Chapter 5

A jasmonate-responsive promoter element from Catharanthus roseus is active in Arabidopsis thaliana and is controlled by the transcription factor AtMYC2

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Abstract

Jasmonates are plant signaling molecules that play key roles in protection against certain pathogens and insects by switching on the expression of genes encoding defense proteins including enzymes involved in the biosynthesis of toxic secondary metabolites. In *Catharanthus roseus*, the AP2/ERF-domain transcription factor ORCA3 is involved in the jasmonate-responsive activation of terpenoid indole alkaloid biosynthetic genes. ORCA3 gene expression is itself induced by jasmonate. Its promoter contains an autonomous jasmonate-responsive element (JRE). Here we describe the jasmonate-responsive activity of the JRE from the ORCA3 promoter in Arabidopsis. We found that it interacts in vitro and in vivo with the basic helix-loop-helix transcription factor AtMYC2. Analysis of JRE-mediated reporter gene expression in an *atmyc2-1* mutant background showed that the activity was strictly dependent on AtMYC2.

Introduction

Jasmonic acid (JA) and its volatile methyl-ester (MeJA), both belonging to the group of jasmonate compounds, are fatty acid derivatives which are synthesized via the octadecanoid pathway (Turner et al., 2002; Wasternack et al., 2006). Jasmonates are involved in the regulation of a number of processes in plants, including certain developmental processes, senescence, and responses to wounding and insect and pathogen attack (Turner et al., 2002; Wasternack et al., 2006). JA is involved in plant protection by switching on the expression of genes encoding antimicrobial and anti-insect proteins including enzymes involved in the biosynthesis of toxic secondary metabolites (Gundlach, 1992; Memelink et al., 2001).

The AP2/ERF-domain transcription factor ORCA3 controls the JA-responsive expression of several genes involved in primary and secondary metabolism, leading to terpenoid indole alkaloid biosynthesis in the plant species *Catharanthus roseus* (van der Fits and Memelink, 2000; Memelink et al., 2001). The expression of the ORCA3 gene itself is rapidly induced by MeJA (van der Fits and Memelink, 2001). By loss- and gain-of-function experiments a 74 bp fragment called “D” was localized within the ORCA3 promoter, which functions as an autonomous jasmonate-responsive element (JRE) (Vom Endt et al., 2007). Block mutation analysis showed that the ORCA3 JRE is composed of two important sequences, a quantitative sequence responsible for a high level of expression, and a qualitative sequence that acts as an on/off switch in response to MeJA. The qualitative
sequence contains a G-box (CACGTG) with one mismatch, and is in fact a T/G hybrid box composed of T-box and G-box half-sites according to the terminology in Foster et al. (1994).

Using yeast one-hybrid screening, DNA-binding proteins belonging to the AT-hook class that interact with the quantitative sequence were isolated (Vom Endt et al., 2007). However those screenings failed to identify proteins that are able to bind specifically to the qualitative sequence. A similar T/G box in the promoter of the leucine aminopeptidase (LAP) gene from tomato (Lycopersicon esculentum) is necessary for its JA-responsive transcriptional activity (Boter et al., 2004). This T/G box interacts with two basic helix-loop-helix (bHLH) proteins called JAMYC2 and JAMYC10. The closest homologue in Arabidopsis thaliana is the bHLH protein AtMYC2 (also called RAP1 or rd22BP1). Knocking out AtMYC2 by T-DNA insertion abolishes the root shortening response to JA and affects JA-responsive gene expression (Boter et al., 2004; Lorenzo et al., 2004). AtMYC2 binds to G-box-like sequences (de Pater et al., 1997), including the G box and the T/G box (Chini et al., 2007). Based on its similarity to the LAP promoter element and its identity as a T/G box, the qualitative sequence in the ORCA3 promoter is likely to bind a bHLH transcription factor with similarity to JAMYC2, JAMYC10 and AtMYC2.

The aim of this study was to investigate the activity of the JRE in Arabidopsis. On the one hand this might confirm that its activity is controlled by a bHLH transcription factor, which would be AtMYC2 in Arabidopsis. On the other hand, the JRE could serve as tool for the identification of additional regulatory proteins involved in JA-responsive gene expression by gain-of-function mutant analysis. In such an approach Arabidopsis transformants containing a selection gene under control of the JRE can be subjected to T-DNA activation tagging (Tani et al., 2004) to identify proteins that activate JRE-mediated gene expression when over-expressed.

