

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

Frank L.H.Menke, Anthony Champion,
Jan W.Kijne and Johan Memelink¹

Institute of Molecular Plant Sciences, Clusius Laboratory,
Leiden University, Wassenaarseweg 64, 2333 AL Leiden,
The Netherlands

¹Corresponding author
e-mail: Memelink@rulbim.leidenuniv.nl

Jasmonate (JA) is an important plant stress hormone that induces various plant defense responses, including the biosynthesis of protective secondary metabolites. The induction of the secondary metabolite biosynthetic gene *Strictosidine synthase (Str)* in *Catharanthus roseus* (periwinkle) cells by elicitor requires JA as a second messenger. A 42 bp region in the *Str* promoter is both necessary and sufficient for JA- and elicitor-responsive expression. This region is unlike other previously identified JA-responsive regions, and contains a GCC-box-like element. Yeast one-hybrid screening identified cDNAs encoding two AP2-domain proteins. These octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA) proteins bind in a sequence-specific manner the JA- and elicitor-responsive element. ORCA2 *trans*-activates the *Str* promoter and its expression is rapidly inducible with JA and elicitor, whereas *Orca1* is expressed constitutively. The results indicate that a GCC-box-like element and ORCA2 play key roles in JA- and elicitor-responsive expression of the terpenoid indole alkaloid biosynthetic gene *Str*.

Keywords: *cis*-element/EREBP/*Catharanthus roseus*/*Strictosidine synthase*/transcriptional regulation

Introduction

Perception of stress or pathogens by a plant triggers rapid defense responses via a number of signal transduction pathways (Yang *et al.*, 1997). A major target of signal transduction is the cell nucleus where the terminal signals lead to the transcriptional activation of numerous genes, and consequently to the *de novo* synthesis of a variety of defense proteins and protective secondary metabolites. Signal transduction is initiated through recognition of stress signals or pathogen-derived molecules called elicitors, which trigger the biosynthesis of one or a combination of the hormone-like compounds jasmonic acid (JA), salicylic acid and ethylene (Reymond and Farmer, 1998). JA, and its volatile methyl-ester (MeJA), collectively called jasmonates, are fatty acid derivatives which are synthesized via the octadecanoid pathway (Mueller, 1997). Jasmonates can be considered global signals of defense gene expression, as many defense-related genes have been shown to be responsive to jasmonates (Creelman and Mullet, 1997; Reymond and Farmer, 1998). *Arabidopsis* plants impaired in JA perception or biosyn-

thesis are unable to mount appropriate defense responses (Penninckx *et al.*, 1996; Pieterse *et al.*, 1998; Vijayan *et al.*, 1998). An important defense response that depends on JA as a regulatory signal is the elicitation of secondary metabolism (Gundlach *et al.*, 1992; Mueller *et al.*, 1993). Elicitors of secondary metabolism induce JA biosynthesis as an early response (Gundlach *et al.*, 1992; Mueller *et al.*, 1993; Menke *et al.*, 1999), and JA subsequently induces the expression of secondary metabolite biosynthetic genes (Gundlach *et al.*, 1992; Menke *et al.*, 1999). How jasmonates regulate gene expression is largely unknown, although sequences involved in JA-responsive expression have been identified in a number of promoters.

Regions containing the bZIP protein-binding motifs TG-ACG or the G-box CACGTG, are present in the *lipoxygenase 1 (Lox1)* promoter of barley (Rouster *et al.*, 1997), in the potato *Pin2* promoter (Kim *et al.*, 1992) and in *as-1*-like elements (Xiang *et al.*, 1996), and have been implicated in JA-responsive gene expression. However, *trans*-acting factors involved in JA signaling have not been identified and, moreover, none of the regions implicated in JA signaling have been linked to elicitor responsiveness.

A number of elicitor-responsive *cis*-acting elements have been identified and this has allowed the characterization of their cognate binding factors (reviewed by Rushton and Somssich, 1998). Well-characterized elicitor-responsive elements are the W-boxes in the promoter of the parsley *PR1-1* gene (Rushton *et al.*, 1996) and tobacco chitinase gene (*CH50*; Fukuda and Shinshi, 1994). However, elicitor-responsive elements in genes involved in secondary metabolism and their cognate interacting factors are not as well characterized (reviewed by Rushton and Somssich, 1998).

Here we describe a novel JA- and elicitor-responsive element (JERE) containing a GCC motif in the terpenoid indole alkaloid (TIA) biosynthetic gene *Strictosidine synthase (Str)*. Expression of the *Str* gene is induced by MeJA and elicitor, and elicitor responsiveness depends on JA as a secondary signal (Menke *et al.*, 1999). Using yeast one-hybrid screening, we have isolated two cDNA classes, encoding AP2-domain proteins. These octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA) proteins bound in a sequence-specific manner to the JERE, and ORCA2 also *trans*-activated the *Str* promoter. Expression of *Orca2* is induced by elicitor and MeJA at the mRNA level. Our results show that a GCC motif and an AP2-domain protein constitute the terminal stage of a JA-responsive signal transduction pathway, that forms an integral part of elicitor-induced expression of a secondary metabolite biosynthetic gene.

Results

Identification of a JA- and elicitor-responsive element in the *Str* promoter

A 396 bp fragment of the *Str* promoter has previously been shown to be responsive to JA and elicitor (Menke

