Jasmonate-responsive transcriptional regulation in Catharanthus roseus

Zhang, H.

Chapter 3

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

Hongtao Zhang [1,†], Sabah Hedhili [2,†], Grégory Montiel [2,†], Guillaume Chatel [2], Martial Pré [1,2], Johan Memelink [1,†,*] and Pascal Gantet [2,‡]

[1] Institute of Biology, Leiden University, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands, and

[2] EA 2106, Biomolécules et Biotechnologies Végétales, Université de Tours, UFR des Sciences et Techniques, Laboratoire de Physiologie Végétale, Parc de Grandmont, 37200 Tours, France

* For correspondence (fax +31 71 5275088; e-mail j.memelink@biology.leidenuniv.nl).

[†] These authors contributed equally to this work.

[‡] Present address: UMR DAP 1098, Université Montpellier 2, Bat 15, CC 002, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France.

Summary

Jasmonates are plant signalling molecules that play key roles in defence against insects and certain pathogens, among others by controlling the biosynthesis of protective secondary metabolites. In *Catharanthus roseus*, the AP2/ERF-domain transcription factor ORCA3 controls the jasmonate-responsive expression of genes encoding enzymes involved in terpenoid indole alkaloid biosynthesis. *ORCA3* gene expression is itself induced by jasmonate. The *ORCA3* promoter contains an autonomous jasmonate-responsive element (JRE) composed of a quantitative sequence responsible for a high level of expression and a qualitative sequence that acts as an on/off switch in response to methyl-jasmonate (MeJA). Here we identify the basic Helix-Loop-Helix (bHLH) transcription factor CrMYC2 as the major regulator of MeJA-responsive *ORCA3* gene expression. The *CrMYC2* gene is an immediate-early jasmonate-responsive gene. CrMYC2 binds to the qualitative sequence in the *ORCA3* JRE *in vitro*, and trans-activates reporter gene expression via this sequence in transient assays. Knock-down of the *CrMYC2* expression level via RNA interference caused a strong reduction in the level of MeJA-responsive *ORCA3* mRNA accumulation. In addition, MeJA-responsive expression of the related transcription factor gene *ORCA2* was significantly reduced. Our results show that MeJA-responsive expression of alkaloid biosynthesis genes in *C. roseus* is controlled by a transcription factor cascade consisting of the bHLH protein CrMYC2 regulating *ORCA* gene expression, and the AP2/ERF-domain transcription factors ORCA2 and ORCA3, which regulate in turn a subset of alkaloid biosynthesis genes.

Introduction

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

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

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

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

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

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

Results

Basic Helix-Loop-Helix proteins from Catharanthus roseus

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

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

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

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

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

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

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

CrMYC2 is an immediate-early jasmonate-responsive gene

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

Fig.1

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1261       TCTCAGAATGCCCAGCAGCCTCAAGGTAGTTTCTTTACAAGGGAATTGAATTTTTCAGAA
359         S  Q  N  A  Q  Q  P  Q  G  S  F  F  T  R  E  L  N  F  S  E
```

```
1321    TACGGGTTTGAAAGGAGTAGTGTAAAGAATGGGAATTGTAAGCCAGAATCGGGAGAAATA
379      Y  G  F  E  R  S  S  V  K  N  G  N  C  K  P  E  S  G  E  I

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2281    GTATGAAGGGTAGATTAAACTCAAGAAAAGTTGGTAGGACTGAGAGTTTCTTTGCAAATT
699      V  *