Our results show that the JRE from the ORCA3 promoter was JA-responsive in Arabidopsis, and that it interacted in vitro and in vivo with AtMYC2. Analysis of JRE-mediated reporter gene expression in an atmyc2-1 mutant background showed that the activity was strictly dependent on AtMYC2.

Results

The JRE from the ORCA3 promoter is JA-responsive in Arabidopsis

A tetramer of a fragment from the ORCA3 promoter indicated as “D” was previously shown to function as an autonomous JRE when fused to the Cauliflower Mosaic Virus (CaMV) 35S minimal promoter and the GUS reporter gene (Vom Endt et al., 2007). This construct was
introduced in Arabidopsis to evaluate whether it has similar activity in this plant species. The T-DNA carries besides the *GUS* gene and the hygromycin resistance gene a *chloramphenicol acetyltransferase* (*CAT*) reporter gene driven by the CaMV 35S promoter, which is separated from the *GUS* gene by 5 kb of spacer DNA (Vom Endt et al., 2007). Besides the wild-type 4D tetramer, also the activity of the 4DM7 mutant, which is affected in the T/G box rendering it completely inactive in *Catharanthus*, was investigated to confirm that possible JRE activity in Arabidopsis also depends on the qualitative sequence. Seedlings from the T2 generation from 9 independent transformed lines were screened for JA-responsive expression of the *GUS* gene by histochemical staining with the substrate X-Gluc. For the wild-type D tetramer 3 lines were found to be active, whereas for the M7 mutant tetramer no GUS-positive seedlings were found. Subsequently, JA-responsive GUS activity was quantified in these three active 4D lines and compared to three randomly chosen 4DM7 lines. As shown in Figure 1A with the 4D lines strong increases in GUS activity were observed upon JA treatment, whereas in the 4DM7 lines only background levels were observed without any response to JA. The most active line was #3, which was used for further experiments.

Histochemical staining with the GUS substrate X-Gluc was performed of seedlings from 4D-*GUS*-47 line #3 to study whether the D tetramer showed tissue- or organ-specific expression. Representative examples of GUS staining are shown in Figure 1B. Without JA treatment seedlings never showed GUS staining in any cell type. Upon treatment with 100 μM JA for 24 hrs weak GUS activity was observed in specific cell types. In leaves GUS activity was mainly localized in the hydathodes, with staining in other parts of the leaves in a fraction of the seedlings. In the roots staining was localized in the area where lateral roots emerged (Figure 1B) with weak staining in other root cell types in a fraction of the seedlings (data not shown). Upon treatment with 200 μM JA for 24 hrs the majority of the seedlings showed the same tissue-specific GUS staining as after treatment with 100 μM JA (not shown). A fraction of the seedlings showed intense GUS staining in extended areas of the leaves extending also in the petioles (Figure 1B). Root staining was similar for both treatments with different concentrations of JA.

The JRE from the *ORCA3* promoter responds specifically to JA independent of de novo protein synthesis

We asked the question whether the gene expression response conferred by the JRE is specific to JA, or whether other defense hormones also affect its activity. Fourteen-days-old T2 seedlings of 4D-*GUS*-47 #3 were treated with different hormones and expression of the
Figure 1. The JRE from the ORCA3 promoter is JA-responsive in Arabidopsis. (A) Fourteen-days-old T2 seedlings from three independent transgenic lines (indicated by numbers) containing either 4D-GUS-47 (4 copies of the wild-type JRE) or 4DM7-GUS-47 (4 copies of the JRE mutant M7) were treated for 24 hrs with 50 μM JA or as a control (C) with DMSO at a final concentration of 0.1% (v/v). GUS activities were related to chloramphenicol acetyltransferase activities encoded by the CAT gene controlled by the CaMV 35S promoter on the T-DNA. Absolute CAT activities were in a similar range for all transgenic lines (data not shown). GUS/CAT ratios are means ± SE determined in three pools of 10 seedlings. (B) Histochemical localization of GUS activity in fourteen-days-old seedlings treated with DMSO or JA at concentrations as indicated for 24 hrs.
GUS gene in comparison with AtMYC2 was analyzed by Northern blot hybridization. The expression of the vegetative storage protein 1 (VSP1) gene was also analyzed as a representative JA-responsive gene in Arabidopsis. As shown in Figure 2A, GUS mRNA accumulated only in response to JA, whereas ethylene, salicylic acid and abscisic acid (ABA) had no visible effect. Ethylene had a strong negative effect on the JA-responsive expression of the GUS gene. Consistent with previous reports (Lorenzo et al., 2004; Boter et al., 2004; Abe et al., 1997) AtMYC2 expression was induced by JA and ABA with a negative effect of ethylene on the JA response. The expression of the VSP1 gene was qualitatively very similar to AtMYC2 expression with the difference that it responded slower to JA and ABA.