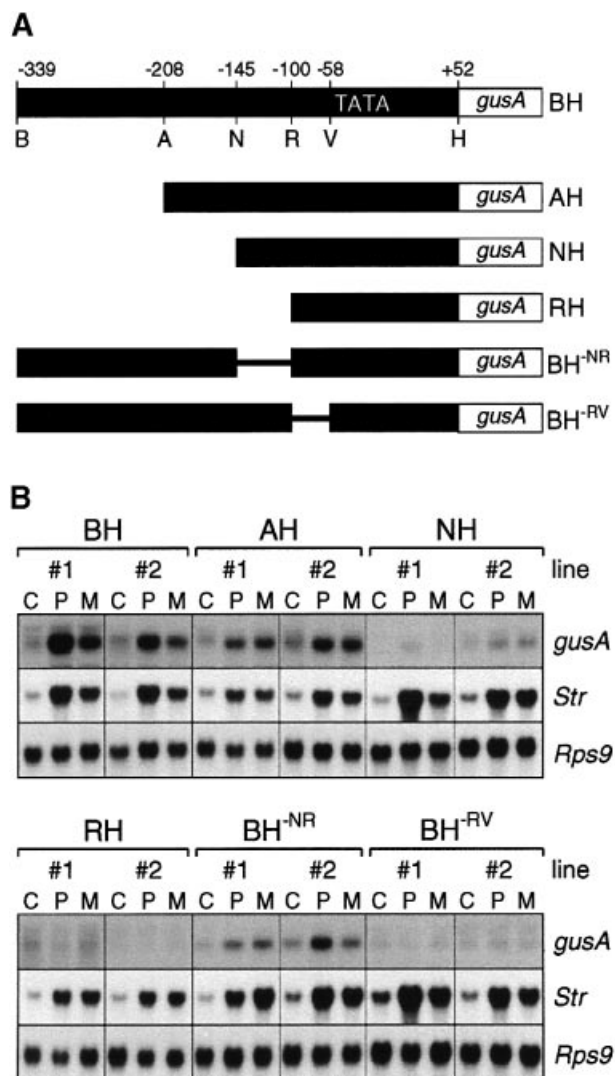


Fig. 1. The RV region of the *Str* promoter is required for MeJA- and elicitor-responsive gene expression. **(A)** Schematic representation of *Str* promoter-*gusA* constructs used for stable transformation. Letters indicate restriction sites used for construction of deletions. Numbers indicate the position relative to the transcriptional start site. The TATA box is indicated. Internal deletions are indicated with a thin line. **(B)** Northern blot showing *gusA*, *Str* and *Rps9* mRNA levels in independent transgenic cell lines (indicated with #) containing constructs shown in (A). Cells were incubated for 6 h with DMSO (C), partially purified elicitor (P) or 10 μM MeJA (M). The Northern blot was sequentially probed with *gusA* gene, *Str* cDNA and *Rps9* cDNA.

et al., 1999). To locate the JERE within this fragment, the effect of progressive 5' deletions and two internal deletions (Figure 1A) on promoter activity was analyzed in stably transformed *Catharanthus roseus* cells. Figure 1B shows the expression of the *gusA* gene driven by deletion derivatives of the *Str* promoter, in comparison with the endogenous *Str* mRNA level in the same RNA samples. In transgenic cells harboring the BH (-339 to +52) or AH (-208 to +52) fragment (Figure 1A), incubation with partially purified yeast extract elicitor (PE) or MeJA resulted in increased *gusA* mRNA accumulation compared with the non-induced control (Figure 1B). The induced accumulation of *gusA* mRNA was qualitatively similar to *Str* mRNA accumulation in these cells. A 5' deletion to

position -145 (construct NH) did not abolish MeJA or PE inducibility, but the expression level was reduced dramatically. Further 5' deletion to -100 (RH) resulted in an unresponsive promoter construct. Analysis of the endogenous *Str* gene expression showed that the induction treatment was effective. Analysis of the level of *Rps9* mRNA, encoding the 40S ribosomal protein S9, showed equal loading of RNA (Figure 1B). Internal deletion of the NR (-145 to -100) fragment within the BH context (BH^{NR}), did not affect PE or MeJA responsiveness. The NR fragment contains a G-box (Pasquali *et al.*, 1999). Our results indicate that this element is not required for JA- and elicitor-responsive gene expression. However, deletion of the RV (-100 to -58) region from the BH promoter rendered this BH^{RV} construct unresponsive to PE or MeJA. These results show that the 42 bp RV region is required for JA- and elicitor-responsive gene expression.

Some variability was observed in the effectiveness of elicitor versus JA induction, with elicitor usually being more effective than MeJA, although occasionally the reverse was observed (e.g. line BH^{NR} #1). This variability was not correlated with the presence of a certain transgenic construct (compare Figure 1 with Figure 3), and could be due to either intrinsic variability between cell lines, or to differential sensitivity of the cell lines due to culture manipulation. These explanations could also account for the small variations observed in the level of endogenous *Str* mRNA in control-treated cultures.

The region upstream of RV is required for a detectable expression level, since deletion of this region resulted in an inactive promoter construct. The NR region functioned as a weak enhancer of gene expression within the NH context, whereas the combination of the AN and NR regions, as in the AH construct, or the combination of the BA and AN regions, as in the BH^{NR} construct, functioned as stronger enhancers of gene expression. These observations indicate that the AN region (-208 to -145) contributes quantitatively to the gene expression level, with additional quantitative elements in the NR and possibly the BA region. However, only the RV region is essential for PE and MeJA responsiveness of the *Str* promoter derivative BH.

Detailed block scanning mutagenesis of the RV region in the context of the BH derivative of the *Str* promoter was performed (Figure 2A), to pinpoint the exact location of the JERE within the RV fragment. The activities of eight mutated versions of the BH promoter were analyzed for two independently transformed *C.roseus* cell lines for each construct. By comparing basal and MeJA-induced *gusA* gene expression for each mutant line to the expression conferred by the wild-type BH promoter, mutants M1, M6, M7 and M8 were found to confer wild-type MeJA responsiveness (Figure 2B). In mutant M2 the expression was only minimally inducible, whereas mutants M3, M4 and M5 were unresponsive to MeJA, similar to the BH^{RV} construct (Figure 2B). Mutations M2, M3, M4 and M5 also abolished elicitor responsiveness of the *Str* promoter in stably transformed *C.roseus* cells (data not shown). Induction of endogenous *Str* gene expression in these transgenic cell lines by MeJA was similar in each independent line (Figure 2B). The results of these loss-of-function experiments showed that the *Str* promoter contains a JERE that is inactivated by mutations M2 to M5, with mutations M3, M4 and M5 having the most

