100 ng of *CrMYC* (M) effector plasmids alone or combined with 100 ng of *CrMYC2* effector plasmid, as indicated. Total effector was adjusted to 6 μg in all transformations using empty vector plasmid pRT101. Bars represent means + SEs (n = 3). GUS activities are shown as fold induction compared with the vector (V) control.

because of 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 performs a dual role in the regulation of the JA-responsive expression of this TIA 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 transactivate gene expression via the G-box tetramer. As shown in Figure 5b, this was indeed the case. Transactivation occurred via a specific interaction with the G-box, and not via binding to sequences flanking or separating the individual G-boxes, as mutation of two nucleotides in the G-box abolished transactivation. Next, we tested whether CrMYC2 can transactivate gene expression via a tetramer of a larger *STR* promoter fragment of 45 nucleotides containing the G-box (from positions −145 to −100; Figure 5a). Surprisingly, a *GUS* reporter gene carrying four copies of this NR fragment was not transactivated by CrMYC2 (Figure 5c). Next we tested −339 (BH) and −145 (NH) versions of the native *STR* promoter for transactivation by CrMYC2. As a positive control we used an *ORCA3* effector plasmid. Whereas ORCA3 transactivated the gene expression of these BH and NH derivatives, as reported earlier (van der Fits and Memelink, 2001; Pauw *et al.*, 2004), CrMYC2 did not transactivate these *STR* promoter *GUS* constructs (Figure 5c).

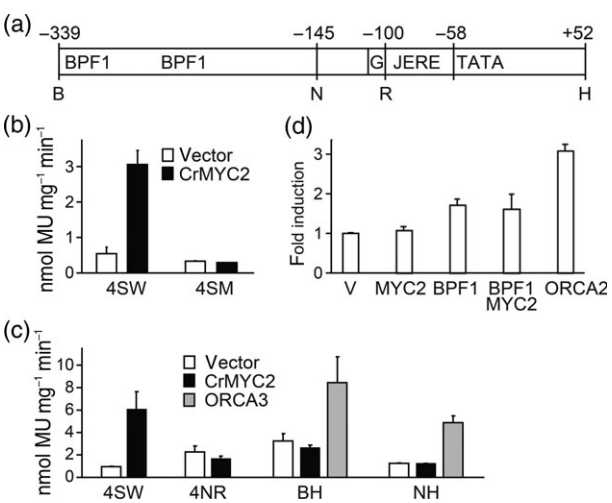

Figure 5. CrMYC2 does not activate expression of the *STR* promoter.
(a) Schematic representation of the *STR* promoter. Letters underneath indicate restriction sites (B, *Bgl*II; N, *Nsi*I; R, *Rsa*I; H, *Hind*III). Numbering is relative to the transcriptional start site. The positions of the TATA box, the G-box, the jasmonate- and elicitor-responsive element (JERE) interacting with the ORCA proteins, and *in vitro* CrBPF1 binding sites (van der Fits *et al.*, 2000) are indicated.
(b) *Catharanthus 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 + SEs (n = 3).
(c) *Catharanthus 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 + SEs (n = 3).
(d) *Catharanthus 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 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 + SEs (n = 4).

In activating promoters from genes encoding enzymes involved in anthocyanin biosynthesis, specific bHLH transcription factors work together with certain members belonging to the R2R3 subgroup of the MYB family of transcription factors (Mol *et al.*, 1998). The MYB-type protein CrBPF1 from *C. roseus* belonging to the family of single-repeat (1R) MYBs has been reported to interact with the *STR* promoter (van der Fits *et al.*, 2000). Although 1R-MYBs generally interact with bHLH proteins to negatively affect gene expression (e.g. Dubos *et al.*, 2008; Matsui *et al.*, 2008), we nevertheless wanted to test the possibility that CrBPF1 functions together with CrMYC2 in activating *STR* promoter activity. Cells of cell line C20D were co-bombarded with the BH-*GUS* reporter gene, and *CrBPF1* 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 5d show that ORCA2 strongly transactivated reporter gene activity via the BH promoter, whereas CrBPF1 gave an intermediate level of transactivation. Also in this cell line CrMYC2 did not transactivate gene expression via the *STR* promoter, and the combination with CrBPF1 had no effect compared with CrBPF1 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 transactivate gene expression via this tetramer in *C. roseus* cells, it did not transactivate gene expression via native *STR* promoter derivatives containing this same G-box either alone or in combination with the 1R-MYB protein CrBPF1.

