A jasmonate-responsive element within the *A. thaliana vsp1* promoter

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Abstract

The *vsp1* gene of *Arabidopsis thaliana* encodes a storage protein that accumulates in vegetative organs. Transgenic plants expressing a *vsp1* promoter–gus (β-glucuronidase) gene fusion were found to contain high GUS activity when challenged with jasmonate, a volatile plant hormone. The induction of *vsp1–gus* expression by jasmonate could be measured in tobacco mesophyll protoplasts, after transient expression. A number of deletions were operated in the *vsp1* promoter in order to locate its jasmonate-responsive element. A 41 bp sequence taken approximately 150 bp upstream of the *vsp1* TATA box could confer jasmonate responsiveness upon a short CaMV 35S promoter. Whereas the deletion of a CAAT box-like element within the 41 bp sequence did not affect promoter activity, mutation of a short palindromic motif completely abolished jasmonate responsiveness. This motif shows no homology with the jasmonate-responsive elements of other promoters.

Key words: *Arabidopsis thaliana*, gene expression, jasmonate, promoter, vegetative storage protein.

Introduction

Jasmonate is a plant hormone involved in the regulation of a number of processes such as development, senescence, secondary metabolism, and response to wounding and pathogen attack (Creelman and Mullet, 1997). In response to wounding, jasmonate is synthesized and activates a subset of defence genes, through a signalling pathway different from the salicylate pathway (Thomma et al., 1998; Pieterse et al., 1998; Schenk et al., 2000; Turner et al., 2002). Jasmonate regulatory elements have been identified in several plant promoters. They often contain a G-box motif and a C-rich region such as in the promoter of the *pin2* gene of potato (Kim et al., 1992), or in the soybean *vspB* (Mason et al., 1993). However, the jasmonate-responsive elements of other promoters do not contain these motifs (Rouster et al., 1997). In *Catharanthus roseus*, jasmonate activates the synthesis of alkaloid compounds. The jasmonate-responsive element of the strictosidine synthase (*str*) gene was shown to be a GCC-box-like element. AP2-domain transcription factors binding this element have been characterized (Menke et al., 1999; van der Fits and Memelink, 2000).

Vegetative storage proteins (VSP) are abundant in flowers and in young seedpods. In soybean, they accumulate in mature leaves after seedpod ablation or in response to jasmonate (Mason and Mullet, 1990). Two homologues of the soybean *vsp* genes have been found in *Arabidopsis*. Northern analysis showed *Arabidopsis vsp* gene expression to be induced by jasmonate, phosphate and sucrose (Berger et al., 1995). Fusions of the *vsp1* and *vsp2* promoters to a reporter gene showed *vsp1* to be expressed primarily in carpels and siliques, whereas the expression of *vsp2* was found in vegetative shoots and petals (Utsugi et al., 1998). The two proteins accumulate in response to herbivore damage (Berger et al., 2002) and VSP2 is produced in response to various oxidative stresses (Mira et al., 2002). It is shown here that the transcription of the *vsp1* gene is induced by jasmonate and that the promoter responsive element is an inverted repeat containing a G-box like element. This inverted repeat is located approximately 150 bp upstream of the TATA box.
**Materials and methods**

**Materials**

*E. coli* strain JM101 was used for cloning and strain DH5-α for producing the plasmid for transient expression, *Nicotiana tabacum* cv. PB6 for protoplast isolation, and *Agrobacterium tumefaciens* strain LBA4404 and *A. thaliana* ecotype C24 for transformation. Restriction enzymes were purchased from New England Biolabs, T4 DNA ligase from Boehringer-Mannheim, and *Taq* polymerase from ATGC Biotechnologie. DNA was sequenced using the Pharmacia T7 sequencing kit. GUS substrate (MUG) and plant tissue culture media (Murashige and Skoog, and Gamborg B5) were purchased from Sigma-Aldrich. Radioisotopes were obtained from ICN. Methyl jasmonate was obtained from Sigma-Aldrich.

**Plasmid construction**

Enzymes and kits were specified by the manufacturers. A genomic library made from ecotype C24 DNA, was screened using a vsp1 EST as a probe and a 4.5 kb EcoRI fragment from a positive clone was subcloned into pUC19, giving pAM1. The vsp1 promoter region was recovered from pAM1 as a 1 kb EcoRI–PstI fragment and inserted into pUC19, to give pAM3.