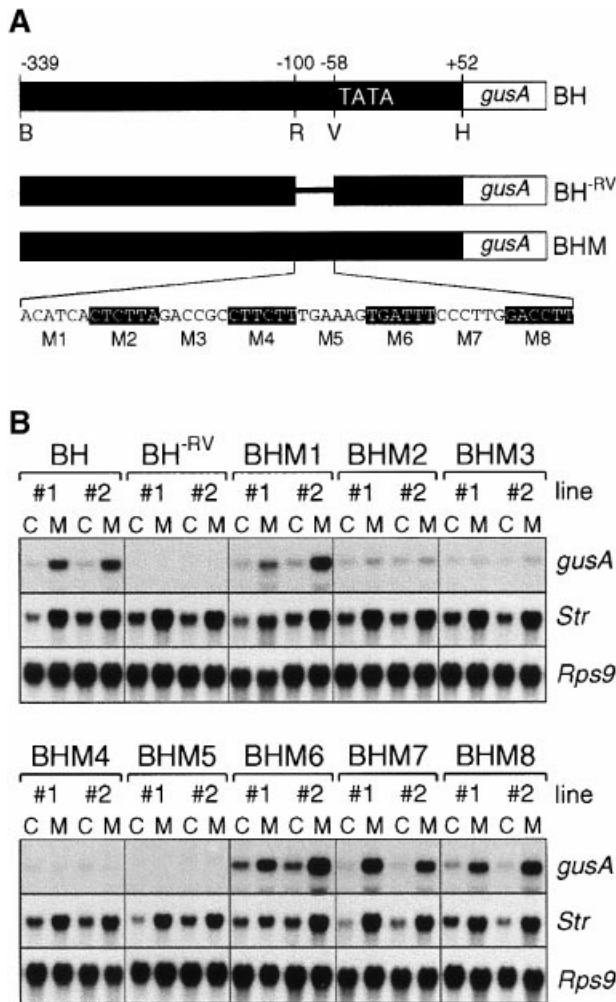


Fig. 2. Block scanning mutagenesis identifies a 24 bp MeJA-responsive element in the RV region of the *Str* promoter. (A) Schematic representation of *Str* promoter-*gusA* fusion constructs used for stable transformation. Numbers and letters are as in Figure 1. The TATA box is indicated. An internal deletion is indicated with a thin line. The wild-type sequence of the RV region is shown. Numbering of mutations is given below the sequence. In each mutant six adjacent nucleotides were mutated into their complementary nucleotides. (B) Northern blot showing *gusA*, *Str* and *Rps9* mRNA levels in independent transgenic cell lines (indicated with #) containing constructs shown in (A). Cells were incubated for 6 h with DMSO (C) or 10 μ M MeJA (M). The Northern blot was sequentially probed with *gusA* gene, *Str* cDNA and *Rps9* cDNA.

dramatic negative effect on the inducibility of the *Str* promoter.

The RV region autonomously confers JA- and elicitor-responsive transcriptional activation

To determine whether the RV region alone can confer JA- and elicitor-responsive gene expression, tetramers of the wild-type RV region and mutant versions were fused to a minimal CaMV 35S promoter (-47) fused to *gusA* (Figure 3A). The RV tetramer conferred JA and elicitor responsiveness onto the *gusA* gene (Figure 3B). The minimal CaMV 35S promoter itself was inactive (Figure 3B). In cell lines carrying the unrelated 6Tcyt-GusSH-47 construct, high basal levels of *gusA* mRNA were found, and expression was not inducible with MeJA

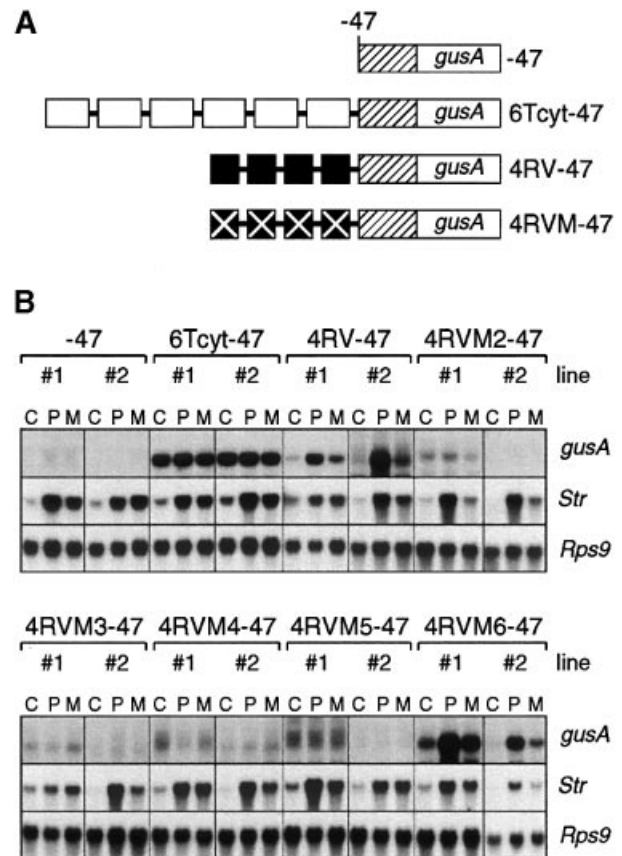


Fig. 3. The RV region is an autonomous MeJA- and elicitor-responsive element. (A) Schematic representation of CaMV minimal promoter-*gusA* derivatives used for stable transformation. Construct 6Tcyt contains six head-to-tail copies of the *cyt-1* element. Construct 4RV contains four head-to-tail copies of the RV region. Constructs 4RVM2 to 4RVM6 contain four head-to-tail copies of the mutated RV regions. Mutations in the RV region, indicated as white crosses, are as in Figure 2. (B) Northern blot showing *gusA*, *Str* and *Rps9* mRNA levels in independent transgenic cell lines (indicated with #) containing constructs shown in (A). Cells were incubated for 6 h with DMSO (C), partially purified elicitor (P) or 10 μ M MeJA (M). The Northern blot was sequentially probed with *gusA* gene, *Str* cDNA and *Rps9* cDNA.

or PE (Figure 3B). The 6Tcyt construct contains six head-to-tail copies of the *cyt-1* cis-acting element from the T-DNA T-cyt gene (Neuteboom *et al.*, 1993; Figure 3A). Expression of *gusA* in cell lines containing mutant constructs 4RVM2, 4RVM3, 4RVM4 or 4RVM5 was not inducible with MeJA or PE (Figure 3B), whereas in cell lines containing the mutant 4RVM6 construct the expression of *gusA* was inducible with both MeJA and PE. In each cell line PE or MeJA induced the expression of the endogenous *Str* gene (Figure 3B). These results show that a tetramer of the RV region is sufficient to confer JA- and elicitor-responsive gene expression onto an otherwise inactive minimal promoter. Within the tetramer context the important sequences are located in the region covered by mutations M2–M5, which is consistent with the results from the mutational analysis within the BH context (Figure 2B). The results with the 6Tcyt lines show that the RV region did not potentiate a silent JERE within the CaMV -47 region or elsewhere in the construct.

