Pathogen- and NaCl-Induced Expression of the SCaM-4 Promoter Is Mediated in Part by a GT-1 Box That Interacts with a GT-1-Like Transcription Factor[1]

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The Ca^{2+}-binding protein calmodulin mediates cellular Ca^{2+} signals in response to a wide array of stimuli in higher eukaryotes. Plants express numerous CaM isoforms. Transcription of one soybean (*Glycine max*) CaM isoform, *SCaM-4*, is dramatically induced within 30 min of pathogen or NaCl stresses. To characterize the cis-acting element(s) of this gene, we isolated an approximately 2-kb promoter sequence of the gene. Deletion analysis of the promoter revealed that a 130-bp region located between nucleotide positions −858 and −728 is required for the stressors to induce expression of *SCaM-4*. A hexameric DNA sequence within this region, GAAAAA (GT-1 cis-element), was identified as a core cis-acting element for the induction of the *SCaM-4* gene. The GT-1 cis-element interacts with an Arabidopsis GT-1-like transcription factor, AtGT-3b, in vitro and in a yeast selection system. Transcription of *AtGT-3b* is also rapidly induced within 30 min after pathogen and NaCl treatment. These results suggest that an interaction between a GT-1 cis-element and a GT-1-like transcription factor plays a role in pathogen- and salt-induced *SCaM-4* gene expression in both soybean and Arabidopsis.

Plant cells, like animal cells, elevate their cytosolic free-calcium levels ($[Ca^{2+}]_{cyt}$) with varying amplitude, frequency, and duration in response to a variety of external stimuli (Thomas et al., 1996; Berridge, 1997; McAinsh and Hetherington, 1998). The stimulus-specific $[Ca^{2+}]_{cyt}$ transients are sensed by intracellular Ca^{2+}-binding proteins, of which calmodulin (CaM) is one of the best characterized (Chin and Means, 2000; Snedden and Fromm, 2001). CaM is a ubiquitous intracellular mediator of Ca^{2+} signals having four helix-loop-helix Ca^{2+}-binding motifs referred to as EF-hands (Babu et al., 1988). The Ca^{2+}-bound CaM transduces the signals into many cellular processes through modulation of a variety of CaM-binding proteins, including enzymes such as kinases, phosphatases, and nitric-oxide synthase, as well as receptors, ion channels, G-proteins, and transcription factors (Liao et al., 1996; Snedden and Fromm, 1998; Lee et al., 1999a; Zuhlke et al., 1999).

In plant cells, in contrast to mammalian cells, multiple CaM genes code for a number of CaM isoforms. This has been shown in wheat (*Triticum aestivum*; Yang et al., 1996), potato (*Solanum tuberosum*; Takezawa et al., 1995; Poovaiah et al., 1996), and soybean (*Glycine max*; Lee et al., 1995a), among others. Over 30 genes encoding CaM isoforms are found in the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000). We have recently cloned five CaM isoforms from soybean (*SCaM-1-5*). Although SCaM-1-3 are more than 90% identical to mammalian CaM, SCaM-4 and SCaM-5 exhibit only a 78% homology with SCaM-1 and are therefore the most divergent isoforms reported thus far in the plant and animal kingdoms. SCaM-4 is considered to be a bona fide CaM isoform based on the following characteristics. In its primary protein structure, SCaM-4 has four conserved putative EF-hands and a central linker region, hallmark structural features of CaM (Lee et al., 1995a). In addition, most of the nonconsensus amino acids occur outside the EF-hands, and the total number of

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amino acid residues is also conserved (Lee et al., 1995a). When compared with the consensus amino acid sequence of EF-hands derived from known Ca^{2+}-binding proteins, the residues in all four Ca^{2+}-binding loops of SCaM-4 conform to the consensus (Falke et al., 1994; Choi et al., 2002), suggesting that SCaM-4 can bind four Ca^{2+} molecules. Furthermore, SCaM-4 has the ability to modulate the activity of many CaM-dependent enzymes.

SCaM-4 can be distinguished from SCaM-1 by the target enzymes that it can activate (Lee et al., 1995a, 2000; Cho et al., 1998; Kondo et al., 1999; Chung et al., 2000). Some enzymes, including phosphodiesterase, plant Ca^{2+}-ATPase, plant Glu decarboxylase, and CaM-dependent protein kinase II, can be activated equally well by either SCaM-1 or SCaM-4. However, other enzymes can only be activated by one isoform. For example, only SCaM-1 activates calcineurin, myosin light chain kinase, red blood cell Ca^{2+}-ATPase, and plant NAD kinase, and only SCaM-4 activates nitric-oxide synthase. SCaM-1 and SCaM-4 also exhibit differences in the Ca^{2+} concentrations required for target enzyme activation (Lee et al., 2000).

All SCaM isoforms, including SCaM-4, are ubiquitously expressed in various plant tissues and show similar subcellular localization patterns to those of SCaM-1 (Lee et al., 1995a, 1999b). Intriguingly, the cellular level of SCaM-4 rapidly and dramatically rises in response to specific stimuli such as pathogens. Moreover, transgenic tobacco and Arabidopsis plants overexpressing SCaM-4 or SCaM-5 under the control of the cauliflower mosaic virus (CaMV) 35S promoter increase their resistance to pathogens by forming spontaneous hypersensitive response-like lesions with elevated expression of systemic acquired resistance-associated genes. This suggests that plant CaM isoforms have different physiological functions in vivo (Heo et al., 1999).