Gene expression analysis using the protein synthesis inhibitor cycloheximide (CHX) showed that in Catharanthus cells D-mediated JA-responsive expression does not require de novo protein synthesis, indicating that the JRE is a primary response element. To establish whether the JRE has similar activity in Arabidopsis, T2 seedlings from 4D-GUS-47 line #3 were treated with CHX or JA or both. Figure 2B shows that GUS expression was relatively weakly induced by JA, strongly induced by CHX and superinduced by the combination. This expression pattern is qualitatively very similar to the response in transgenic Catharanthus cells (Vom Endt et al., 2007). The expression of the AtMYC2 gene was very similar to the GUS gene with the difference that it responded faster to JA. The VSP1 gene showed a totally different expression pattern without a response to CHX alone and a strong negative effect of CHX on JA-responsive expression.

**AtMYC2 binds to the G-box like sequence in the JRE in vitro**

As a first experiment to test whether the activity of the JRE from the ORCA3 promoter is controlled by AtMYC2, electrophoretic mobility shift assays were performed with recombinant AtMYC2 protein expressed in Escherichia coli. As shown in Fig.3B, AtMYC2 bound to the monomeric D fragment in vitro. Block mutations M1 to M5 (Figure 3A) did not affect complex formation whereas block mutations M6 and M7 completely abolished AtMYC2 binding.

**AtMYC2 trans-activates the JRE via the G-box-like sequence in vivo**

Next, the ability of AtMYC2 to trans-activate the GUS reporter gene via interaction with the JRE from the ORCA3 promoter was tested. Arabidopsis protoplasts were co-transformed with combinations of GUS reporter genes controlled by tetramerized wild-type or mutant versions of the D fragment and an overexpression plasmid which was empty or expressed AtMYC2 from the CaMV 35S promoter (Figure 4A). As shown in Figure 4B, AtMYC2 trans-activated the GUS reporter gene driven by the wild-type D tetramer 2-3 fold. Reporter constructs
Figure 2. The JRE from the ORCA3 promoter responds specifically to JA independent of de novo protein synthesis. (A) Fourteen-days-old seedlings of 4D-GUS-47 line #3 were treated for the indicated number of hours with 50 μM JA, 1 mM ethephon (ET; an ethylene releaser), 1 mM salicylic acid (SA), 1 mM abscisic acid (ABA) or 0.1% of the solvent DMSO, or (B) with 50 μM JA, 100 μM cycloheximide (CHX) or both. RNA gel blots were hybridized with the indicated probes. The TUB probe was used to verify RNA loading. In (A) all panels for each probe are from the same blot and exposed to film for the same time allowing direct comparison of expression levels.
Figure 3. AtMYC2 binds to the G-box like sequence in the JRE in vitro. (A) Part of the wild-type sequence of the JRE is shown. Numbering of mutations is given above the sequence. In each mutant, boxed nt were changed into their complementary nt. The quantitative sequence responsible for maximum expression levels and the qualitative sequence forming the JA-responsive on/off switch are underlined. (B) In vitro binding of AtMYC2 to wild-type or mutated derivatives of fragment D as shown in (A).

carrying the DM1 to DM5 mutant tetramers were similarly trans-activated between 2 to 4 fold by AtMYC2. However, the mutant tetramers DM6 and DM7 did not confer trans-activation by AtMYC2. Thus there is a perfect correlation between the effects of mutations on in vitro binding of AtMYC2 to the D fragment, and the ability of AtMYC2 to trans-activate reporter gene expression in vivo. This indicates that AtMYC2 trans-activated the 4D-GUS-47 reporter construct in vivo via direct binding to the qualitative sequence containing the T/G box.