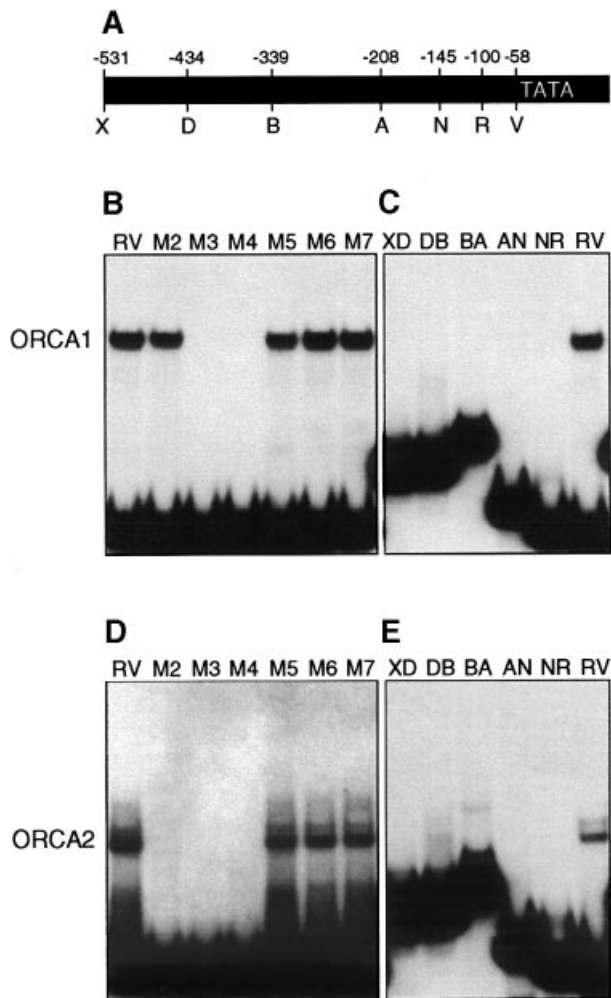


Fig. 5. Binding of ORCA1 and ORCA2 to the RV region of the *Str* promoter. EMSAs were performed with *in vitro* expressed ORCA1 protein or ORCA2 protein expressed in *E. coli* and with labeled *Str* promoter fragments or mutant RV derivatives. (A) Schematic representation of the *Str* -531 promoter. The TATA box is indicated. Numbers and letters are as in Figure 1. (B) Sequence-specific binding of ORCA1 to the JERE in the RV region. (C) Binding of ORCA1 to the RV fragment of the *Str* promoter. (D) Binding-specific of ORCA2 to the JERE in the RV region. (E) Binding of ORCA2 to the RV fragment of the *Str* promoter. Fragments used as probes are indicated at the top of the panels. M2, M3, M4, M5, M6 and M7, indicate mutant RV fragments (mutations as in Figure 2).

(1.3-fold) compared with the vector control (Figure 6A). Expression of *Orca1* in antisense orientation did not affect *Str* promoter activity. Expression of *Orca2* in sense orientation resulted in a 2.9-fold induction of *Str* promoter activity, whereas expression of *Orca2* in antisense orientation had no significant effect compared with the vector control. These results show that transient overexpression of ORCA2 activated the *Str* promoter, whereas overexpression of ORCA1 had little effect on *Str* promoter activity. The differential activation of the *Str* promoter by ORCA1 and ORCA2 may reflect differences in the intrinsic capacity of these proteins to activate the *Str* promoter, or may reflect differences in transient expression level between ORCA1 and ORCA2.

Since ORCA1 had little effect on *Str* promoter activity, a more detailed analysis of *Str* promoter activation was performed only for ORCA2. To test whether ORCA2

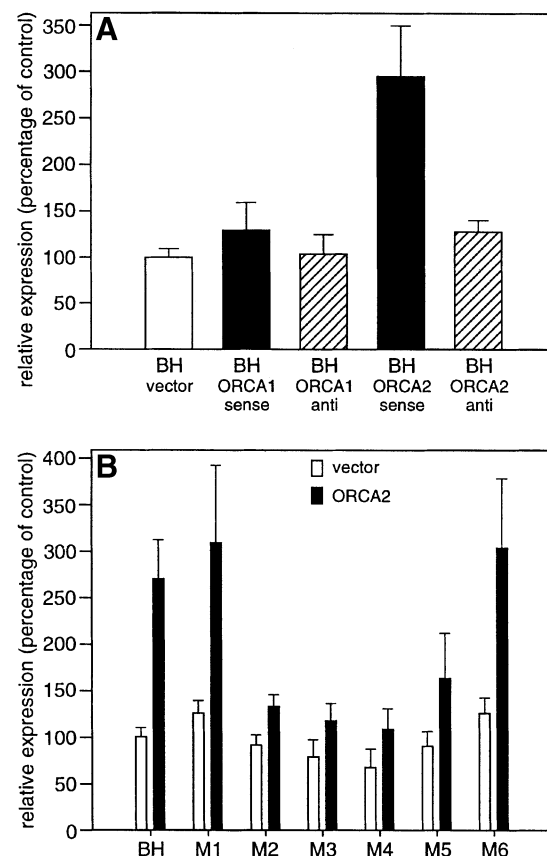


Fig. 6. The *Str* promoter is trans-activated by ORCA2.

(A) *Catharanthus roseus* cells were transiently co-transformed with *Str*-promoter-*gusA*, CaMV 35S promoter-*cat* and overexpression vectors containing *Orca1* or *Orca2* cDNA fused in sense or antisense orientation to the CaMV 35S promoter. Bars represent SE ($n = 12$, except for antisense, $n = 3$). (B) *Catharanthus roseus* cells were transiently co-transformed with wild-type or mutant *Str*-promoter-*gusA* construct, CaMV 35S promoter-*cat* and an overexpression vector containing *Orca2* cDNA fused in sense orientation to CaMV 35S. Bars represent SE ($n = 9$). GUS expression was related to CAT expression to correct for transformation efficiency, and expressed as percentage of vector control. Vector, empty expression vector; BH, wild-type *Str* -339 promoter; M1, M2, M3, M4, M5 and M6, indicate mutant BH promoter derivatives (mutations as in Figure 2).

activates the *Str* promoter via interaction with the RV region, co-transformations were done using wild-type and mutant versions of the BH fragment. ORCA2 strongly enhanced the expression of the *gusA* reporter gene through interaction with wild-type and mutant versions M1 and M6 of the *Str* promoter (Figure 6B). Mutations M2, M3, M4 and M5 severely reduced the activation of BH by ORCA2 (Figure 6B). The activation of mutant constructs M2, M3, M4 and M5 was statistically significantly different from the wild-type, whereas activation of mutant constructs M1 and M6 was not significantly different from the wild-type construct ($P < 0.05$). These results show that ORCA2 acts as a transcriptional activator, that interacts in a sequence-specific manner with the JERE in the *Str* promoter. Three mutations that abolished binding of ORCA2 to the RV fragment *in vitro* also affected trans-activation of the *Str* promoter *in vivo*, indicating that ORCA2 activates the *Str* promoter via direct binding. Whereas mutation M5 did not affect *in vitro* binding of ORCA2, it significantly reduced ORCA2-mediated trans-