Although we know that the expression of *CaM* isoforms is differentially regulated by specific external stimuli, the cis- and trans-acting elements involved in plant *CaM* gene expression have not been well characterized. In this study, we have isolated and characterized the promoter sequence of the *SCaM-4* gene. Core cis-acting elements that regulate expression of the *SCaM-4* gene in response to pathogen infection or salt stress were identified within the *SCaM-4* promoter between −1,215 and −1,150 bp, and between −858 and −728 bp. Here we report that an interaction between a GT-1 cis-element and a GT-1-like transcription factor plays a role in pathogen- and salt-induced *SCaM-4* gene expression.

RESULTS

Isolation of the *SCaM-4* Promoter and Analysis of Tissue-Specific Expression of the *ScaM-4* Promoter-*β*-Glucuronidase Reporter Gene

To characterize the regulatory mechanisms controlling transcription of the *SCaM-4* gene, we isolated its promoter region. Figure 1 shows the sequence of the *SCaM-4* promoter (−1,286 bp to +765 bp), which extends into the 5′-untranslated region (GenBank accession no. AY052528). For comparative purposes,

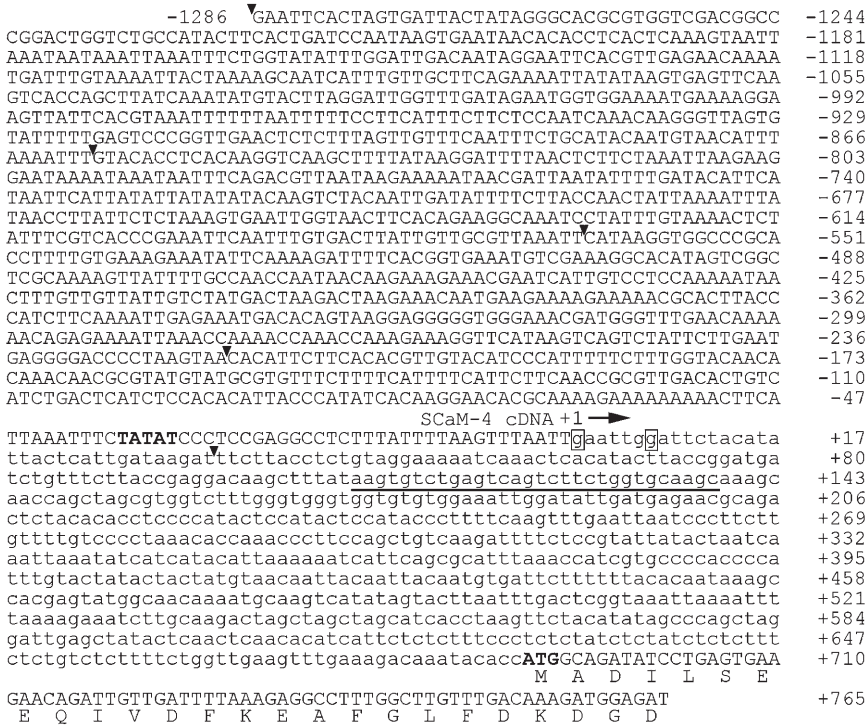

Figure 1. Nucleotide sequence of the promoter region of the *SCaM-4* gene. Sequences of the 5′-flanking region and the first exon of the *SCaM-4* gene are shown together with a partial amino acid sequence from the 5′-end of the *SCaM-4* coding region. The numbering of nucleotides relative to the site of initiation of transcription (+1) is shown on the right. The starting points of the 5′-deleted derivatives of the fragments used in the loss-of-function experiments are indicated by triangles (see Fig. 4A). The putative TATA box is in bold letters. The 3′ oligonucleotides for primer extension mapping are underlined. The 5′ upstream sequence of *SCaM-4* was submitted to GenBank under the accession number AY052528.

2.4 kb of the 5′-upstream region of *SCaM-1* was isolated from a soybean genomic library using SCaM-1 cDNA as a probe (GenBank accession no. AY052527; data not shown). We used a primer extension analysis to map the start site of *SCaM-4* transcription. Two long extension products were detected 689 bp and 683 bp upstream of the first ATG site, suggesting heterogeneity in the mRNA 5′ ends or premature arrest of the reverse transcriptase (data not shown). The G residue corresponding to the longer extension product was taken to be the transcription start site and was numbered +1. As shown in Figure 1, a putative TATA box sequence is located upstream (nucleotides −33 to −37) of the transcription start site.

We then examined the tissue-specific expression pattern of an *ScaM-4* promoter-*β*-glucuronidase (*GUS*) reporter in transgenic Arabidopsis to see whether it matched the expression pattern of *ScaM-4* gene in soybean (Lee et al., 1995a, 1999b). Figure 2 shows representative examples of the tissue-specific expression of the *SCaM-4* promoter-*GUS* gene. The *SCaM-4* promoter-*GUS* was expressed primarily in the apical meristem (Fig. 2D) and hypocotyl regions of transgenic Arabidopsis seedlings (Fig. 2, C and F). The GUS staining patterns in transgenic Arabidopsis seedlings were similar to the expression patterns of *SCaM-4* mRNA and SCaM-4 protein in soybean seedlings (Lee et al., 1995a, 1999b).