CrMYC2 is essential for MeJA-responsive *ORCA* expression

To determine whether CrMYC2 is required for the 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 S6). Levels of *CrMYC2* and *ORCA3* mRNA were tightly correlated among independent control (V) or RNAi (RM) lines. Among the lines transformed with the *CrMYC2* silencing construct, lines

RM3, RM7 and RM19 showed a marked decrease in *CrMYC2* and *ORCA3* mRNA levels. No differences were observed between control lines and the RNAi lines in the levels of *RPS9* mRNA (encoding ribosomal protein S9; Figure S6). No differences were observed between control line V7 and the RNAi lines RM7 and RM19 in the levels of *CrMYC1* or *CrMYC4* mRNA (Figure 6). This showed that the RNAi construct did not affect mRNA levels in general, and that it specifically knocked down *CrMYC2* mRNA levels.

RNAi-*MYC2* lines RM7 and RM19 were analysed in more detail for the effect of knocking down *CrMYC2* expression on MeJA-responsive gene expression by using a time course of MeJA treatment (Figure 6). Lines RM7 and RM19 showed a strong reduction in the levels of MeJA-responsive *ORCA2* and *ORCA3* mRNA accumulation compared with the vector control lines V2 and V7. MeJA-induced *STR* and *TDC* mRNA levels were not negatively affected.

The RNAi experiment was repeated by generating independent sets of cell lines transformed with the vector or the RNAi-CrMYC2 construct. Screening of 18 cell lines only yielded a single RNAi-CrMYC2 cell line RM#15, with strongly reduced *CrMYC2* and *ORCA3* mRNA levels (Figure S7a). Also in this screening experiment levels of *CrMYC2* and *ORCA3* mRNA were tightly correlated among independent control or RNAi lines. No differences were observed between control line V#15 and the RNAi line RM#15 in the levels of *RPS9* mRNA, or of *CrMYC1* or *CrMYC4* mRNAs (Figure S7b), confirming that the RNAi construct did not affect mRNA levels in general, and that it specifically knocked down *CrMYC2* mRNA levels. RNAi-*CrMYC2* line RM#15 showed a strong reduction in the levels of MeJA-responsive *ORCA3* and *ORCA2* mRNA accumulation compared with the vector control line V#15 (Figure S7b). MeJA-induced *STR* and *TDC* mRNA levels were not negatively affected, although they appeared to be slightly lower in the RNAi line.

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

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 the 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 with control lines (Figure 7a). No 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 eight independent control lines none showed an elevated *ORCA3* mRNA level, and only one showed an elevated *ORCA2* mRNA level, whereas among 16 independent

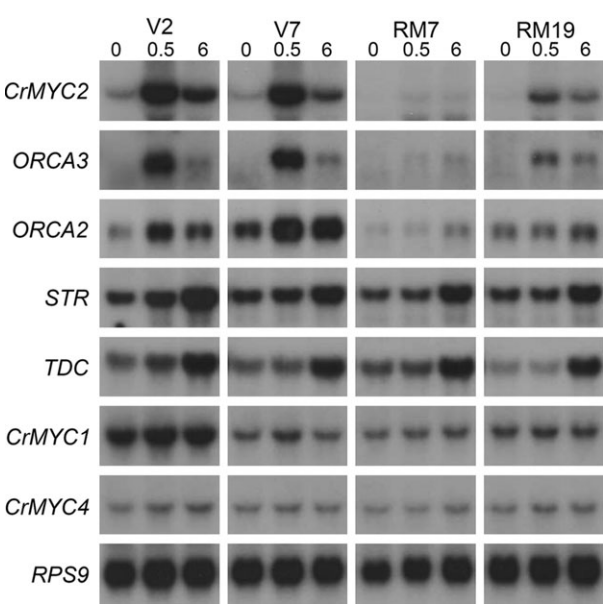

Figure 6. CrMYC2 is essential for MeJA-responsive *ORCA* expression.
Time-course analysis of gene expression in control lines V2 and V7, and in RNAi-*CrMYC2* lines RM7 and RM19. Cell lines were treated with 10 μM MeJA for the number of hours indicated. The *CrMYC2* probe corresponds to an N-terminal fragment that does not contain sequences present in the RNAi construct.