2341    AGTTTTAGGCTCTAGGATATTTTAAGGCTCCCTGGAAGAATCCCTGTAAACTATTTCCTT
2401    TTCAACTAGGGTTTCCTAAAATTCTAGCAAGAGTCAGAAGTTGAGGTCTCTCTTAGTTGA
2461    AAACCTAAGAGAATTCAGCTTTCATCAAATTTTTGTCTTTTAACTTTTGGGGTTTATTTT
2521    TATGTCTAGATTGTATATATATTTGTAATCAATCAATAAACCTTGTCTACTCTGGTGCTT
2581    AGTTGTGCTGTTAGATTGGAGGTTTGCTAAAAAAAAAAAAAAA
```

Figure 1. Nucleotide sequence of *CrMYC2* cDNA from *C. roseus* and deduced amino acid sequence. (To be continued on the next page)

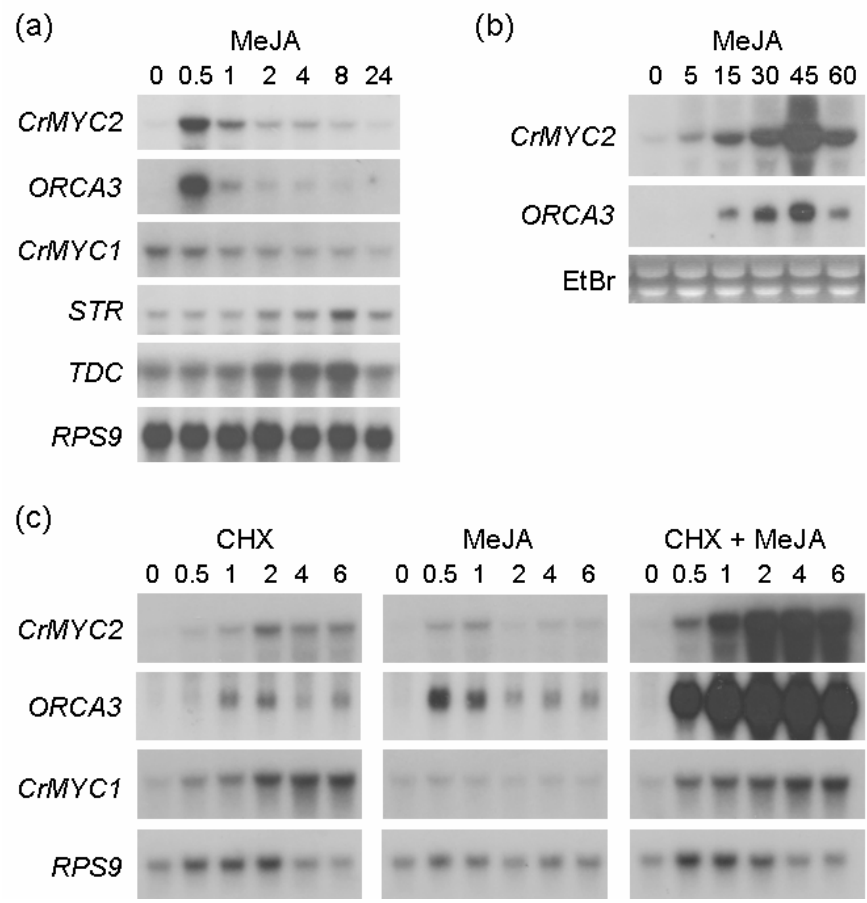

Figure 2. *CrMYC2* is an immediate-early jasmonate-responsive gene.

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

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

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

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

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

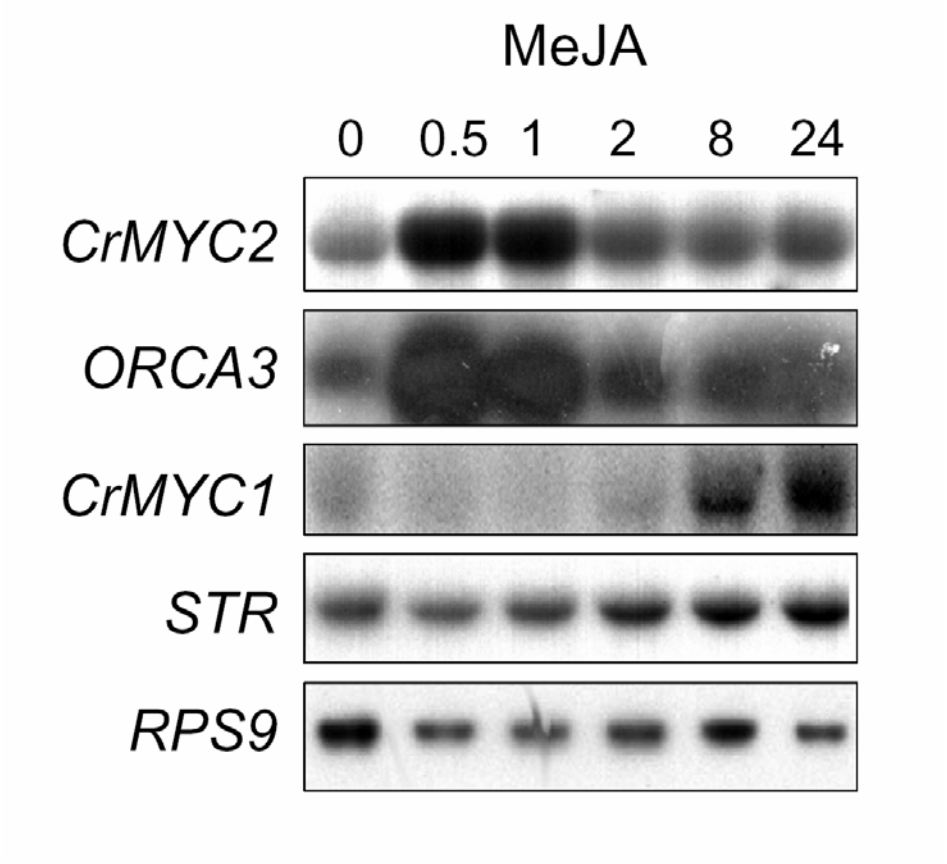

Figure 3. *CrMYC2* is rapidly induced by MeJA in *C. roseus* cell line C20D.
C20D cells were treated 4 days after transfer to medium without hormones with 100 µM MeJA for the number of hours indicated. Northern blots were hybridized with cDNAs as indicated on the left.

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

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