To construct pAM19, an EcoRI–HaeIII 800 bp fragment from pAM3 containing the vsp1 promoter and the first 37 bp of the coding sequence was fused in frame with the gus gene of a pJII62 (Guérineau and Mullineaux, 1993) derivative from which the 35S promoter had been deleted. Under the same strategy, pAM20 was constructed by inserting upstream of the gus gene, the 400 bp HincII–HaeIII fragment harbouring the vsp1 promoter.

To obtain a transcriptional fusion between the vsp1 promoter and the gus coding sequence, a Bal31 deletion was initiated from the PstI site of pAM3 and the products were subsequently digested with EcoRI, and subcloned into pUC12. A clone where the deletion had occurred 12 bp upstream of the vsp1 first ATG sequence was selected and the vsp1 promoter was then inserted as a HindIII–PstI fragment to replace the CaMV 35S, upstream of the gus gene of a pJII62 derivative (Guérineau and Mullineaux, 1993), giving pAM35. From the same Bal31 deletion experiment, a clone having a deletion extending to 18 bp upstream of the vsp1 TATA box was selected for fusion with the 35S promoter TATA box: to obtain pAM41, the vsp1 sequence was cloned as a HindIII–PstI fragment into the same sites of a pBI221 derivative (Jefferson et al., 1987) from which the 35S promoter upstream of position –46 with respect to the transcription start had been deleted.

Plasmid pAM69 was constructed as follows. Oligonucleotides OL69R and OL69F (Table 1) were annealed and inserted into the XhoI and Smal sites of pHIS1 (from Stratagene). Three subsequent copies of the oligonucleotides were sequentially added using SmaI and XbaI and the tetramer was recovered from the resulting plasmid and inserted into a pBI221 derivative (Jefferson et al., 1987) from which the 35S promoter sequence upstream of position –46 with respect to the transcription start had been deleted. The nucleotide sequence across the vsp1 elements was checked.

Plasmids pAM50, pAM51, pAM57, pAM58, pAM74, pAM77, and pAM78 were constructed as follows. Different PCR reactions were performed using the primer OLGUS homologous to part of the gus coding sequence and different primers (OL50, OL51, OL57, OL58, OL74, OL77, OL78) (Table 1) homologous to parts of the vsp1 promoter (Fig. 1) and having an extension to create a HindIII site at the 5′ end of the amplified fragment. In all cases, the PCR fragments were digested with HindIII and PstI and ligated into the same sites of pAM35, thus replacing the whole vsp1 fragment with shorter versions. The regions upstream of the vsp1 TATA box in all these constructs were checked by nucleotide sequencing. The vsp1–gus–CaMV polyadenylation signal were recovered from pAM57 and pAM58 as HindIII–XhoI fragments and inserted into the HindIII and SalI sites of the binary vector pDR400 (Datia et al., 1992), to create pAM60 and pAM61, respectively. Plasmid pAM57 was obtained by cloning the annealed oligonucleotides OL75F and OL75R (Table 1) into the HindIII site of pAM58. The proper orientation and the sequence of the cloned oligonucleotides were checked by nucleotide sequencing. The *E. coli* strains were transformed using a heat shock protocol (Nishimura et al., 1990) and the binary constructs were transferred to *A. tumefaciens* using electroporation.

**Expression experiments**

Tobacco mesophyll protoplasts were isolated from young tobacco plants grown in a greenhouse at 25 °C under long-day light as described previously (Guérineau et al., 1991). Approximately 2×10⁶ protoplasts were used for incubation with each construct. The PEG-based incubation protocol was as in Guérineau et al. (1991). After incubation with the various constructs, each sample of protoplasts was divided into two identical batches, one incubated in 1 ml of culture medium, the other in 1 ml of culture medium containing 2 µM of methyl jasmonate. The protoplasts were incubated at 25 °C under light for 24 h. They were harvested by centrifugation at 100 g for 5 min. The protoplasts were then lysed in 150 µl of GUS extraction buffer (Jefferson et al., 1987) not containing any sarkosyl, by five passages through a 25G needle. Cell debris was eliminated by centrifugation at 15 000 g for 5 min and fluorometric GUS assays were performed on the supernatant, as in Jefferson et al. (1987). Fluorescence was quantified using a Labsystem Fluoroscan II fluorometer. Proteins were assayed using the Bradford reagent from Biorad, with BSA as a standard. Specific activities were expressed in pmol methyl-umbelliferone min⁻¹ mg⁻¹ protein. The jasmonate induction factor was calculated for each transfection.