Analysis of the Effect of Signaling Molecules on Expression of the *SCaM-4* Promoter-GUS Reporter Gene

Expression of plant *CaM* genes has been shown to respond to various environmental stresses including light, phytohormones, touch, wounding, high salinity, and pathogens (Jena et al., 1989; Braam and Davis, 1990; Botella and Arteca, 1994; Harding et al., 1997). The expression of the two soybean *CaM* genes encoding the SCaM-1 and SCaM-4 isoforms was examined after treatment of soybean suspension-culture cells (W82) with a soybean pathogen, *Pseudomonas syringae* pv *glycinea A* (*Psg*), or with 150 mM NaCl (Fig. 3A). *SCaM-4* mRNA levels peaked at 0.5 h following pathogen or NaCl treatment and then slowly declined to basal levels by 12 h. In contrast, the expression of *SCaM-1* was not activated by the same treatments (Fig. 3A). Similarly, 4-week-old transgenic Arabidopsis plants carrying the *SCaM-4* 2-kb promoter-*GUS* reporter were treated with a pathogen, *P. syringae* pv *tomato DC3000* (*PsD*), or with 150 mM NaCl to examine the expression pattern of the *SCaM-4* promoter in a heterologous system (Fig. 3B). Gel blots of total RNA isolated from the transgenic Arabidopsis plants were probed with *GUS* cDNA. *GUS* mRNA levels appeared at high levels by 0.5 h after application of the pathogen or NaCl, but returned to nearly basal levels by 24 h, despite the continued presence of the stressor (Fig. 3B). In addition, the expression of the *SCaM-4* gene in soybean suspension-culture cells (W82) was dramatically induced within 1 h after treatment with *Psg*, glycol chitin, NaCl, or Ca^{2+}-ionophore A23187 (Fig. 3C). The application of exogenous KCl, mannitol, hydrogen peroxide (H_2O_2), salicylic acid, jasmonic acid, or abscisic acid (ABA) did not induce the expression of *SCaM-4* (Fig. 3C).

We then examined the effects of these treatments on GUS reporter gene expression in Arabidopsis leaf protoplasts, a plant transient expression system. After treatment with various biotic and abiotic signals, we determined the level of induction of GUS activity with reference to luciferase (LUC) activity. The GUS activity of the *SCaM-4* promoter-*GUS* reporter was enhanced about 3- to 7-fold when treated with *PsD*, glycol chitin, NaCl, or Ca^{2+}-ionophore A23187 (Fig. 3D). However, the other treatments did not increase GUS activity. We also examined the effects of the treatments on the expression of 2.4-kb *SCaM-1* promoter-*GUS* construct. No GUS induction was observed for the *SCaM-1* promoter in Arabidopsis protoplast cultures, similar to the expression pattern found in soybean seedlings (Fig. 3E). Overall, these experiments show that Arabidopsis can be a useful system in which to study the pathogen and NaCl responsive regulatory elements of the *ScaM-4* promoter.

Deletion Analysis of the *SCaM-4* Promoter

To determine the specific regions of the promoter that are involved in *SCaM-4* induction by pathogen or NaCl treatments, a series of 5′ deletions were made in the *SCaM-4* promoter region (Fig. 4A). Each construct was transiently introduced into Arabidopsis protoplasts by polyethylene glycol-mediated transformation, and GUS activity was assayed after treatment with 150 mM NaCl, or *PsD* for 12 h. The *GUS* reporter gene was strongly induced by pathogen or NaCl in constructs containing deletions up to −1,286 (*pBI 4D1*) or −858 (*pBI 4D2*), but this induction was completely lost in the construct containing a deletion up to −566 (*pBI 4D3*). Furthermore, the *pBI 4delA* construct, containing nucleotides −1,286 to −728, showed maximal GUS induction after treatment with pathogen or NaCl (about 14- and 19-fold, respectively), a pattern of induction very similar to that of the *SCaM-4* 2-kb promoter (Fig. 4B).

To determine the region(s) within the −1,286 to −728 bp region that are responsible for induction by pathogen and NaCl treatments, the *SCaM-4* promoter region was further divided into six overlapping fragments of 100 to 200 bp in length, and the fragments were fused to the upstream region of the TATA minimal promoter contained within the *pDel. 151-8* vector (Sundaresan et al., 1995; Fig. 4C). These constructs were then tested in transient expression assays in Arabidopsis protoplasts treated for 12 h with water (control), pathogen, or NaCl (Fig. 4D). The −1,286 to −728 construct (*pBI 4delA*) showed GUS induction of