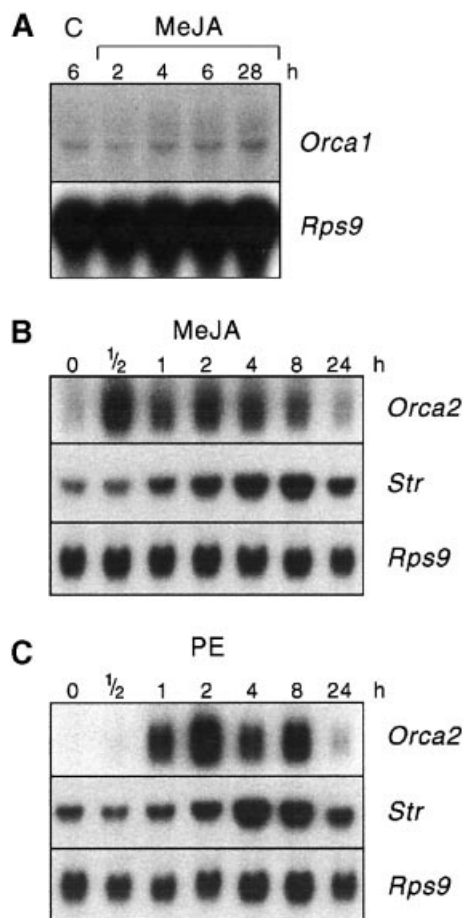


Fig. 7. *Orca2* mRNA, but not *Orca1* mRNA, is rapidly induced by MeJA and elicitor. *C.roseus* cells were exposed to MeJA (10 μ M) or partially purified elicitor (PE) for the number of hours (h) indicated at the top of the figure. (A) *Orca1* and *Rps9* mRNA levels after addition of MeJA. (B) *Orca2*, *Str* and *Rps9* mRNA levels after addition of MeJA. (C) *Orca2*, *Str* and *Rps9* mRNA levels after addition of PE. Northern blots were hybridized with *Orca1*, *Orca2*, *Str* and *Rps9* cDNAs as indicated. C, control incubation with DMSO.

activation, which is consistent with its effect on JA and elicitor responsiveness of the *Str* promoter.

Elicitor and MeJA rapidly induce *Orca2*, but not *Orca1* mRNA levels

We analyzed *Orca1* and *Orca2* mRNA levels after induction with MeJA and PE. Figure 7A shows that a basal level of *Orca1* expression was present in the control sample, which was not significantly induced by MeJA (Figure 7A) or PE (data not shown). Analysis of *Str* gene expression showed that the induction treatment was effective (data not shown). In an independent experiment *Orca1* expression was also not inducible by MeJA at 30 min or 1 h after addition (data not shown). A single mRNA species, with an estimated length of ~1800 nucleotides was detected for *Orca1*. For *Orca2*, a single mRNA species was detected with an estimated length of 900 nucleotides. *Orca2* gene expression was rapidly and transiently induced by MeJA, with high expression levels as early as 30 min after addition (Figure 7B). A biphasic induction pattern, with less *Orca2* mRNA at 1 h than at 30 min and 2 h, was consistently observed in independent experiments and was not due to differences in loading of

RNA or inadequate induction by MeJA, since *Str* and *Rps9* mRNA levels were not reduced at 1 h. Furthermore, biphasic kinetics of *Orca2* expression were also observed after induction by PE, with an increase of the mRNA level within 1 h, maximum accumulation at 2 h followed by a decrease in the mRNA amount at 4 h and a second peak in mRNA accumulation at 8 h. At 24 h the level of *Orca2* mRNA returned to the control level. As shown previously (Menke *et al.*, 1999), the expression of the *Str* gene is induced by MeJA and PE. *Str* gene expression was induced at 2 h after the addition of PE and MeJA and continued to rise up to 8 h (Figure 7B and C). These results show that the *Orca2* mRNA level was induced rapidly by MeJA and PE, whereas *Orca1* expression was not induced. The first peak of the induced expression of *Orca2* preceded the maximum induced expression of *Str* by at least 2 h. Interestingly, the delay in *Orca2* induction kinetics by elicitor compared with MeJA is similar to the time required for elicitor to induce high levels of JA biosynthesis, which peaks at 2–3 h (Menke *et al.*, 1999).

Discussion

We have shown by loss- and gain-of-function experiments that a 24 bp sequence with a GCC core acts as an important *cis*-element required for JA- and elicitor-responsive *Str* gene expression. We have previously demonstrated that elicitor-induced *Str* gene expression requires a transient flux through the octadecanoid pathway (Menke *et al.*, 1999). The fact that both JA- and elicitor-induced *Str* gene expression is mediated through the same *cis*-element and is similarly affected by the same mutations further supports the intermediary role for JA in elicitor-induced signalling. Furthermore, the JERE is the first *cis*-element reported to be responsive to both JA and elicitor. JA-responsive sequences characterized in other promoters do not resemble the JERE present in the *Str* promoter. A common feature in other promoters is the presence of a TGACG motif or a G-box (as discussed by Rouster *et al.*, 1997). However, the G-box present in the *Str* promoter does not function as a JA-responsive element in periwinkle. Mutation of the G-box-containing fragment within the *Str* promoter also did not affect elicitor responsiveness in transgenic tobacco (Pasquali *et al.*, 1999). Within the JERE of the *Str* promoter, a GCC-box-like sequence is present, that bears similarity to an ethylene-responsive element present in the promoter of the β -1,3-glucanase gene *gln2* from tobacco (Ohme-Takagi and Shinshi, 1995). DNA-binding proteins that interact with ethylene-responsive GCC-like sequences belong to the AP2-domain family of transcription factors (Riechmann and Meyerowitz, 1998). These proteins are characterized by the presence of the AP2-domain, that was first identified in the APETALA2 protein. The APETALA2 gene of *Arabidopsis* controls important processes during flower development (Jofuku *et al.*, 1997). The cDNAs, isolated in the yeast one-hybrid screening with the JERE-containing RV region of the *Str* promoter, encoded two AP2-domain proteins. Both ORCA1 and ORCA2 contain highly conserved AP2-domains, but outside this domain little homology exists between the two ORCAs. Outside the AP2-domain, ORCA1 has substantial homology to the N-terminal region of *Arabidopsis* DREB2A and DREB2B (Liu *et al.*, 1998),