**Table 1. Nucleotide sequence of the oligonucleotides used for plasmid construction**

<table>
<thead>
<tr>
<th>OLGUS</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>OL50</td>
<td>5′-GAGAAGCTTAGCAGTCTCAACTGTC-3′</td>
</tr>
<tr>
<td>OL51</td>
<td>5′-GAGAAGCTTTGTTCAACAGTATGCGAC-3′</td>
</tr>
<tr>
<td>OL57</td>
<td>5′-GAGAAGCTTCAAGCACGGCCCAAATTCT-3′</td>
</tr>
<tr>
<td>OL58</td>
<td>5′-GAGAAGCTTCTCTTAAACAGTTGTTG-3′</td>
</tr>
<tr>
<td>OL69F</td>
<td>5′-CAAGAGGACGCGCCAAATTCTTAAAATTTAGCACAGTCT-3′</td>
</tr>
<tr>
<td>OL69R</td>
<td>5′-CTAGACGCCCCCGCCCCAGTTGACGTCTGCGTTGAGCTC-3′</td>
</tr>
<tr>
<td>OL74</td>
<td>5′-GAGAAGCTTGCAAAATTCTTAAATTACAGTCAGTC-3′</td>
</tr>
<tr>
<td>OL75F</td>
<td>5′-GAGCTTAAAGCACGCAATTCTTACTAGACATGAGTCTCTC-3′</td>
</tr>
<tr>
<td>OL75R</td>
<td>5′-AGCTAGAGACTGTCGTAATTAGATTTGGCCTGTCGTTAG-3′</td>
</tr>
<tr>
<td>OL77</td>
<td>5′-GAGAAGCTTCAATTTAGACAGTCTCAAC-3′</td>
</tr>
<tr>
<td>OL78</td>
<td>5′-GAGAAGCTTAATCTCATAATTCTATGCTCAACGTGCTC-3′</td>
</tr>
</tbody>
</table>
experiment by dividing the specific GUS activity in the batch of protoplasts incubated with methyl jasmonate by the activity in the batch incubated without methyl jasmonate. The 0.95 confidence intervals for the mean induction factors were calculated from the results of at least six transfection experiments. The significance of the difference between the means of induction factors given by two constructs was assessed using the non-parametric Mann–Whitney test. The calculations were done using the VassarStats on line facilities at http://faculty.vassar.edu/lowry/VassarStats.html.

Arabidopsis roots were transformed as in Clarke et al. (1992). Seeds were harvested on putative transformants and the presence of the gus gene in their progeny was assessed by PCR using specific primers. For measuring the induction of gus gene expression by methyl jasmonate, transformed seedlings were grown in vitro on 0.5× Murashige and Skoog basal media (Sigma M5519) containing 1% sucrose and 0.8% BactoAgar (Difco). Two leaves from each 2-week-old plant were cut off and incubated in liquid medium (composition as above), with or without 50 μM methyl jasmonate. Specific GUS activities were measured in each of the two leaves after a 24 h incubation period under continuous light at 25 °C.

Results

The vsp1 promoter is activated by jasmonate

The vsp1 gene was cloned from a genomic library and its nucleotide sequence was determined (Genbank accession number AF043343). The sequence of the promoter region is shown on Fig. 1. Translational fusions with the gus coding sequence were made using a long and a short version of the vsp promoter, giving pAM21 and pAM22, respectively. The long version contains approximately 0.8 kb upstream of the putative TATA box and the short version only 0.34 kb. These were introduced into Arabidopsis thaliana. Two transgenic lines showing a 3:1 segregation on kanamycin-containing media were obtained and homozygous plants were challenged with jasmonate. GUS activity in these plants was approximately 100 times higher when the plants were treated with...
jasmonate (Fig. 2). The jasmonate regulation by the short version of the vsp1 promoter present in pAM22 was as effective as that of the long version in pAM21, indicating that the jasmonate-responsive elements are located downstream of position 407 (Fig. 1).