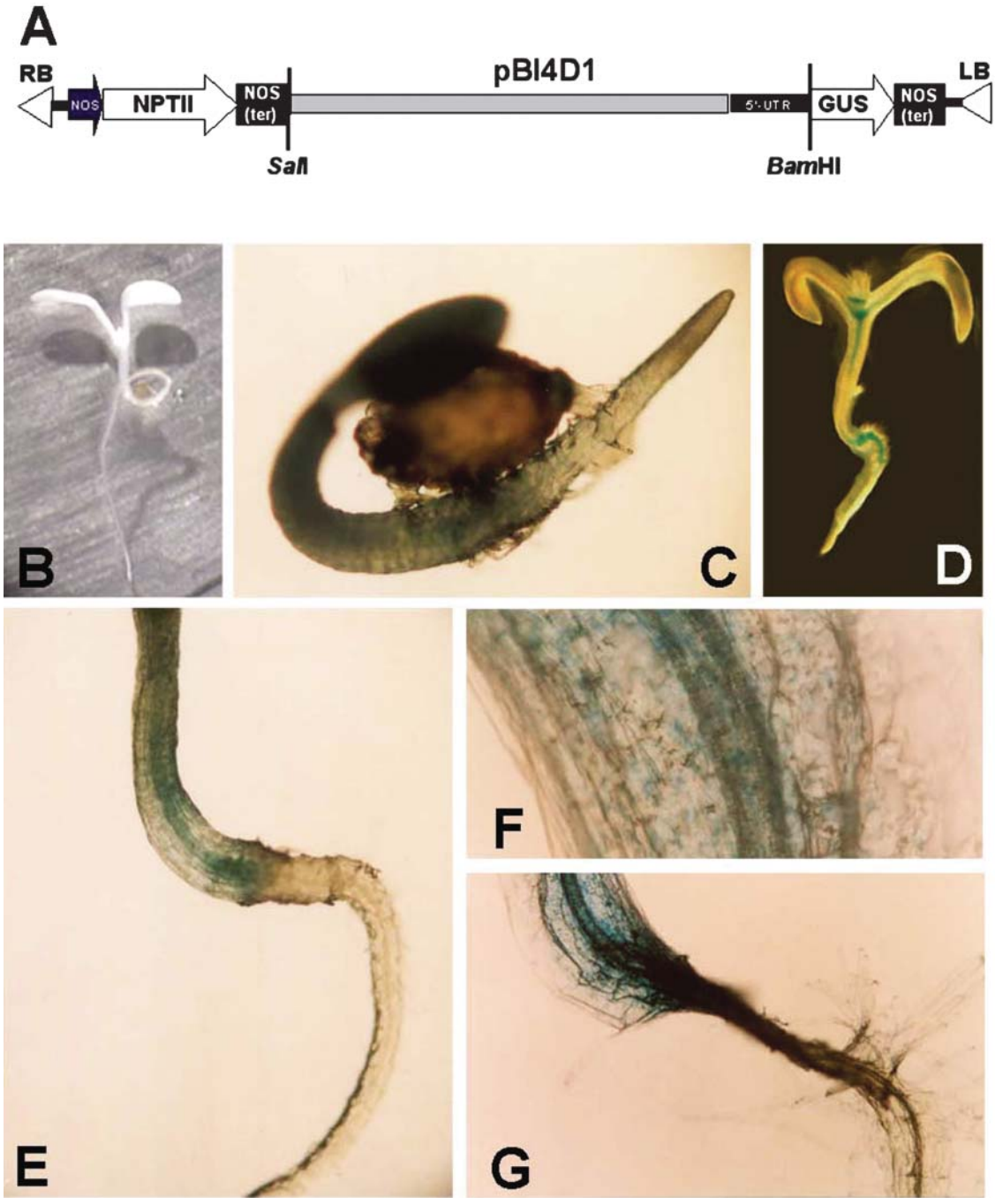

Figure 2. Histochemical localization of the expression of GUS fused to the *SCaM-4* 2-kb promoter in transgenic Arabidopsis seedlings. A, Structure of the *pBI 4D1*, a binary vector used for *SCaM-4* promoter-*GUS* expression. The bacterial neomycin phosphotransferase II gene (NPTII) served as a selectable marker. LB and RB, left and right T-DNA border. Two-day-old (C) and 4-d-old transgenic Arabidopsis seedlings (D–G) containing the *SCaM-4* 2-kb promoter-*GUS* fusion vector (*pBI 4D1*), as well as a 4-d-old transgenic Arabidopsis seedlings containing the promoterless *GUS* vector (*pBI 101*; B), were stained for GUS activity with GUS staining solution. E and G show the lower part (root region) of a seedling. F shows the hypocotyl region of a seedling.

about 8- and 11-fold after treatment with pathogen and NaCl, respectively. The constructs containing the regions −1,286 to −1,065 (*pBI 4delB*), −858 to −728 (*pBI 4delD*), −1,065 to −728 (*pBI 4delE*), and −1,286 to −858 (*pBI 4delG*) showed GUS activities that were approximately one-half of those of the *pBI 4delA* construct. In the construct containing the regions −1,286 to −1,065 and −858 to −728 (*pBI 4delF*), the GUS activity was increased about 6- and 9-fold after treatment with pathogen and NaCl, respectively.