whereas ORCA2 is most homologous to the tobacco EREBP1 (Ohme-Takagi and Shinshi, 1995) and tomato Pti4 (Zhou *et al.*, 1997) proteins. This indicates that ORCA1 and ORCA2 are members of different subfamilies. All functionally characterized members of the subfamily of AP2-domain proteins with a single AP2-domain are involved in stress-responsive gene expression (Riechmann and Meyerowitz, 1998). Yeast two-hybrid screening identified three Pto-interacting (Pti4, 5 and 6) tomato proteins, each of which contained one AP2-domain (Zhou *et al.*, 1997). Pto is a receptor kinase that is involved in the recognition of *Pseudomonas* avirulence gene product AvrPto (Martin *et al.*, 1993). Therefore, Pti4, 5 and 6 could be involved in elicitor-induced activation of defense responses in tomato. In tobacco carrying a *Pto* transgene, expression of the AP2-domain protein EREBP1 was specifically enhanced upon infection with a bacterial strain harboring the *AvrPto* avirulence gene, and this preceded induction of PR gene expression (Zhou *et al.*, 1997). Another family of AP2-domain proteins, represented by DREB2A and DREB2B is involved in drought-responsive gene expression (Liu *et al.*, 1998), whereas the CBF1/DREB1 family includes central regulators of cold-regulated gene expression in *Arabidopsis* (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). In *C. roseus* the expression of TIA biosynthetic genes is coordinately regulated in response to elicitor and jasmonates (Menke *et al.*, 1999). Therefore, analogous to CBF1, ORCA proteins are potential central regulators of TIA biosynthetic gene expression.

ORCA2 was shown to act as a transcriptional activator of the *Str* promoter. *In vivo* co-expression of ORCA2 with wild-type and mutant versions of the *Str* promoter demonstrated that *trans*-activation occurs via direct interaction with the JERE in the RV region of the *Str* promoter. This differential *trans*-activation of wild-type and mutated *Str* promoter derivatives by ORCA2 is consistent with the results of the mutational analysis of the RV region of the *Str* promoter in stable transformants. Thus, these results provide evidence for involvement of ORCA2 and the JERE in octadecanoid signaling leading to TIA biosynthetic gene expression. However, the *in vitro* binding specificity of ORCA2 for the JERE-containing RV fragment is not completely consistent with the *in vivo* results. Sequence specificity of DNA binding by AP2-domain proteins *in vitro* is well documented. EREBPs and Pti5 and 6 specifically interact with GCC-boxes present in the promoters of PR genes (Ohme-Takagi and Shinshi, 1995; Zhou *et al.*, 1997). Detailed analysis of the *in vitro* binding requirements of EREBP2, 4 and AtERF1, revealed three critical residues (G⁸ G¹¹ C¹³) located in the GCC-box (Hao *et al.*, 1998). Two of these critical residues (G¹¹ C¹³) are present in most GCC-box-like elements characterized, and are also present in the JERE of the *Str* promoter. EMSAs with mutant RV fragments clearly show that ORCA2 can not interact with mutants RVM3 and RVM4, in which these residues are mutated. Furthermore, ORCA2 could also not interact with mutant RVM2. *In vivo*, JA- and elicitor-responsive activation of the *Str* promoter was severely reduced by mutation M2 and abolished by M3, M4 and M5. The apparent discrepancy between the effect of mutation M5 *in vivo* and its effect on *in vitro* binding of ORCA2 is puzzling. Possible explanations include the interaction of an additional factor with the sequence

covered by M5, or the creation of an artificial repressor binding site by the M5 mutation, that disrupts the function of the JERE *in vivo*. Alternatively, the *in vitro* and *in vivo* binding specificity may well be different for ORCA2. Nonetheless, the results indicate that ORCA2 is the likely transcription factor involved in JA- and elicitor-responsive *Str* gene expression. For ORCA1 the *trans*-activation data and its binding specificity are not as compatible with the *in vivo* mutational analysis of the *Str* promoter. Based on homology with the DREB2 proteins from *Arabidopsis* (Liu *et al.*, 1998), one could speculate that ORCA1 is involved in drought-responsive gene expression in *C. roseus*.

Lack of induction of *Orca1* expression by MeJA and elicitor may be taken as additional evidence that ORCA1 is not involved in JA- and elicitor-induced *Str* gene expression. In contrast, *Orca2* expression has a low basal level and is rapidly induced by MeJA and elicitor. The lack of induction at the transcriptional level does not exclude the involvement of ORCA1 in the regulation of *Str* gene expression. In soybean, elicitation did not induce the accumulation of bZIP factor G/HBF-1 mRNA, but G/HBF-1 was phosphorylated upon elicitation and this enhanced binding to its target DNA (Dröge-Laser *et al.*, 1997). We have previously demonstrated that downstream of JA a protein kinase is involved in induction of *Str* expression (Menke *et al.*, 1999). Although it is unlikely that phosphorylation is required for DNA binding of ORCA1, since ORCA1 expressed *in vitro* is able to bind, it may well enhance its binding, similar to G/HBF-1. Induction of *Orca2* gene expression by JA and elicitor may indicate that ORCA2 is not a primary response transcription factor. Regulation of *Str* gene expression by ORCA2 may include a transcriptional cascade as has been described for ethylene-regulated genes by ERF1. Expression of *ERF1*, an *Arabidopsis* EREBP family member, is controlled by a novel DNA-binding protein encoded by the *EIN3* gene, indicating that ERF1 acts downstream of EIN3 in ethylene signaling (Solano *et al.*, 1998). *Orca2* gene expression may be regulated by a *trans*-acting factor similar to EIN3. Alternatively, ORCA2 may be activated by phosphorylation and enhance its own expression via an autoregulatory loop. An autoregulation mechanism in which activated ORCA2 is removed quickly via degradation could explain the biphasic induction of *Orca2* expression in response to MeJA and elicitor. Biphasic kinetics of gene expression are a common feature of mammalian oncogene induction by various stimuli, for example *c-jun* induction by thrombin (Trejo *et al.*, 1992).

This report concerns the first identification of a plant transcription factor involved in JA signal transduction and identifies this factor as a member of the AP2-domain protein family. Recently, a number of reports have shown that certain defense responses are dependent on both JA and ethylene signaling (O'Donnell *et al.*, 1996; Penninckx *et al.*, 1996; Pieterse *et al.*, 1998). The involvement of AP2-domain family members in both ethylene and JA signaling suggests that ethylene and JA may cross-talk via these transcription factors. Furthermore, through the identification of ORCA2, the spectrum of defense genes regulated by AP2-domain proteins, which includes ethylene- and elicitor-responsive PR genes (Ohme-Takagi and Shinshi, 1995; Zhou *et al.*, 1997) and cold- and drought-

stress-responsive genes (Stockinger *et al.*, 1997; Liu *et al.*, 1998), can now be extended to include a JA-responsive secondary metabolite biosynthetic gene.