**Jasmonate induction of the expression of a vsp1–gus fusion can be recorded after transient expression in tobacco mesophyll protoplasts**

A transcriptional fusion between the vsp1 promoter and the gus coding sequence was constructed. The sequence corresponding to position 110 to 852 (Fig. 1) was present in this construct, namely pAM35. Tobacco protoplasts were incubated with pAM35 or with a 35S–gus construct as a control. Each lot of incubated protoplasts was divided into two identical batches. One was treated with 2 μM jasmonate and the other was used as a control. GUS activities in the two batches of protoplasts were assayed 24 h after incubation. The ratio between the GUS activities in the two aliquots represented the jasmonate induction factor. There was no induction of GUS activity by jasmonate when protoplasts were incubated with the 35S–gus construct (Fig. 3). By contrast, GUS activity in jasmonate-treated protoplasts was 1.75 times higher than the activity in untreated protoplasts, when incubated with the vsp1–gus construct. The induction factors recorded in transient expression experiments were low compared with the ones measured in Arabidopsis transformants. This discrepancy is likely to be due to an activation of vsp1–gus expression in protoplasts, possibly following the release of jasmonate by the plant cells during the process of protoplast isolation. However, added jasmonate still enhanced gene expression, allowing the effect of deletions in the vsp1 promoter to be monitored.

**Deletions in the promoter delimited a jasmonate-responsive element**

Transcriptional fusions were made between various lengths of vsp1 promoter sequence and the gus gene (Fig. 3). The constructs were incubated with tobacco mesophyll protoplasts and the induction factor by jasmonate was determined for each construct. Deletions of the sequence upstream of position 605 did not affect the induction of gene expression by jasmonate, whereas the deletion extending to position 645 completely abolished the effect of jasmonate. The deletion upstream of position 627 gave an induction factor reduced to 1.33. The GUS activity in protoplasts transfected by pAM35 and incu-
bated in jasmonate-containing medium was 3027±866 pmol MU min⁻¹ mg⁻¹ protein (0.99 CI, n=25). By contrast, the activity given by pAM57 was approximately five times lower.

The constructs corresponding to deletions upstream (pAM60) or downstream (pAM61) of the 41 bp element delimited after the transient expression experiments, were introduced into Arabidopsis. Leaves from T₂ plants from five independent transgenic lines were challenged with jasmonate (Fig. 4). The pAM60 transformants gave results similar to the pAM21 or pAM22 transformants. The GUS activity in leaves treated with jasmonate was many times higher than in untreated leaves. By contrast, there was no induction by jasmonate in pAM61 transgenic leaves. This confirmed the results of the transient expression experiments.

Both the transient expression analysis and the expression in stable transformants suggest that the jasmonate responsive element of the vsp1 promoter is located in the 41 bp sequence extending from position 605 to position 645 (Fig. 1).

A 41 bp sequence from vsp1 confers jasmonate regulation upon a heterologous TATA box

A tetramer of the 41 bp sequence defined above was fused to the 35S promoter TATA box element beginning at position –46 with respect to the transcription start, giving pAM69. Alternatively, the vsp1 promoter sequence extending from position 110 to 777 (Fig. 1) was inserted upstream of the 35S –46 region, giving pAM41. Protoplasts were transfected with the chimeric promoters fused to the gus coding sequence, or with the 35S (--46)–gus construct or with pAM35 containing the full vsp1 promoter. The jasmonate induction factors given by the vsp1-containing constructs were significantly higher from that given by the 35S TATA box-gus control (Fig. 5A). The 41 bp tetramer sequence fused to the 35S TATA box gave an induction factor similar to that given by the complete vsp1 promoter of pAM35, indicating that the 41 bp vsp1 is able to confer full jasmonate responsiveness upon a heterologous TATA box. Specific GUS activities given by plasmids containing the full-length vsp1 regulatory region were much higher than the ones given by plasmids harbouring the 41 bp vsp1 sequence or the 35S TATA box alone (Fig. 5B).