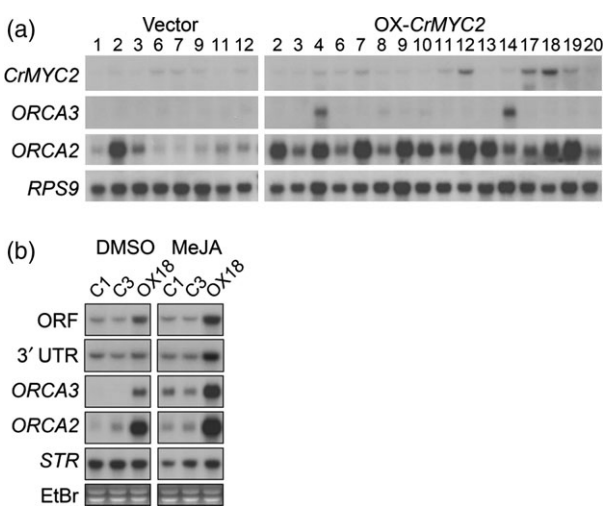

Figure 7. Overexpression of *CrMYC2* induces *ORCA* gene expression.
(a) Screening of independent transgenic MP183L cell lines transformed with a *CrMYC2* overexpression (OX) construct or the corresponding vector control.
(b) Control lines C1 and C3 and overexpression line OX18 were treated for 30 min with 0.1% (v/v) of the solvent DMSO or 50 nM methyl-jasmonate (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 construct.

overexpression lines two showed elevated *ORCA3* mRNA levels and nine showed elevated *ORCA2* mRNA levels.

CrMYC2 overexpression line OX18, showing the highest *CrMYC2* mRNA level and an elevated *ORCA2* mRNA level in the initial screening, was further analysed in comparison with control lines C1 and C3 (Figure 7b). In this second gene expression analysis, line OX18 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. No effect of *CrMYC2* overexpression was observed on the *STR* mRNA level. To determine whether an elevated *CrMYC2* expression level might make the cells more sensitive to MeJA, line OX18 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 7b). Cells overexpressing *CrMYC2* were more sensitive to MeJA, as they showed increased levels of *ORCA2*, *ORCA3* and endogenous *CrMYC2* mRNAs. Induction of endogenous *CrMYC2* mRNA accumulation indicates that CrMYC2 autoregulates its own MeJA-responsive gene expression.

CrMYC2 is essential for MeJA-responsive alkaloid accumulation

Next we investigated whether downregulation of *CrMYC2* by RNAi affected alkaloid accumulation. Cell line MP183L does not produce any alkaloids under normal growth conditions, even when ORCA3 is overexpressed (van der Fits and Memelink, 2000). Alkaloid production is only observed when the terpenoid intermediate loganin is fed. This indicates that in cell line MP183L there is a block in the terpenoid branch of the pathway (Figure S8). Generally, in cell lines vindoline biosynthesis and the biosynthesis of dimeric vinblastine (derived from the condensation of vindoline and catharanthine; Figure S8) do not occur, whereas an accumulation of tabersonine, ajmalicine and/or catharanthine has been observed (Canel *et al.*, 1998; van der Fits and Memelink, 2000).

In a pilot experiment we investigated whether MeJA stimulates the production of alkaloids in control line V5 (Figure S6). In the absence of loganin feeding, no alkaloids were measured with or without MeJA addition after 48, 72 or 96 h (data not shown). When the alkaloid precursors tryptamine and loganin (Figure S8) were added, an accumulation of alkaloids was observed with a positive effect of MeJA. Catharanthine and ajmalicine production was stimulated by MeJA after 72 and 96 h, whereas tabersonine production was stimulated by MeJA at all three time points (Figure S9).

Based on these observations we chose the treatment time of 72 h with MeJA combined with tryptamine and loganin feeding for investigating the effect of downregulating *CrMYC2* expression by comparing alkaloid accumulation in control lines V2 and V7, and in RNAi lines RM7 and RM19 (Figure 8). The RNAi lines had strongly reduced levels of catharanthine and tabersonine. The effect on ajmalicine production was less clear, although the levels appeared to be slightly lower in the RNAi lines.