Materials and methods

Construction of plasmids, cell culture and transformation

Cell suspension cultures of *C.roseus* were grown as described by Pasquali *et al.* (1992). *Str* (DDBJ/EMBL/GenBank accession number Y10182) promoter derivatives with 5' and internal deletions were fused to the β -glucuronidase (*gusA*) reporter gene in the vector GusSH (Pasquali *et al.*, 1994), as described by Pasquali *et al.* (1999). These GusSH derivatives were used to make transgenic *C.roseus* MP183L cell lines by particle bombardment, according to the method described by van der Fits and Memelink (1997). Each transgenic cell line was a mixed population that was estimated to consist of hundreds of independent transformants. The transgene expression level therefore reflects an average, and can be considered to be largely independent of the chromosomal position or copy number. For *Agrobacterium*-mediated transformation, the GusSH derivatives were introduced into the binary vector pMOG22 λ CAT, a derivative of pMOG λ CAT (Pasquali *et al.*, 1994) containing the hygromycin resistance gene, as described previously (Menke *et al.*, 1999). The GusSH derivatives of pMOG22 λ CAT were introduced into *C.roseus* BIX cell lines via an improved *Agrobacterium* strain (L.van der Fits and J.Memelink, unpublished results). Eight block mutations were introduced in the RV fragment via PCR, covering the entire fragment from the *RsaI* site to the *AvaII* site (M1 to M8). In each mutant six adjacent nucleotides were changed into their complementary nucleotides, i.e. changing A to T and G to C (Figure 2A). The eight mutated versions of the BH fragment were each fused to *gusA* in GusSH. The wild-type RV region and mutated derivatives M2 to M6 were cloned as *RsaI*–*AvaII* (filled in with the Klenow fragment of DNA polymerase I) fragments into the *SmaI*–*EcoRV* sites of pIC20H, and tetramerized using the enzymes *BamHI*–*BglII*. The head-to-tail tetramers were cloned as *BamHI*–*BglII* fragments into the *BamHI* site of GusSH-47 (Pasquali *et al.*, 1994).

Elicitor and JA treatment

Yeast extract (YE; Difco) was dissolved in water, autoclaved and used at a final concentration of 400 μ g/ml to elicit *C.roseus* cells. PE was prepared from YE through ultra-filtration and a number of chromatographic steps, as described in Menke *et al.* (1999). The amount of PE used for induction experiments was calibrated using a semi-quantitative alkalization response assay as described previously (Menke *et al.*, 1999). Methyl-jasmonate (Bedoukian Research Inc.) was diluted in dimethylsulfoxide (DMSO).

RNA extraction and Northern blot analysis

RNA extraction and Northern blot analysis were performed as described previously (Menke *et al.*, 1999), loading 20 μ g RNA samples onto the gels. All Northern blots were probed using 32 P-labeled DNA probes. For *Orca1*, full-length cDNA was used as a probe, whereas for *Orca2* a 450 bp fragment, located 3' of the sequence encoding the AP2-domain, that was specific for *Orca2* was used. DNA probes for *Rps9*, *Str* and *gusA* have been described previously (Menke *et al.*, 1999).

Yeast one-hybrid screening

RV wild-type and mutant tetramers were cloned as *BamHI*–*BglII* fragments into the *BamHI* site of pHIS3NX (Meijer *et al.*, 1998). The tetramer–*HIS3* gene fusions were transferred as *NotI*–*XbaI* fragments to pINT1 (Meijer *et al.*, 1998). The resulting plasmids were linearized with *NcoI* and introduced into yeast strain Y187 (Clontech). Recombinants were selected on YPD medium containing 150 μ g/ml G418, and the occurrence of single recombination events between the pINT1 derivative and the chromosomal *PDC6* locus was verified via Southern blotting. To construct the library, cDNA was synthesized using a Stratagene kit on two different poly(A)⁺ RNA preparations, mixed in a 1:1 ratio, isolated from cell suspensions that were elicited with 0.04 % YE for 1 and 4 h, respectively. The cDNA was cloned in the *EcoRI*–*XhoI* sites of λ ACTII. The amplified lambda library, consisting of 3.5×10^6 independent primary transformants was converted to a pACTII (Clontech) plasmid library via *in vivo* excision in the *Cre*-expressing *E.coli* strain BNN132. The missing portion of ORCA2 cDNA was isolated via PCR using the pACTII cDNA library as a template. A prominent 0.6 kb PCR band was digested with *EcoRI* and *BamHI*, and cloned in pBluescript II

SK+. A complete clone was constructed in pBluescript II SK+ by fusion of the PCR fragment with the cDNA fragment from ORCA2-class plasmid RV210 using a unique *BamHI* site.

EMSA

A *BamHI*–*BglII* cDNA fragment encoding ORCA1 was cloned in pBluescript II SK+ cut with *BamHI*, such that the 5' end faced the T7 promoter. ORCA1 protein was produced by *in vitro* transcription/translation using the TnT coupled reticulocyte lysate system (Promega). A PCR-amplified ORCA2 open reading frame (ORF) was cloned into pET16b (Novagen) cut with *NdeI*–*XhoI*. His-tagged ORCA2 protein was expressed in *E.coli*, and purified via Ni²⁺ affinity chromatography. Wild-type and mutant RV fragments (M2–M7) were excised from BH promoter derivatives using *RsaI*–*AvaII* (filled in with the Klenow fragment of DNA polymerase I), and cloned in pIC20H digested with *SmaI*–*EcoRV*. Fragments were isolated following digestion with *XhoI*–*XbaI*, and labeled by filling in with the Klenow fragment of DNA polymerase I. DNA-binding reactions contained 0.1 ng of end-labeled DNA probe, 100 ng of poly(dIdC)–poly(dIdC), 500 ng of poly(dAdT)–poly(dAdT), 25 mM HEPES–KOH pH 7.2, 100 mM KCl, 0.1 mM EDTA, 10 % glycerol and protein extract in a 10 μ l volume. Following addition of protein extract, reactions were incubated for 30 min at room temperature before loading on 5% acrylamide/bisacrylamide (37:1)–0.5 $\times$ TBE gels under tension. After electrophoresis at 125 V for 1 h, gels were dried on Whatmann DE81 paper and autoradiographed.