Mutation of an inverted repeat sequence suppressed jasmonate responsiveness

The analysis of the 41 bp sequence using the PlantCARE database of transcriptional regulatory elements (http://sphinx.rug.ac.be:8080/PlantCARE/index.htm) revealed the presence of two G box-like elements and of a CAAT box-like motif (Fig. 6). The distal G box-like element (CACGCC) is degenerated with only 4 bp conserved with the G-box hexamer core (CACGTG) (Williams et al., 1992). This element was deleted in pAM74 and the CAAT box-like motif was also removed in pAM77. None of these deletions significantly affected the jasmonate induction factor (Fig. 6). By contrast, mutation of the proximal G box-like sequence (AAGTG), which has 5 bp conserved

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Fig. 3. Transient expression of various vsp1–gus constructs in tobacco mesophyll protoplasts. The coordinates at the 5’ ends of the vsp regions refer to Fig. 1. The jasmonate induction factors were calculated as indicated in the text. n: Number of transfections. a, b, c: Different letters indicate statistically different results (Mann–Whitney test; P <0.01).
with the G-box core, completely abolished the regulation (Fig. 6). Interestingly, some of this G-box-like element is present in the right part of an inverted repeat within the 41 bp fragment (Fig. 6). Mutation of the left part of the inverted repeat, in pAM78, greatly reduced the induction by jasmonate (Fig. 6).

Discussion

VSP mRNAs were shown to be abundant in jasmonate-treated leaves (Berger et al., 1995). However, due to the extensive homology between the vsp1 and vsp2 coding sequences, this northern analysis could not discriminate between the transcripts encoded by the two genes. High GUS activities were found in the plants transformed with vsp1–gus constructs when challenged with jasmonate (Fig. 2), whereas low activities were recorded in untreated plants, showing that the expression of the vsp1 gene was induced by jasmonate. Jasmonate had no effect on the expression of the gus gene placed under the control of the CaMV 35S promoter, used as a control. This result confirmed a northern analysis, which did not reveal the presence of any vsp mRNA in the leaves of Arabidopsis plants at the rosette stage (Berger et al., 1995). To ease the promoter deletion analysis and to avoid the T-DNA position effect on quantifying gene expression, transient
expression experiments were performed using tobacco mesophyll protoplasts. Protoplasts have been used to locate the phosphate response domain in the soybean *vspB* promoter (Tang et al., 2001). The authors also reported a failed attempt to use their transient expression system to study jasmonate responsiveness of the *vspB* promoter. The cause was likely to be an activation of *vspB* expression during the process of making protoplasts. Vsp genes were shown to be activated by wounding (Mason and Mullet, 1990) and it is therefore not surprising that they are activated during the digestion of the cell wall that is required for making protoplasts. As a result, treatment of the protoplasts by jasmonate did not result in an increase of *gus* gene expression, after incubation with *vspB–gus* constructs (Tang et al., 2001). By contrast, it is shown here that a transient expression system could be used to monitor jasmonate activation of *vsp1* expression. Indeed, the GUS activity in protoplasts incubated with a *vsp1–gus* construct was high, even in the absence of jasmonate, but the addition of the compound resulted in a further increase of GUS activity, allowing the calculation of an induction factor. This indicated that the jasmonate induction pathway was not fully activated during the isolation of protoplasts. However, because of the partial activation of the jasmonate pathway in protoplasts, the induction factors calculated from the transient expression experiments are probably a gross underestimation of the induction factors that would be recorded in transgenic plants. Some variation of the induction factor was also dependent on the age of the plants used for isolating protoplasts. Younger plants gave higher induction factors (data not shown). This possibly explains the difference in induction factors given by pAM35 in various experiments (Figs 3, 5). Nevertheless, the analysis allowed the jasmonate respon-
sive element to be identified in a 41 bp sequence (Fig. 3). These data were confirmed in Arabidopsis transgenic plants (Fig. 4). GUS activity was highly variable between independent transgenic lines. This probably reflects differences in transgene copy numbers or in the sites of integration in the genome. In any case, the activation of vsp1-gus by jasmonate in tobacco protoplasts suggests that the Arabidopsis DNA motif for jasmonate induction was similarly recognized by its cognate protein in tobacco cells.