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

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

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

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

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

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

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

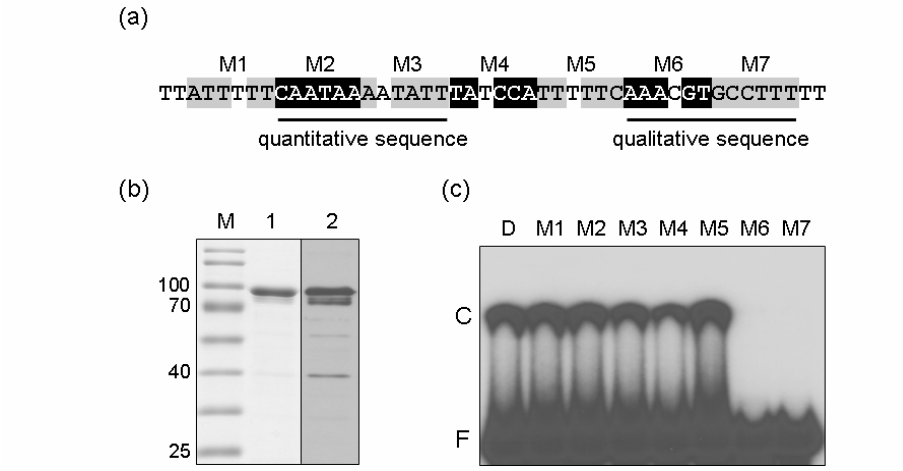

Figure 4. CrMYC2 binds *in vitro* to the qualitative sequence within the *ORCA3* JRE.

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

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

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

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

CrMYC2 activates gene expression via interaction with the qualitative sequence

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

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

Next we investigated whether CrMYC2 also functioned as an activator of reporter genes carrying native *ORCA3* promoter derivatives. CrMYC2 activated reporter genes carrying the Δ828 (-826 to -53 relative to the start codon) or Δ264 (-264 to -53) promoter derivatives to similar levels as the D tetramer construct (Figure 6c). Deletion of 33 nucleotides including the qualitative sequence within the Δ264 promoter context caused a significant reduction in the level of trans-activation by CrMYC2.