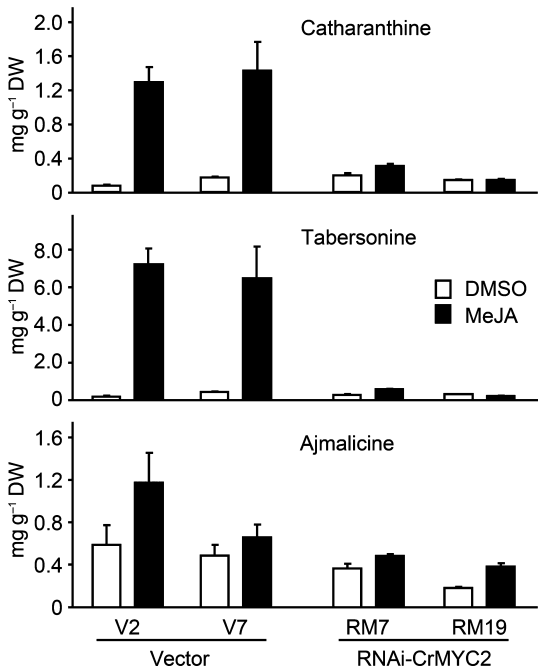

Figure 8. CrMYC2 is essential for MeJA-responsive alkaloid accumulation. Alkaloids were measured in cells of control lines V2 and V7 and RNAi-CrMYC2 lines RM7 and RM19. Cells were grown for 72 h in medium containing 1 mM of the alkaloid precursors tryptamine and loganin, and were treated at the same time with either 0.1% (v/v) of the solvent DMSO or 10 μM methyljasmonate (MeJA). Bars represent means + SEs of triplicate treatments.

DISCUSSION

We show here that CrMYC2 is the activator of MeJA-responsive *ORCA* gene expression. This crucial role for CrMYC2 is deduced from the observations that the *CrMYC2* gene was an immediate-early MeJA-responsive gene, that the CrMYC2 protein bound *in vitro* to the qualitative sequence within the *ORCA3* JRE, that it transactivated gene expression via the same sequence in *C. roseus* cells, that CrMYC2 overexpression caused an increase in *ORCA* mRNA levels and that knock down of the *CrMYC2* mRNA level caused a significant reduction in the level of MeJA-responsive *ORCA* gene expression.

Downregulation of *CrMYC2* expression had no effect on the MeJA-responsive expression of the early alkaloid biosynthesis genes *TDC* and *STR*. This suggests that the basal *ORCA* expression levels are sufficiently high to support normal induction levels. This is in agreement with the observation that *de novo* ORCA protein biosynthesis is not necessary for MeJA-responsive *TDC* and *STR* expression (van der Fits and Memelink, 2001). Despite the lack of effect on *TDC* and *STR* expression, downregulation of *CrMYC2*

expression had a strong effect on alkaloid accumulation. This indicates that one or more downstream biosynthesis genes are tightly controlled by CrMYC2, either directly or indirectly via other unknown transcription factors.

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 model is that the activity of AtMYC2 is regulated by repressors belonging to the family of Jasmonate ZIM-domain (JAZ) proteins (Chini *et al.*, 2007; Hou *et al.*, 2010). 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 also shown to be degraded in response to MeJA, and a stable JAZ derivative was shown to repress the expression of nicotine biosynthesis genes (Shoji *et al.*, 2008).

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, as its induction is 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 the activation of pre-existing CrMYC2 protein. In analogy to the regulation of AtMYC2 by JAZ repressors, this activation step probably consists of the degradation of JAZ proteins that repress CrMYC2 activity in the absence of MeJA.

In *CrMYC2* overexpression lines *ORCA3* mRNA levels did not correlate with *CrMYC2* mRNA levels. At first thought, tight correlation would perhaps be expected in a model where CrMYC2 directly controls *ORCA* gene expression. However, at second thought, the lack of correlation is not unexpected, when one takes into consideration that CrMYC2 activity is probably regulated by JAZ repressors. The expression of *JAZ* genes in Arabidopsis is induced by jasmonates (Chini *et al.*, 2007; Thines *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 existence of a similar oscillator in *C. roseus* cells would cause unpredictable and unstable CrMYC2 activity upon overexpression, as its activity at any given time point would depend on the relative ratios of the activator CrMYC2 and the JAZ repressors. The existence of such an unstable 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.