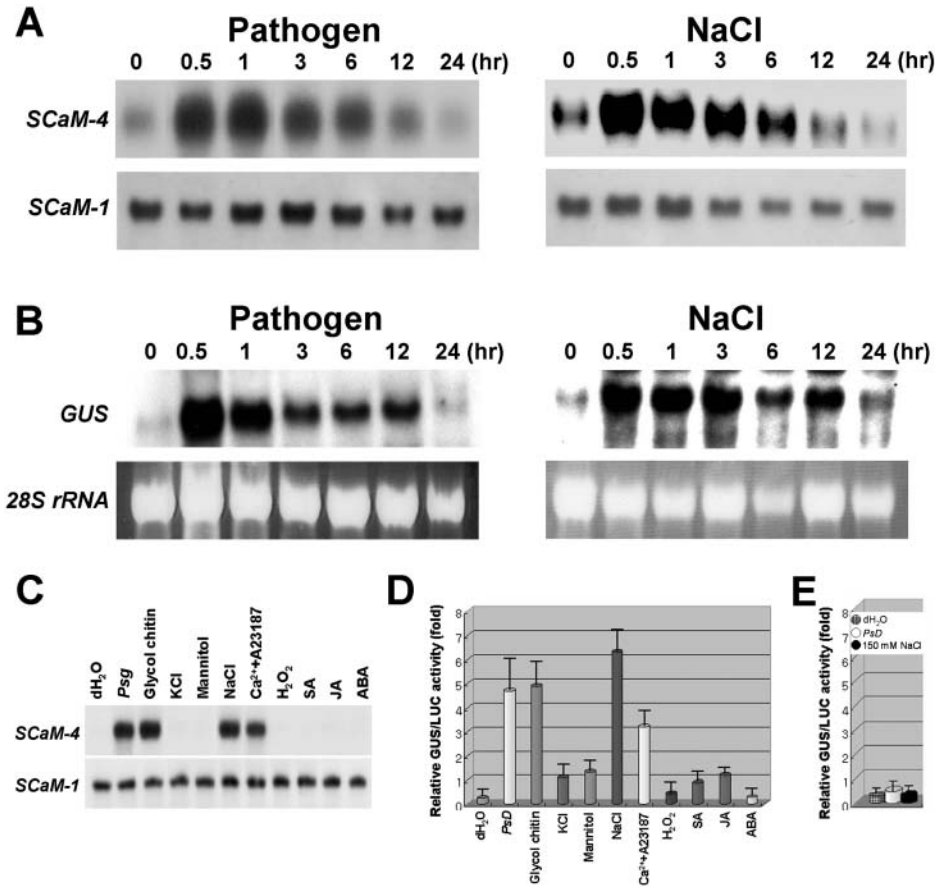

Figure 3. Expression pattern of the *SCaM-4* and *SCaM-1* genes in soybean and *GUS* reporter expression driven by the *SCaM-4* promoter in response to various treatments. A, Time-course of accumulation of *SCaM-4* and *SCaM-1* transcripts after pathogen or NaCl treatment in soybean suspension culture cells. Each lane was loaded with 20 μg of total RNA prepared from soybean suspension-culture cells (W82) treated with pathogen (*Psg*) or NaCl (150 mM) for the times indicated. The gel blots were hybridized with ^{32}P-labeled gene-specific probes derived from the 3′-untranslated regions of the *SCaM-1/-4* genes. B, Time-course of accumulation of *GUS* transcripts in transgenic Arabidopsis plants after pathogen or NaCl treatment. Each lane was loaded with 20 μg of total RNA prepared from transgenic Arabidopsis plants carrying the *SCaM-4* 2-kb promoter-*GUS* construct treated with pathogen (*PsD*) or NaCl (150 mM) for the times indicated. The gel blots were hybridized with a ^{32}P-labeled 1.87-kb *GUS* cDNA. Equal loading of total RNA was verified by ethidium bromide staining to detect rRNA (lower sections). C, Effect of various signaling molecules on the expression of *SCaM-4* and *SCaM-1* genes in soybean suspension-culture cells. Each lane was loaded with 20 μg of total RNA prepared from W82 cells treated for 1 h as indicated, and the mRNA levels of the *SCaM-4* and *SCaM-1* genes were examined with ^{32}P-labeled 3′-untranslated regions specific probes. dH_2O, water control; *Psg, P. syringae* pv *Glycinea avrA*; Glycol chitin, 0.05% glycol chitin; KCl, 150 mM KCl; Mannitol, 300 mM mannitol; NaCl, 150 mM NaCl; Ca^{2+}+A23187, 25 μM Ca^{2+} ionophore A23187 plus 5 mM $CaCl_2$; H_2O_2, 2 mM hydrogen peroxide; SA, 2 mM salicylic acid; JA, 100 μM jasmonic acid; ABA, 100 μM ABA. D, Analysis of GUS expression driven by the *SCaM-4* 2-kb promoter after treatment with various signaling molecules in Arabidopsis protoplasts. GUS activity was calculated relative to LUC activity. The data are presented as mean ± SE of three independent samples. E, Analysis of GUS expression driven by the *SCaM-1* 2.4-kb promoter-GUS (*pBI 1D1*) after pathogen and NaCl treatment.