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

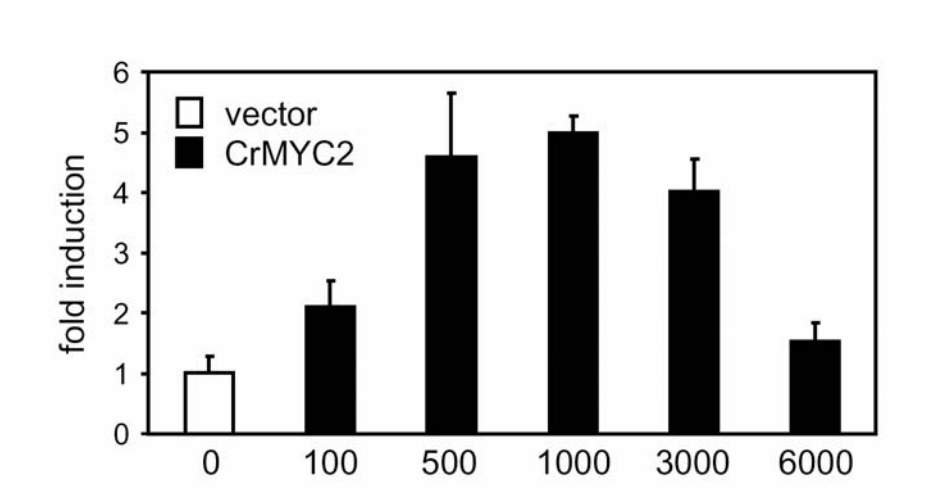

Figure 5. Dose-response relationship for trans-activation of gene expression via the D tetramer by CrMYC2.

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

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

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

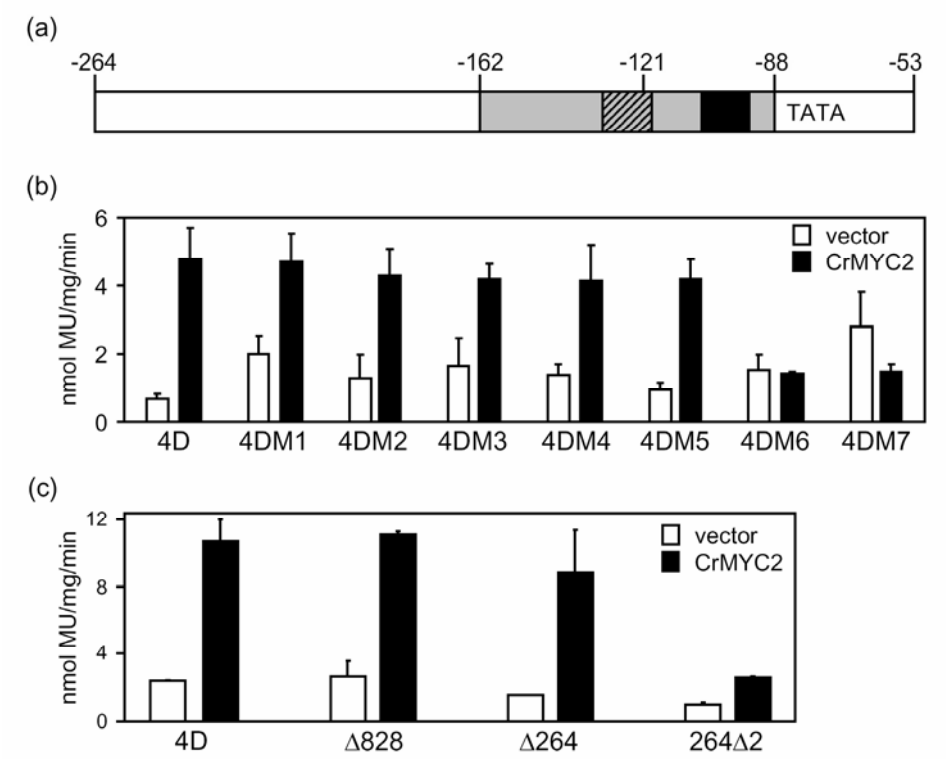

Figure 6. CrMYC2 activates gene expression via interaction with the qualitative sequence within the *ORCA3* JRE.

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

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

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

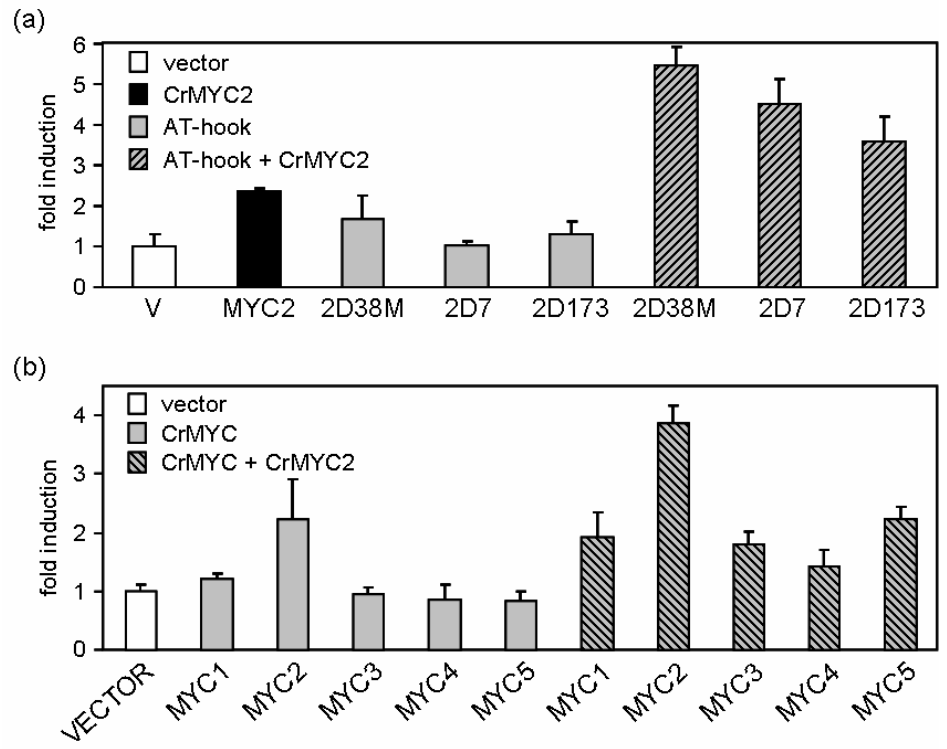

Figure 7. CrMYC2 activates gene expression synergistically with AT-hook proteins but not with other CrMYCs.

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

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

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

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

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