To determine whether the 41 bp element could work as an autonomous determinant of jasmonate induction of gene expression, the sequence was fused to a heterologous TATA box. Four copies of the element were inserted upstream of the CaMV 35S TATA box region extending to position -46, with respect to the transcription start site. Low GUS activities were measured in protoplasts incubated with the basal CaMV promoter of pBI (-46), due to the absence of an activator sequence upstream of the TATA box (Fig. 5B). When the putative vsp1 jasmonate-responsive element was present upstream of the TATA box, GUS activity was approximately twice as high, indicating an activation of transcription by the added element. Besides, GUS activity was even higher in protoplasts treated with jasmonate, indicating that the 41 bp element was able to confer jasmonate-induction upon the CaMV basal promoter (Fig. 5B). The induction factor was similar to that given by a full vsp1 promoter (Fig. 5A), suggesting that the 41 bp element contained all the information for the induction of transcription by jasmonate. The fact that GUS activities were much lower than the ones given by the full vsp1 promoter must be a consequence of the presence of several other activating regions in the vsp1 promoter, since VSP mRNAs were shown to be more abundant in plants treated with light, sucrose and phosphate as well as jasmonate (Berger et al., 1995). This also explains why GUS activity in pAM57-transfected protoplasts was approximately five times lower than that in pAM35-transfected protoplasts.

In silico analysis of the 41 bp sequence containing the jasmonate-responsive element revealed the presence of three motifs, one resembling a CAAT box and two G box-like elements. Deletion of the first two motifs had no effect on the jasmonate induction factor. By contrast, it has been shown elsewhere that a CAAT box was required for optimal induction of the nos promoter by jasmonate (Kim et al., 1993). As mutation of the proximal G box-like element completely abolished the induction, this sequence must be a major determinant of jasmonate regulation of vsp1 transcription. A range of motifs have been found to be responsible for jasmonate induction of transcription of various genes. The jasmonate-responsive domain of the soybean vspB promoter is made of a G box followed by a C-rich region (Mason et al., 1993). Such a C-rich sequence was not identified downstream of the G box-like element of the Arabidopsis vsp1 sequence (Fig. 1). Two jasmonate-responsive elements, JASE1 (5'-CGTCAATGAA-3') and JASE2 (5'-CATACGTCGTCAA-3'), were identified in the promoter of the OPR1 gene in Arabidopsis (He and Gan, 2001). No homology was found between the sequence of

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**Fig. 6.** Effect of deletions or mutations in the 41 bp region of the vsp1 promoter important for jasmonate induction of gene expression. The coordinates above the sequence refer to Fig. 1. The G box-like elements are between bars. The CAAT box-like is boxed. Arrows below the sequence show the inverted repeats. The thick horizontal lines indicate sequence identity with the upper line sequence. Each construct was fused to the gus gene and transfected into tobacco mesophyll protoplasts. The jasmonate induction factors were calculated as indicated in the text. n: Number of transfections. a, b, c: Different letters indicate statistically different results (Mann–Whitney test; P <0.02).
these two elements and the 41 bp sequence found here. In the barley \textit{LOX1} promoter, a short inverted repeat (CGTCA/TGACG) was responsible for jasmonate induction (Rouster et al., 1997). It is striking that although no homology exists with the primary 41 bp \textit{vsp1} sequence involved in regulation by jasmonate, a similar palindromic sequence is found in this region (Fig. 6). The right half of the repeat is part of the G box-like sequence whose mutation completely abolishes the induction by jasmonate. The mutation of the left part of the repeat considerably reduced the induction factor. These observations suggest that the inverted repeat is the jasmonate responsive element of \textit{vsp1}. The presence of similar palindromic motifs in other jasmonate-induced promoters has been underlined (Rouster et al., 1997). The reduced regulation given by pAM50, as compared to that of pAM77 (Fig. 6), suggests that a few nucleotides upstream of the inverted repeat are required for optimal recognition of this motif. The diversity of promoter elements identified so far as responsible for the activation of transcription by jasmonate is to be considered in connection with the various processes in which this hormone is involved. A number of transcription factors activated by several transduction signal pathways must contribute to these regulations. So far, such transcription factors have only been identified in \textit{Catharanthus roseus} (van der Fits and Memelink, 2000). Knowing their cognate binding sequence in jasmonate-regulated promoters should help the identification of transcription factors involved in the control of gene expression by jasmonate in \textit{Arabidopsis}.

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