Pathogen and NaCl-Induced Expression of *SCaM-4* Promoter-*GUS* Involves a GT-1 cis-Acting Regulatory Element

From GUS assays in Arabidopsis protoplasts containing the deletion constructs of the *SCaM-4* promoter in vivo, we identified nucleotides −1,286 to −1,065 and −858 to −728 as important elements for pathogen and NaCl responses. To test whether these regions interact specifically with nuclear proteins, we divided the 1.3-kb promoter region into five fragments (fragments A–E), as shown in Figure 5A. Each of the five double-stranded fragments was used in an initial series of electrophoretic mobility shift assays (EMSAs) with soybean nuclear extracts from W82 cells treated for 1 h with 10 mM $MgCl_2$ (control), pathogen (*Psg*), or NaCl. Fragments containing the −1,286 to −1,065 (A) and −858 to −549 (C) regions each gave one major retarded band when incubated with *Psg*- or NaCl-treated nuclear extracts (Fig. 5A). Fragment C (−858 to −549) was further subdivided into three overlapping fragments,

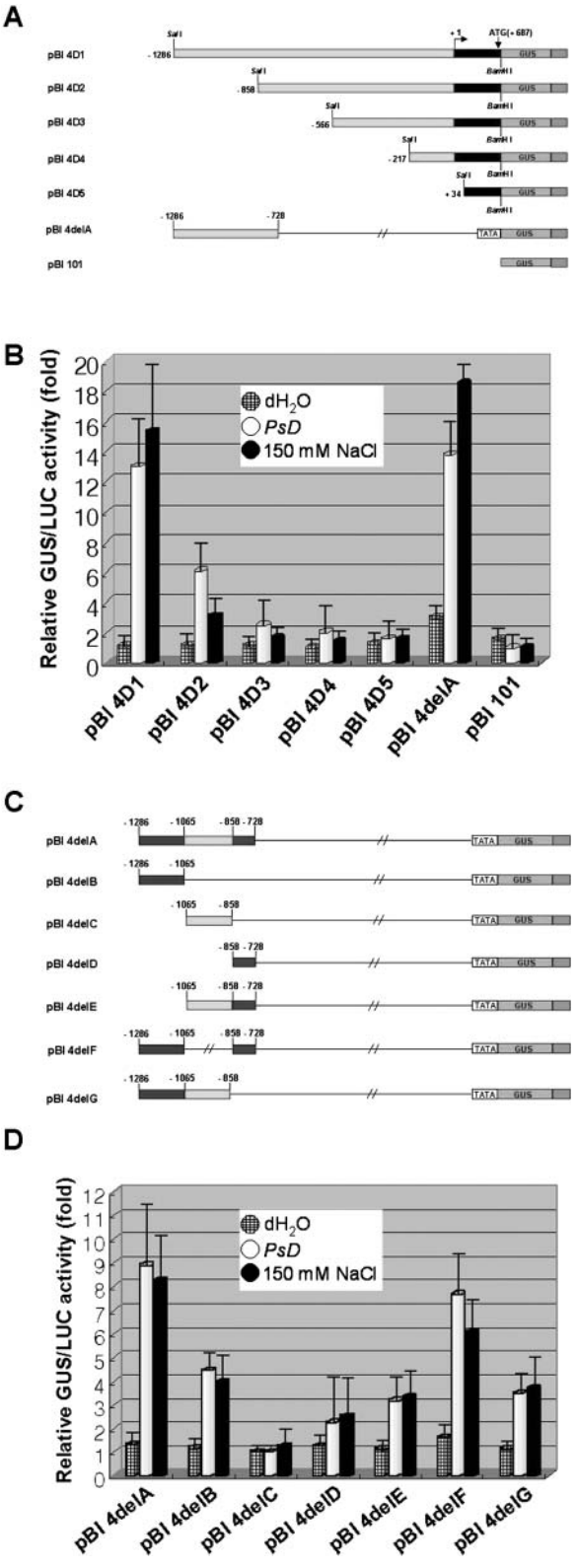

Figure 4. Quantitative fluorometric assays for GUS activity driven by various *SCaM-4* promoter deletion constructs. A, Diagram of various deletion derivatives of the *SCaM-4* promoter. Deletion end points are indicated in bp from the transcription start site (indicated with an arrow in Fig. 1). All promoter derivatives were fused to a *GUS* reporter vector, *pBI 101*. B, GUS activity of DNA constructs prepared from A in the Arabidopsis protoplast transient expression system. The transfected Arabidopsis protoplasts were treated with distilled water, *PsD*, or 150 mM NaCl, for 12 h. GUS activity was calculated relative to LUC activity. The data are presented as mean ± SE of three independent samples. C, Diagram of deletion constructs in the −1,286 to −728 bp region of the *SCaM-4* promoter. All deletion fragments were ligated upstream of the TATA minimal promoter of the *pDel. 151-8* vector. D, GUS activity of DNA constructs prepared from C in an Arabidopsis protoplast transient expression system. The transfected Arabidopsis protoplasts were treated with distilled water, 150 mM NaCl, or *PsD* for 12 h. GUS activity was calculated relative to LUC activity. The data are presented as mean ± SE of three independent samples.