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). Block mutation scanning of the D region from the *ORCA3* promoter did not uncover a MYB binding site important for MeJA-responsive expression levels (Vom Endt *et al.*, 2007). In addition, CrMYC2 was able to transactivate 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). In *C. roseus* cells CrMYC2 was able to activate gene expression via this tetramer. Surprisingly, CrMYC2 was unable to activate gene expression via native *STR* promoter derivatives. Functional analysis of *STR* promoter deletion derivatives showed that the G-box is not involved in MeJA-responsive *STR* promoter activity (Menke *et al.*, 1999). Therefore, it seems unlikely that CrMYC2 directly regulates *STR* expression via the 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 in turn regulate a subset of alkaloid biosynthesis genes, including *TDC* and *STR*.

The transcriptional regulation of TIA biosynthesis in *C. roseus* shows similarities to the regulation of the biosynthesis of nicotine and related alkaloids in the genus *Nicotiana*, including *Nicotiana tabacum* (tobacco) and *Nicotiana benthamiana*. Nicotine biosynthesis is controlled by the *NIC2* locus, which comprises a cluster of *AP2/ERF* genes that are very similar to *ORCA3* (Shoji *et al.*, 2010). Members of this cluster are induced by MeJA. The encoded proteins bind to a GCC box in the promoter of the nicotine biosynthesis gene encoding putrescine *N*-methyltransferase (PMT) (Shoji *et al.*, 2010), and activate the *PMT* promoter in transient assays (De Sutter *et al.*, 2005; Shoji *et al.*, 2010). It is not known how the MeJA-responsive expression of the tobacco *AP2/ERF* genes is regulated. The observation that JAZ proteins control nicotine biosynthesis (Shoji *et al.*, 2008) suggests that bHLH transcription factors play a role, but these have not been identified in tobacco. In *N. benthamiana* bHLH transcription factors were shown to function as positive regulators of nicotine biosynthesis (Todd *et al.*, 2010). However, it is unknown whether these NbbHLH proteins function in jasmonate signalling, and whether their activity is controlled by JAZ proteins. Interestingly, these NbbHLH proteins were shown to bind *in vitro* to a G-box in

the *PMT* promoter (Todd *et al.*, 2010), suggesting that *PMT* expression is regulated both by bHLH and AP2/ERF-domain proteins. This notion is corroborated by the observation that the MeJA-responsive activity of the *PMT* promoter in tobacco depends both on a G-box and a GCC box (Xu and Timko, 2004). Therefore, a difference between *C. roseus* and tobacco alkaloid biosynthesis may be that in *C. roseus* bHLH and AP2/ERF-domain transcription factors act in a transcriptional cascade to regulate biosynthesis genes, whereas in tobacco, bHLH and AP2/ERF-domain transcription factors seem to act directly on the promoters of biosynthesis genes in a cooperative manner. Notwithstanding this possible difference, it remains possible that tobacco bHLH transcription factors regulate the MeJA-responsive expression of *AP2/ERF* genes in a similar manner as observed in *C. roseus*.

Transcription factors may form useful tools for engineering the production of valuable secondary metabolites (Gantet and Memelink, 2002). CrMYC2 is potentially a very valuable tool, as 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 for metabolic engineering is limited if its activity is negatively regulated by JAZ repressors. Therefore, it is important to determine whether CrMYC2 activity is indeed controlled by JAZ repressors, and if so, whether it can be uncoupled from this negative regulation.

EXPERIMENTAL PROCEDURES

Cell cultures, stable transformation, treatments and GFP analysis

Catharanthus roseus cell lines were grown, treated with MeJA and transformed as previously described (van der Fits and Memelink, 1997; Gantet *et al.*, 1998; Appendix S1). GFP fluorescence was examined with a Leica (http://www.leica-microsystems.com) inverted microscope (DM IRBE), equipped with a Leica SP1 confocal scanhead, with an argon laser at an excitation wavelength of 488 nm, and with the collection of emitted fluorescence after passage through a broad bandpass filter (500–550 nm). The resulting signal was amplified, digitalized and the consistent picture reconstituted by Leica software.