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

CrMYC2 does not activate expression of the STR promoter

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

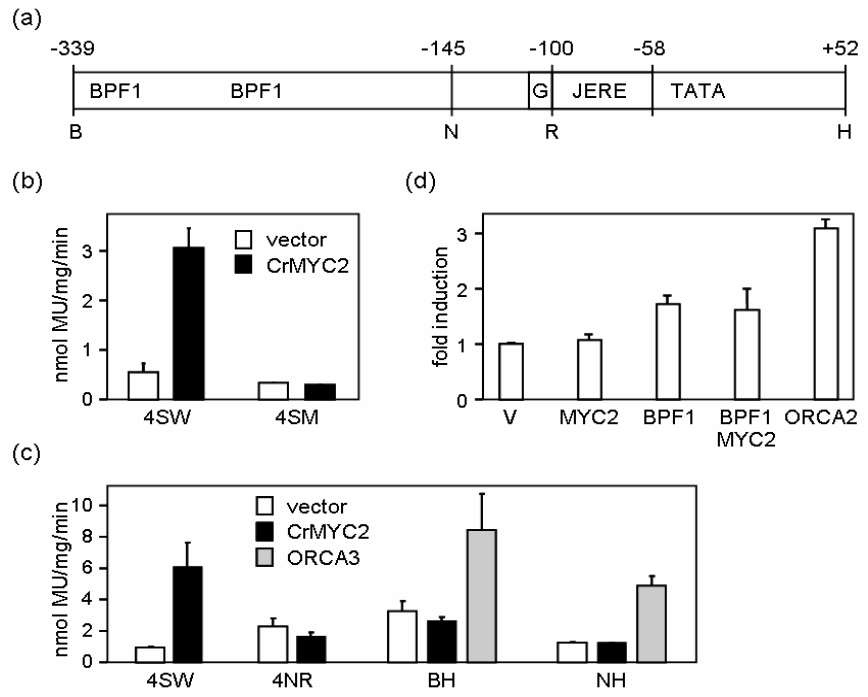

Figure 8. CrMYC2 does not activate expression of the *STR* promoter.

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

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

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

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

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

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

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

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

CrMYC2 is essential for MeJA-responsive ORCA expression

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

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

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

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

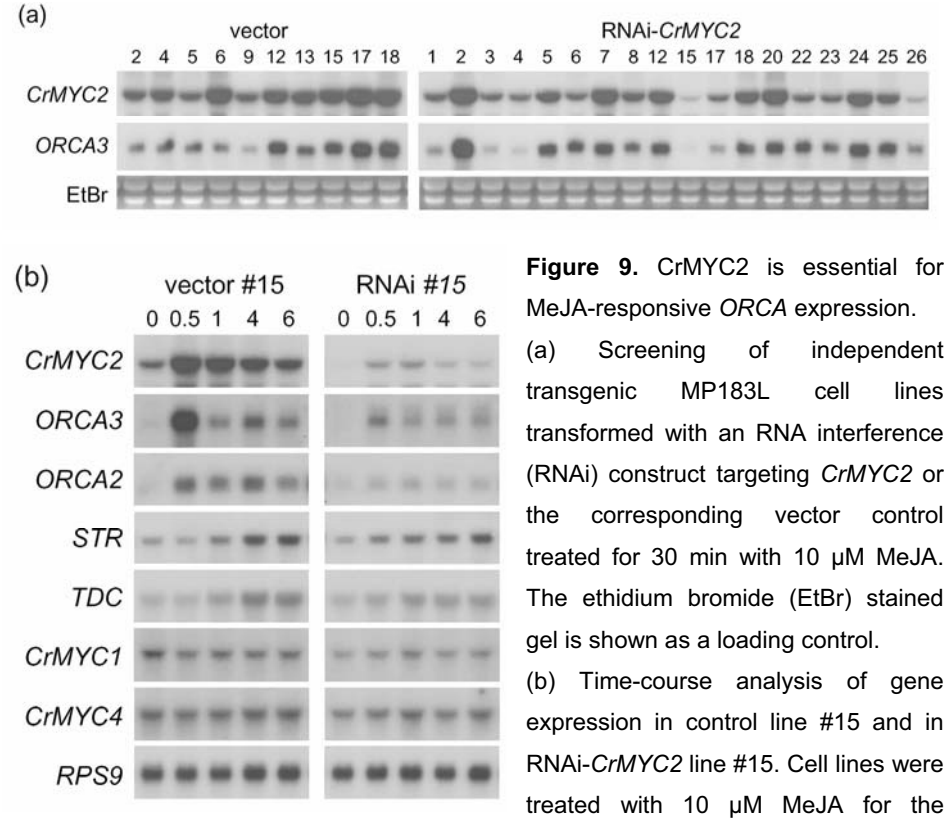

Figure 9. CrMYC2 is essential for MeJA-responsive *ORCA* expression.