C-1, C-2, and C-3, and each was used in EMSAs with the nuclear extracts described above. Only the C-1 region fragment (−858 to −728) showed a strong mobility shift when incubated with *Psg*-treated nuclear extracts (Fig. 5B). The mobility shift was completely blocked by the addition of a 50-fold molar excess of unlabeled C-1 but not by an excess of unlabeled C-2 or C-3. EMSAs using the C-1 region as a probe with heat-treated (65°C, 5 min) or proteinase K-treated nuclear extracts, showed that the DNA-binding complex of the C-1 region was heat stable but sensitive to proteinase K digestion (data not shown).

To more precisely define the position of the protein binding site, we designed nine double-stranded oligonucleotides of 15 to 30 bp in length (E1–E9, Fig. 5C), which were used as C-1 competitors in EMSAs. The E4 oligonucleotide completely blocked nuclear protein binding to the C-1 fragment (Fig. 5D). Examination of the sequence of the E4 fragment revealed that it contains a GT-1 cis-element (GAAAAA). DNase I footprinting assays using the C-1 fragment and pathogen-treated nuclear extracts confirmed that the GT-1 element is indeed recognized by nuclear factors (data not shown). The involvement of the GT-1 cis-element in binding to pathogen-treated nuclear extracts was tested using a subset of oligonucleotides derived from E4 fragments (Fig. 5E). While E4-1 (TAAGAAAAA-TAA) effectively bound to pathogen treated nuclear extracts, the mutations E4-1(M1; TAA**C**AAAAATAA) and E4-1(M2; TAA**CC**AAAATAA) caused significant reductions in protein binding (Fig. 5E).

To examine whether the GT-1 cis-element of the *SCaM-4* promoter, identified by in vitro DNA binding, actually plays a role in the cellular responses to pathogen and NaCl-induction, we generated a mutant *ScaM-4* promoter (−1,286 to −728)-*GUS* construct that contains a GA to CC mutation in the GT-1 element (Fig. 6A). The *pBI 4delA* (−1,286 to −728) showed a 7- to 8-fold induction of the *GUS* reporter gene after treatment with NaCl or pathogen. However, the *pBI 4delA M2* mutant construct repeatedly showed 4- to 5-fold induction by the same treatment, approximately a 30% reduction compared to the wild type promoter (Fig. 6B). This result shows that while the GT-1 element is involved in the expression of *ScaM-4*, the −1,065 to −1,286 region also plays a role in the NaCl- and pathogen-induced expression of the *SCaM-4* gene (Fig. 5A).