JA-responsive activity of the JRE from the ORCA3 promoter is strictly dependent on AtMYC2 in vivo

AtMYC2 could be just one of several bHLH proteins in Arabidopsis that can bind to the JERE and can act as an activator of gene expression when overexpressed. Therefore we wanted to establish whether the activity of the JRE from the ORCA3 promoter in Arabidopsis really depended on AtMYC2. To introduce the 4D-GUS-47 construct in an atmyc2 mutant background, line #3 was crossed with the mutant line SALK_040500 from the SALK collection (Alonso et al., 2003) obtained via the Nottingham Arabidopsis Stock Centre. This
Figure 4. AtMYC2 transactivates the JRE via the G-box-like sequence in vivo. (A) Schematic representation of reporter and effector plasmids. Reporter plasmids contained 4 head-to-tail copies of the wild-type D element (containing the JRE) or one of the mutants shown in Fig. 3A fused to the CaMV 35S minimal promoter and the GUS gene. (B) Arabidopsis protoplasts were co-transformed with combinations of reporter and effector plasmids as indicated. A reference plasmid carrying the Renilla LUC gene fused to the CaMV 35S promoter was co-transformed in all experiments to correct for transformation and protein extraction efficiencies. Bars represent average GUS/LUC activity ratios ± SE of triplicate measurements. D, JRE containing element from ORCA3 promoter; DM, corresponding element with mutated JRE.

atmyc2-1 (or jin1-7) mutant has a T-DNA insertion at nt 57 within the protein coding region of the AtMYC2 gene which causes insensitivity in the JA root growth inhibition assay (Lorenzo et al., 2004) and affects JA-responsive gene expression (Boter et al., 2004).

The PCR analysis of wild-type and mutant AtMYC2 alleles is shown in Figure 5A and confirmed that the two offspring lines selected from the cross by a first PCR screening were indeed homozygous for the T-DNA insertion. Analysis of GUS activity in the atmyc2-1 mutant seedlings showed that the JA responsive activity was almost completely abolished compared to the activity in the parental wild-type line (Figure 6A). Analysis of the GUS mRNA level also showed that JA-responsive accumulation was strongly reduced in the atmyc2-1 background (Figure 6B). With the AtMYC2 probe the accumulation of a JA-responsive RNA was still detected, but consistent with a previous report (Boter et al., 2004) the size was slightly larger than the wild-type AtMYC2 transcript, and it encodes a non-functional protein.
Figure 5. Molecular analysis demonstrating that the selected atmyc2-1/4D-GUS-47 offspring is homozygous for the T-DNA insertion allele and contains a functional GUS reporter gene. (A) PCR detection of wild-type and T-DNA insertion alleles of AtMYC2. Lanes 1-4 contain samples of the genomic DNA used as templates for PCR. Lanes 5, 7, 9, and 11 contain PCR reactions performed with primers specific for the wild-type allele resulting in a fragment of 1037 bp. Lanes 6, 8, 10 and 12 contain PCR reactions performed with primers specific for the T-DNA insertion allele giving a fragment of 600 bp. Numbers 1 and 3 represent 2 different offspring selected from a cross between 4D-GUS-47 line #3 and the atmyc2-1 T-DNA knockout line. Lane M contains phage lambda DNA digested with EcoRI/HindIII. Fragment sizes in kb are indicated at the left. (B) The 4D-GUS-47 gene in the atmyc2-1 mutant is functional. Leaf protoplasts prepared from wild-type plants, or plants containing 4D-GUS-47 in the wild-type or atmyc2-1 background were transformed with the AtMYC2 effector plasmid shown in Fig.4A and the 35S-LUC reference plasmid. GUS/LUC ratios are means ± SE from triplicate transformations.
Figure 6. JA-responsive activity of the JRE from the ORCA3 promoter is strictly dependent on AtMYC2 in vivo. (A) Fourteen days-old seedlings containing 4D-GUS-47 either in a wild-type or in the atmcy2-1 mutant background were treated for 24 hrs with 50 μM JA or with DMSO at a final concentration of 0.1% (v/v). GUS activities were related to CAT activities. Absolute CAT activities were in a similar range for all transgenic lines (data not shown). GUS/CAT ratios are means ± SE determined in three pools of 10 seedlings. (B) RNA gel blot analysis of 14 days-old seedlings treated with 50 μM JA or 0.1% DMSO (D) for 2 or 24 hrs. with probes as indicated. All panels for each probe are from the same blot and exposed to film for the same time allowing direct comparison of expression levels.
containing only the first 17 amino acids of AtMYC2. JA-responsive VSP1 expression was also reduced in the atmyc2-1 background, although it appeared to be less affected than the GUS expression level.