(a) Screening of independent transgenic MP183L cell lines transformed with an RNA interference (RNAi) construct targeting *CrMYC2* or the corresponding vector control treated for 30 min with 10 µM MeJA. The ethidium bromide (EtBr) stained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

Overexpression of CrMYC2 induces ORCA gene expression

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

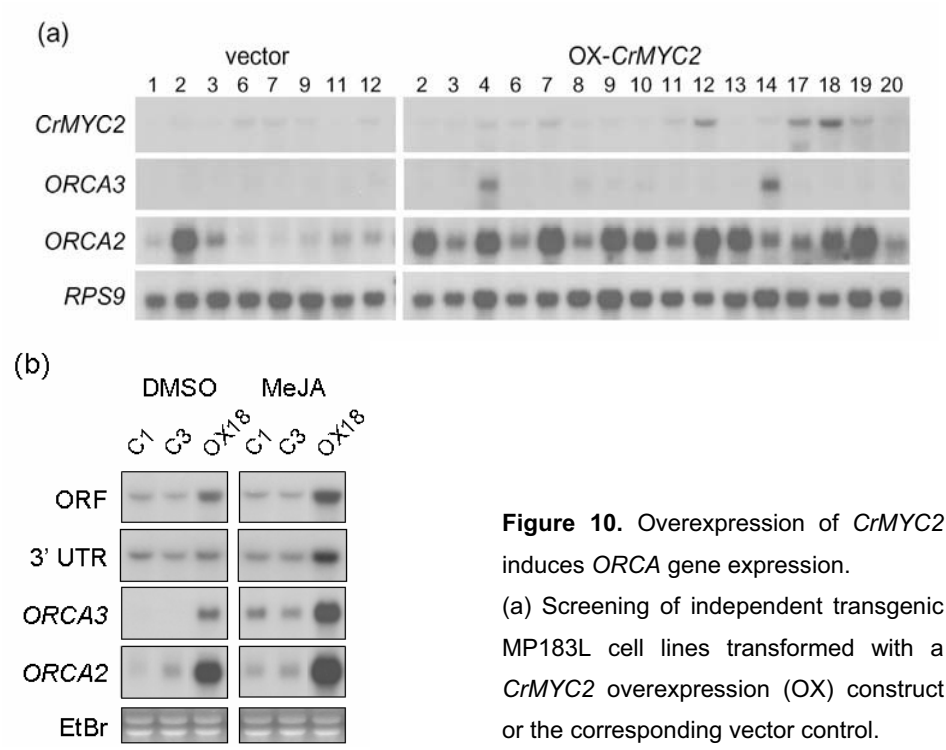

Figure 10. Overexpression of *CrMYC2* induces *ORCA* gene expression.
(a) Screening of independent transgenic MP183L cell lines transformed with a *CrMYC2* overexpression (OX) construct or the corresponding vector control.
(b) Control lines (C) #1 and #3 and overexpression line #18 were treated for 30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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

We have shown here that MeJA-responsive expression of alkaloid biosynthesis genes in *C. roseus* is controlled by a transcription factor cascade consisting of the bHLH protein CrMYC2 regulating *ORCA* gene expression, and the AP2/ERF-domain transcription factors ORCA2 and ORCA3, which regulate in turn a subset of alkaloid biosynthesis genes including *TDC* and *STR*.

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

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

Experimental procedures

Cell cultures, stable transformation, and treatments

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

Isolation of full-length cDNA clones

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

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

Plasmid constructs

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

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

Transient expression assays

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

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

RNA extraction and Northern blot analyses

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

Isolation of recombinant CrMYC2 protein and EMSA

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

Acknowledgements

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. P.G. was supported by grants from the French Ministry of National Education, Research and Technology and by the University François

Rabelais of Tours. S.H. and G.M. held doctoral fellowships from the Région Centre. M.P. and G.C. held Erasmus grants. The collaboration between the Tours and the Leiden groups was supported by a European Van Gogh 2000–2001 exchange grant (grant # 00106NH).

References

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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