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 *C. roseus* MP183L cells treated with yeast extract (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

Detailed cloning procedures can be found in Appendix S1.

Transient expression assays

Transient expression assays were performed as described previously (van der Fits and Memelink, 1997; Appendix S1).

RNA extraction and northern blot analyses

Total RNA extraction and northern blot hybridization was performed essentially as previously described (Memelink *et al.*, 1994; Menke *et al.*, 1999; Appendix S1).

Isolation of recombinant CrMYC2 protein and EMSA

Double Strep/His-tagged CrMYC2 protein was expressed from plasmid pASK-IBA45plus in *E. coli* strain BL21 (DE3) pLysS and purified by sequential Ni-NTA agarose (Qiagen, http://www.qiagen.com) and Strep-Tactin sepharose (IBA Biotagnology, http://www.iba-go.com) 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-horseradish peroxidase (HRP) 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 labelled 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. Competition reactions contained 0.5 ng of end-labeled D fragment without or with 10 ng of unlabeled D fragment or mutant derivatives. Following the addition of protein extract, reactions were incubated for 30 min at room temperature before loading on 5% w/v acrylamide/bisacrylamide (37:1) gel with half-strength Tris-Borate-EDTA under tension. After electrophoresis at 125 V for 1 h, the gel was dried on Whatman DE81 paper and autoradiographed.

Alkaloid measurements

Four days after transfer, 20-ml aliquots of cell cultures were transferred to 100-ml Erlenmeyer flasks in triplicate for each treatment, and were treated with final concentrations of 1 mM tryptamine (Sigma-Aldrich, http://www.sigmaaldrich.com), 1 mM loganin (Extrasynthese, http://www.extrasynthese.com) and 0.1% (v/v) DMSO or 10 μM MeJA dissolved in DMSO. Alkaloid extracts were analysed by HPLC coupled to a photodiode array detector, and identified and quantified by comparison of retention times, UV spectra and peak areas with those of reference standards (Appendix S1).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *AF283507* (*CrMYC2*), *FJ004233* (*CrMYC3*), *FJ004234* (*CrMYC4*) and *FJ004235* (*CrMYC5*).

ACKNOWLEDGEMENTS

We thank Ghulam Hasnain for help with alkaloid measurements. HZ 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 no. 812.06.002). YZ was supported by a scholarship from the China Scholarship Council. JM was supported by a van der Leeuw grant from NWO. PG was supported by grants from the French Ministry of National Education, Research and Technology, and by the University François Rabelais

of Tours. SH and GM held doctoral fellowships from the Région Centre. MP and GC held Erasmus grants. The collaboration between the Tours and the Leiden groups was supported by a European Van Gogh 2000–2001 exchange grant (grant no. 00106NH).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Nucleotide sequence of *CrMYC2* cDNA from *Catharanthus roseus* and deduced amino acid sequence.

Figure S2. CrMYC2 is a constitutively nuclear protein in *Catharanthus roseus* cells.

Figure S3. *CrMYC2* is rapidly induced by MeJA in *Catharanthus roseus* cell line C20D.

Figure S4. CrMYC2 binds *in vitro* to the qualitative sequence within the *ORCA3* JRE.

Figure S5. Dose–response relationship for the transactivation of gene expression via the D tetramer by CrMYC2.

Figure S6. Screening for effective downregulation of CrMYC2 mRNA accumulation in RNAi lines.

Figure S7. CrMYC2 is essential for MeJA-responsive *ORCA* expression.

Figure S8. Biosynthetic pathway for terpenoid indole alkaloids in *Catharanthus roseus*.

Figure S9. Effect of MeJA on alkaloid accumulation in *Catharanthus roseus* cell line MP183L.

Table S1. *Catharanthus roseus* CrMYC proteins and their most similar counterparts in Arabidopsis.

Appendix S1. Detailed experimental procedures.

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Accession numbers: *CrMYC2* (*AF283507*); *CrMYC3* (*FJ004233*); *CrMYC4* (*FJ004234*); *CrMYC5* (*FJ004235*).