Since the presence of the GUS reporter gene in the atmyc2-1 mutant seedlings was deduced only from the fact that the seedlings were hygromycin-resistant, we wanted to prove that indeed the mutants contain a functional 4D-GUS-47 gene. Therefore leaf protoplasts prepared from plants carrying the reporter gene in the wild-type and mutant backgrounds were transformed with the AtMYC2 overexpression plasmid and the Renilla reniformis luciferase reference gene. GUS activity analysis showed that introduction of the AtMYC2 overexpression plasmid caused a strong induction of GUS activity in the selected mutant lines which was similar to the GUS activity level in the wild-type background (Figure 5B). This demonstrates that the GUS gene is functional and is able to respond to AtMYC2, which together with the other results shows that the activity of the JRE from the ORCA3 promoter in Arabidopsis depended strictly on AtMYC2.

Discussion

Here we have demonstrated that the JRE from the ORCA3 promoter is functional in Arabidopsis. In addition we have shown via various approaches that the JA-responsive activity of the JRE is controlled by the bHLH transcription factor AtMYC2. The most convincing evidence is the observation that the JRE is inactive in the atmyc2-1 T-DNA knockout mutant.

The expression of the VSP1 gene was not completely abolished in the atmyc2-1 mutant. In addition, the expression of VSP1 is strongly affected by the protein synthesis inhibitor cycloheximide. Both observations indicate that the promoter of VSP1 does not directly interact with AtMYC2, although it was reported to contain a G-box-like sequence (Guerineau et al., 2003) that is a potential binding site for AtMYC2.

As in Catharanthus cells (Vom Endt et al., 2007), the JA-responsive activity of the JRE did not depend on de novo protein synthesis in Arabidopsis. This also is consistent with the notion that the JRE is directly interacting with AtMYC2. In addition, it shows that pre-existing AtMYC2 is activated in response to JA without requirement for newly synthesized proteins, which is consistent with the recent discovery of AtMYC2 activation by COI1- and proteasome-dependent degradation of members of the JAZ family of repressor proteins (Chini et al., 2007; Thines et al., 2007).
Our results show that AtMYC2 is a transcriptional activator when overexpressed in protoplasts in the absence of JA. One option is that an excess of AtMYC2 protein out-titrates the negative regulators from the JAZ family. Alternatively it is possible that the JA signal transduction pathway is activated due to protoplast preparation or to the transformation procedure.

Previously it was attempted to isolate the Catharanthus transcription factor interacting with the qualitative sequence in the JRE by yeast one-hybrid screening (Vom Endt et al., 2007). Although many JRE-binding transcription factors were isolated, none of them bound to the qualitative sequence. In that paper it was speculated that the Catharanthus protein is a bHLH transcription factor with similarity to AtMYC2. The results described here corroborate this hypothesis. Since bHLH proteins bind as homo- or heterodimers to their target sites in DNA, it was speculated by Vom Endt et al. (2007) that the Catharanthus bHLH transcription factor binds as an obligatory heterodimer and therefore escaped recovery in the yeast one-hybrid screening. AtMYC2 appears to bind as a homodimer when expressed in E.coli (de Pater et al., 1997; this report). The tomato proteins JAMYC2 and JAMYC10 were identified by yeast one-hybrid screening using the tomato LAP promoter as bait (Boter et al., 2004). It cannot be excluded that in planta the JAMYC proteins and AtMYC2 bind as heterodimers with a specific bHLH partner that strongly increases the DNA-binding affinity of the heterodimer compared to the homodimer. If AtMYC2 binds as a heterodimer in Arabidopsis, then its partner is not functionally limiting since in trans-activation experiments and in T-DNA knockout mutants AtMYC2 appears to be the only limiting factor (this report; Lorenzo et al., 2004; Boter et al., 2004).