Transient expression assays

Catharanthus roseus cells were transiently co-transformed with a *Str*-promoter–*gusA* (BH–GusSH) construct, an overexpression vector carrying *Orca1* or *Orca2* cDNAs fused in sense or antisense orientation to the CaMV 35S promoter, and a plasmid carrying the *chloramphenicol acetyl transferase* gene (*cat*) under the control of the CaMV 35S promoter. As a control, co-transformation of BH–GusSH with an empty overexpression vector (pMOG184) and the *cat* expression vector was carried out. Cells were transformed through particle bombardment as described previously (van der Fits and Memelink, 1997), using the three constructs in a ratio of 1:1:3 (*gus:cat:Orca*). Twenty-four hours after transformation cells were harvested and frozen in liquid nitrogen. GUS and CAT activity assays were performed as described by van der Fits and Memelink (1997). *GusA* reporter gene expression was related to *cat* expression to correct for transformation efficiency and depicted as relative expression compared with the vector control. Statistical analysis of the results was performed using the nonparametric Wilcoxon–Mann–Whitney test.

Acknowledgements

The authors would like to thank Ward de Winter and Marga Harteveld for excellent technical assistance and Leslie van der Fits for valuable discussions. For critical reading of the manuscript we like to thank Sylvia de Pater. A.C. was sponsored by a Leonardo Grant of the European Community.

References

- Creelman, R.A. and Mullet, J.E. (1997) Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **48**, 355–381.
- Dröge-Laser, W., Kaiser, A., Lindsay, W.P., Halkier, B.A., Loake, G.J., Doerner, P., Dixon, R.A. and Lamb, C. (1997) Rapid stimulation of a soybean protein-serine kinase which phosphorylates a novel bZIP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependent defenses. *EMBO J.*, **16**, 726–738.
- Fukuda, Y. and Shinshi, H. (1994) Characterization of a novel *cis*-acting element that is responsive to a fungal elicitor in the promoter of class I chitinase gene. *Plant Mol. Biol.*, **24**, 485–493.
- 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.
- Hao, D., Ohme-Takagi, M. and Sarai, A. (1998) Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. *J. Biol. Chem.*, **273**, 26857–26861.
- Jaglo-otosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. and Thomashow, M.F. (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science*, **280**, 104–106.

- Jofuku, K.D., den Boer, B.G.W., Van Montagu, M. and Okamoto, J.K. (1997) Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2*. *Plant Cell*, **11**, 437–451.
- Kim, S.-R., Choi, J.-L., Costa, M.A. and An, G. (1992) Identification of G-box sequence as an essential element for methyl-jasmonate response of potato proteinase inhibitor II promoter. *Plant Physiol.*, **99**, 627–631.
- Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*, **10**, 1391–1406.
- Martin, G.B., Brommonschenkel, S., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D. and Tanksley, S.D. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*, **262**, 1432–1436.
- Meijer, A.H., Ouwerkerk, P.B.F. and Hoge, J.H.C. (1998) Vectors for transcription factor cloning and target site identification by means of genetic selection in yeast. *Yeast*, **14**, 1407–1416.
- Menke, F.L.H., Parchmann, S., Mueller, M.J., Kijne, J.W. and Memelink, J. (1999) Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. *Plant Physiol.*, **119**, 1289–1296.
- Mueller, M.J. (1997) Enzymes involved in jasmonic acid biosynthesis. *Physiol. Plant.*, **100**, 653–663.
- Mueller, M.J., Brodschelm, W., Spannagl, E. and Zenk, M.H. (1993) Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. *Proc. Natl Acad. Sci. USA*, **90**, 7490–7494.
- Neuteboom, S.T.C., Stoffels, A., Hulleman, E., Memelink, J., Schilperoort, R.A. and Hoge, J.H.C. (1993) Interaction between the tobacco DNA-binding activity CBF and the *cyt-1* promoter element of the *Agrobacterium tumefaciens* T-DNA gene T-CYT correlates with *cyt-1* directed gene expression in multiple tobacco tissue types. *Plant J.*, **4**, 525–534.
- O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, D.J. (1996) Ethylene as a signal mediating the wound response of tomato plants. *Science*, **274**, 1914–1917.
- Ohme-Takagi, M. and Shinshi, H. (1995) Ethylene-inducible DNA-binding proteins that interact with an ethylene-responsive element. *Plant Cell*, **7**, 173–182.
- Pasquali, G., Goddijn, O.J.M., de Waal, A., Verpoorte, R., Schilperoort, R.A., Hoge, J.H.C. and Memelink, J. (1992) Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors. *Plant Mol. Biol.*, **18**, 1121–1131.
- 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.
- Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samlanx, G.W., Buchala, A., Métraux, J.-P., Manners, J. and Broekaert, W.F. (1996) Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell*, **8**, 2309–2323.
- Pieterse, C.M.J., van Wees, S.C.M., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. and van Loon, L.C. (1998) A novel signalling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell*, **10**, 1571–1580.
- Reymond, P. and Farmer, E.E. (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.*, **1**, 404–411.
- Riechmann, J.L. and Meyerowitz, E.M. (1998) The AP2/EREBP family of plant transcription factors. *Biol. Chem.*, **379**, 633–646.
- Rouster, J., Leah, R., Mundy, J. and Cameron-Mills, V. (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. *Plant J.*, **11**, 513–523.
- Rushton, P.J. and Somssich, I.E. (1998) Transcriptional control of plant genes responsive to pathogens. *Curr. Opin. Plant Biol.*, **1**, 311–315.
- Rushton, P.J., Tovar Torres, J., Parniske, M., Wernert, P., Hahlbrock, K. and Somssich, I.E. (1996) Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoter of parsley *PR1* genes. *EMBO J.*, **15**, 5690–5700.
- 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.
- Stockinger, E.J., Gilmour, S.J. and Thomashow, M.F. (1997) *Arabidopsis thaliana* CBF1 encodes an AP2-domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl Acad. Sci. USA*, **94**, 1035–1040.
- Trejo, J., Chambard, J.-C., Karin, M. and Brown, J.H. (1992) Biphasic increase in *c-jun* mRNA is required for induction of AP1-mediated gene transcription: differential effects of muscarinic and thrombin receptor activation. *Mol. Cell. Biol.*, **12**, 4742–4750.
- 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.
- Vijayan, P., Shockey, J., Lévesque, C.A., Cook, R.J. and Browse, J. (1998) A role for jasmonate in pathogen defense of *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **95**, 7209–7214.
- Xiang, C., Miao, Z.-H. and Lam, E. (1996) Coordinated activation of *as-1* type elements and tobacco glutathione S-transferase gene by auxins, salicylic acid, methyl-jasmonate and hydrogen peroxide. *Plant Mol. Biol.*, **32**, 415–426.
- Yang, Y., Shah, J. and Klessig, D.F. (1997) Signal perception and transduction in plant defense responses. *Genes Dev.*, **11**, 1621–1639.
- Zhou, J., Tang, X. and Martin, G. (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *EMBO J.*, **16**, 3207–3218.

Received May 7, 1999; revised and accepted June 30, 1999