The JRE is potentially useful as a tool for the isolation of new regulatory proteins involved in JA-responsive gene expression. It can for example be coupled to an antibiotic resistance gene. Transgenic plants or cells containing such a construct can then be super-transformed with an activation tagging T-DNA (Tani et al., 2004). In this way genes can be identified that activate the JRE when over-expressed. The advantage of a multimerized JRE over a natural JA-responsive promoter is that the presence of relatively few cis-elements reduces the likelihood that proteins that are not directly involved in the activity of JA-responsive sequences are activation-tagged. One concern with this JRE is that its activity is not very strong in Arabidopsis. In Catharanthus cells, the tetramerized JRE confers mRNA levels that are similar to those driven by the CaMV 35S promoter (Vom Endt et al., 2007), whereas in Arabidopsis relatively low mRNA levels were found even in the highest expressing transgenic line. This potential problem could be solved by screening more lines to find a higher expresser, or by using higher order multimers of the JRE.
Materials and methods

Biological materials, growth conditions and treatments

*Arabidopsis thaliana* wild-type plants, the *atmyc2-1* mutant and all transgenic plants were in the genetic background of ecotype Col-0. Seeds were surface-sterilized by incubation for 1 minute in 70% ethanol, 15 min in 25% commercial bleach (1.25% sodium hypochlorite), and five rinses with sterile water. Alternatively, seeds were surface-sterilized in a closed container with chlorine gas for three hours. Surface-sterilized seeds were transferred to plates containing MA medium supplemented with 0.6% agar (Masson and Paszkowski, 1992). Transgenic plants were selected on solid MA medium containing 20 mg/L hygromycin. Mutant *atmyc2-1* plants were selected on solid MA medium containing 25 mg/L kanamycin. Following stratification for 3 days at 4°C, seeds were incubated at 21°C in a growth chamber (16 h light/ 8 h dark, 2500 lux) for 10 days. For each treatment, 15 to 20 seedlings were then transferred to 50 ml polypolypropylene tubes (Sarstedt, Nümbrecht, Germany) containing 10 mL MA medium and incubated on a shaker at 120 rpm for 4 additional days. Seedlings were treated for different time periods with 50 μM JA (Sigma-Aldrich, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO, 0.1% v/v final concentration), 1 mM of the ethylene releaser ethephon (Sigma-Aldrich) dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration), a combination of JA and ethephon, 1 mM salicylic acid dissolved in 50 mM sodium phosphate pH 7 (0.5 mM final concentration) or 100 μM abscisic acid dissolved in water. As a control, seedlings were treated with a combination of 0.1% DMSO and 0.5 mM sodium phosphate pH 7. Alternatively, seedlings were treated for 48 hrs with 100 μM JA dissolved in DMSO (0.2% v/v final concentration). As a control, seedlings were treated with 0.2% DMSO. For cycloheximide experiments, seedlings were first treated for 10 min with 100 μM CHX dissolved in DMSO (0.1% final concentration) and then JA dissolved in DMSO was added to 50 μM for times as indicated. The 4D-GUS-47 gene was introduced in the *atmyc2-1* mutant background by pollinating homozygous *atmyc2-1* mutant plants (T-DNA insertion line SALK_040500 from the SALK collection obtained from the Nottingham Arabidopsis Stock Centre) with pollen from 4D-GUS-47 #3 plants. Seeds were selected on solid MA medium containing 20 mg/L hygromycin and 25 mg/L kanamycin and plants were transferred to soil and were allowed to self-pollinate. Offspring was again selected on hygromycin and kanamycin and was allowed to self-pollinate and seed was collected. Seedlings from those seed batches were selected on antibiotics and screened for T-DNA insertions in both *AtMYC2* alleles using the primers 040500RP; 5’-TTT TCC TGT ACT CCT GAT CCG-3’ and 040500LP; 5’-TGA ATT ATT AGC AAC GAC TCA CG-3’, for detection of the wild-type allele, the primers 040500RP and LBB1; 5’-GCGTGGACCGCTTGCTGCAACT-3’ (LBB1 primer is recommended by the SALK institute and 040500RP/040500LP were designed using the SALK iSECT tool online software) for detection of the T-DNA insertion allele. Genomic DNA was isolated from 20 mg of frozen leaves and buds. Frozen tissues were ground for 30 seconds in a microcentrifuge tube containing small metal beads using a Retsch Miller MM301 shaker. To the powder 400 μL of extraction buffer (0.3 M NaCl, 0.05 M Tris pH 7.5, 20 mM EDTA pH 8.0, 2% w/v sarkosyl, 0.5% w/v SDS, 5 M urea, 5% v/v Tris-saturated phenol) and 400 μL of phenol-chloroform-isoamylalcohol (25:24:1) were then added. After homogenization, tubes were centrifuged for 20 min at 6,000 rpm and the aqueous phase was transferred to a new tube. DNA was precipitated using 0.8 volume isopropanol and centrifuged for 5 min at 13,000 rpm. DNA pellets
were then washed with 70% ethanol and resuspended in 50 μL TE buffer containing 10 μg/mL RNase A. After incubation for 30 min at 37°C samples were ready for PCR analysis.

Plant transformation

Binary vectors containing tetramerized derivatives of the D fragment from the ORCA3 promoter fused to the GUS gene were described previously (Vom Endt et al., 2007). Agrobacterium-mediated transformation of Arabidopsis plants was performed via the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MA medium containing 100 mg/L timentin and 20 mg/L hygromycin.

RNA extraction and Northern blot analyses

Total RNA was extracted from frozen tissues by hot phenol/chloroform extraction followed by overnight precipitation with 2 M lithium chloride and two washes with 70% ethanol, and resuspended in water. As described by Memelink et al. (1994), 10 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, Boston, MA). All probes were 32P-labeled by random priming. Pre-hybridization of blots, hybridization of probes and subsequent washings were performed as described (Memelink et al., 1994) with minor modifications. Blots were exposed to X-ray films (Fuji, Tokyo, Japan). DNA fragments used as probes were PCR amplified from Arabidopsis genomic DNA. The following primer sets were used: 5’-ATGACTGATTACCGGCTACAA-3’ and 5’-CCGATTTTTTGAATCAAAGTTG-3’ for AtMYC2 (At1g32640); 5’-CGG GAT CCA TGA AAA TCC TCT CAC TTT-3’ and 5’-CCC TCG AGT TAA GAG ACG TAG TAG G-3’ for Vegetative Storage Protein 1 (VSP1, At5g24780); 5’-CGG AAT TCA TGA GAG AGA TCC TTC ATA TC-3’ and 5’-CCC TCG AGT TAA GTC TCG TAC TCC TTC TAC TC-3’ for TUB (At5g44340).

Electrophoretic mobility shift assays

A PstI/NcoI fragment amplified on a Rap-1 cDNA from ecotype C24 (Genbank acc.No. X99548, de Pater et al., 1997) with the primers 5’-CTG CAG GAT GAC TGA TTA CCG GCT ACA A-3’ and 5’-CCA TGG TAC CGA TTT TTG AAA TCA AAC TTG-3’ containing the complete AtMYC2 open reading frame was cloned into pASK-IBA45 (IBA, Göttingen, Germany). Double Strep/His-tagged protein was expressed in E. coli strain Rosetta-gami B (DE3) pLysS (Novagen) and purified by sequential Ni-NTA agarose (Qiagen) and Strep-Tactin sepharose (IBA) chromatography. D wild-type and mutant fragments (Vom Endt et al., 2007) were isolated from pIC-20H with XbaI/XhoI and labeled by filling in the overhangs with the Klenow fragment of DNA polymerase I and α32P-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.
Transient expression assays

The AtMYC2 gene was excised from the Rap-1 cDNA in pBluescript SK (De Pater et al., 1997) with Xmal and cloned in the CaMV 35S cassette of the overexpression plasmid pRT101 (Töpfer et al., 1987). Protoplasts prepared from Arabidopsis cell suspension ecotype Col-0 were co-transformed with plasmids carrying the different D element derivatives fused to the GUS gene, the effector plasmid pRT101 carrying the AtMYC2 gene and the p2rL7 plasmid (De Sutter et al., 2005) carrying the Renilla reniformis luciferase (LUC) gene under the control of the CaMV 35S promoter. As controls, co-transformations of the different D element derivatives fused to the GUS gene with an empty pRT101 plasmid and p2rL7 were carried out. Protoplasts were transformed in triplicate using polyethylene glycol as described previously (Schirawski et al., 2000) with the three constructs in a ratio 2:2:6 (μg GUS/LUC/effector plasmid). Protoplasts were harvested 18 hours after transformation and frozen in liquid nitrogen. Alternatively, Arabidopsis leaf protoplasts were prepared and transformed as described (Sheen, 2002) using a ratio 2:6 (μg LUC/effector plasmid). GUS and LUC or CAT activity assays for each transformation were performed as described (van der Fits and Memelink, 1997; Dyer et al., 2000) with minor modifications. GUS activities were related to CAT or LUC activities to correct for differences in transformation and protein extraction efficiencies.

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References