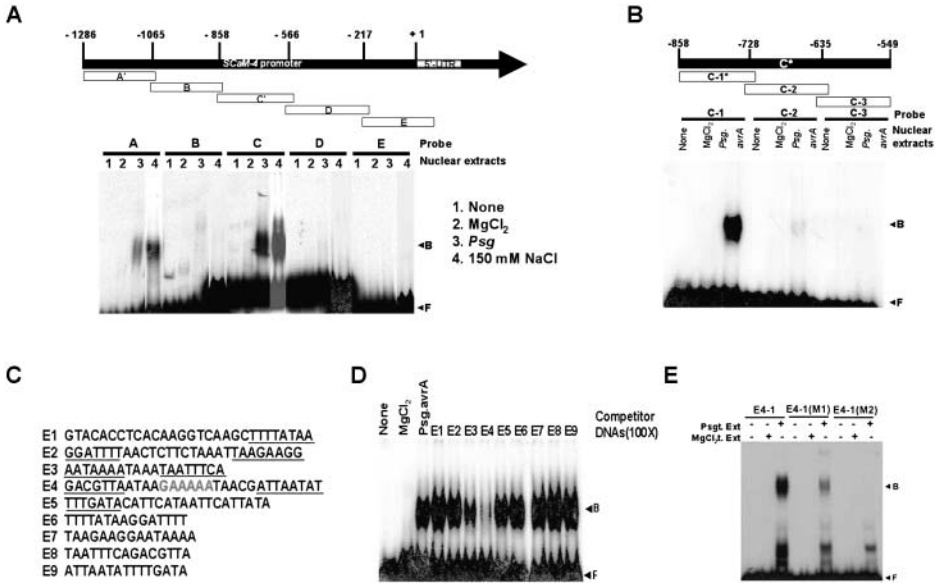

Figure 5. Identification of a GT-1 cis-element involved in *SCaM-4* gene expression in response to pathogen or NaCl. The binding reaction mixture of each experiment (20 μL) contained ^{32}P-labeled DNA probe (40 kcpm), poly[dI/dC] (2 μg), and nuclear extracts (4 μg) from W82 cells treated with $MgCl_2$ (control), pathogen (*Psg.avrA*), or 150 mM NaCl. Free (F) and protein-complexed (B) DNA fragments were separated on a 5% or 8% polyacrylamide gel and visualized by autoradiography. A, Schematic diagram of the DNA fragments of the *SCaM-4* promoter used for DNA probes in the EMSAs (upper section) and their binding patterns (lower section) with nuclear extracts from W82 soybean suspension-culture cells treated with $MgCl_2$ (control, lane 2), pathogen (*Psg*, lane 3), or 150 mM NaCl (lane 4). "None" (lane 1) represents free DNA probe. Fragment A and C that interact with nuclear factors are indicated as asterisks. B, Schematic diagram of the DNA fragments from the −858 to −549 bp region in the *SCaM-4* promoter used for DNA probes in the EMSAs and their binding patterns with nuclear extracts from W82 cells treated with $MgCl_2$ (control) or pathogen (*Psg.avrA*). "None" represents each free DNA probe. Fragment C-1 that interacts with nuclear factor is indicated as asterisk. C, Sequences of nine overlapping oligonucleotides (E1–E9) within the C-1 fragment used for competitive EMSAs. D, Competitive EMSAs using the nine oligonucleotides (E1–E9) derived from the C-1 fragment shown in C. The DNA binding reaction was performed by preincubating unlabeled competitors (E1–E9) before adding the ^{32}P-labeled C-1 fragment as a probe. A 100-fold molar excess of competitor DNA was added to each reaction mixture. E, EMSAs using synthetic oligonucleotides derived from E4. E4-1, a tetramer of TAAGAAAAATAA, containing wild-type GT-1 cis-element (underlined); E4-1(M1), a tetramer of TAA**C**AAAAATAA; E4-1(M2), a tetramer of TAA**CC**AAAATAA. Each oligonucleotide was incubated with nuclear extracts from soybean W82 cells treated with $MgCl_2$ (control) or pathogen (*Psg*).

Isolation of a Transcription Factor Interacting with the *SCaM-4* GT-1 cis-Element

As a first approach to isolate the transcription factor that interacts with the GT-1 cis-element within the *SCaM-4* promoter, we searched the complete genome of Arabidopsis. Seventeen sequences encoding trihelix DNA-binding factors (or GT transcription factors) have been found in the Arabidopsis genome (Ayadi et al., 2004). The analysis identified four GT-1 related transcription factors with a single trihelix motif: AtGT-1 (At1g13450), AtGT-4 (At3g25990), AtGT-3a (At5g01380), and AtGT-3b (At2g38250). AtGT-3a and AtGT-3b showed low homology to AtGT-1 (less than 36% sequence identity). However, both these factors contain a conserved trihelix DNA-binding domain that is also found in the N-terminal region of AtGT-1 (Fig. 7A).

The full-length cDNA clones for the three AtGT-1-related proteins (AtGT-1, AtGT-4, and AtGT-3b) were isolated by the reverse transcription (RT)-PCR method. The expression patterns of the three *AtGT-1*-related genes were examined in pathogen- and NaCl-treated Arabidopsis plants by northern-blot analysis. As shown in Figure 7B, treatment of the plants with pathogen or NaCl resulted in a rapid increase in the transcription of *AtGT-3b*. However, the expression levels of the two other AtGT-1-related transcription factors were not changed by treatment with pathogen or NaCl. Therefore, AtGT-3b was selected as a good candidate for a transcription factor which binds to the GT-1 cis-element of the *SCaM-4* promoter during the plant response to pathogen attack and NaCl stress.

The *AtGT-3b* gene has an open reading frame of 870 bp, which would encode a protein of 289 amino acids with a molecular mass of approximately 31.8 kD (Fig. 7A). The deduced amino acid sequence of *AtGT-3b* contains two different nuclear localization signal sequences, one of which corresponds to a bipartite-type nuclear localization signal within a trihelix domain (KRNKLLWEVISNKMRDK) located between amino acids 65 and 81. The other corresponds to a simian virus 40 (SV 40)-type nuclear localization signal located in the C-terminal region (KKRK) encompassing amino acids 185 to 188.

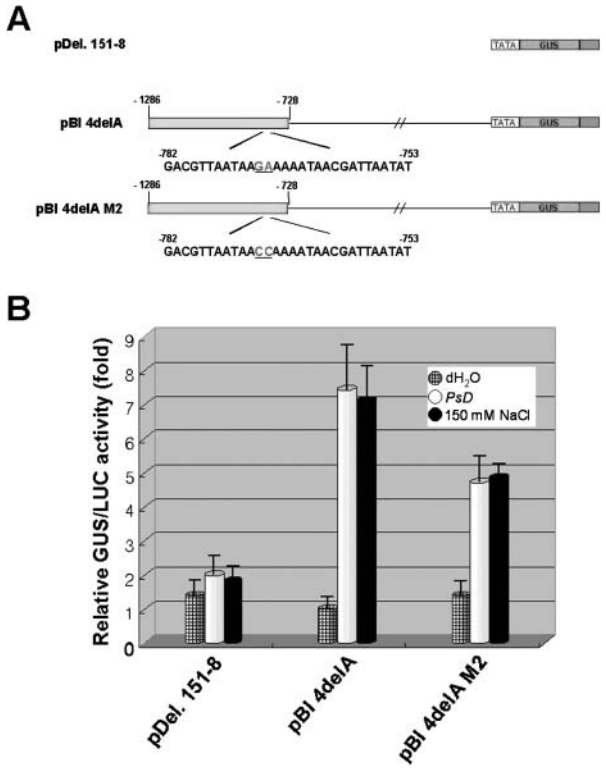

Figure 6. Effect of mutation of the GT-1 cis-element within the *SCaM-4* promoter on expression of the *SCaM-4* 2 kb promoter-*GUS* reporter gene in Arabidopsis protoplasts. A, Schematic diagram of a *SCaM-4* promoter construct of the −1,286 to −728 bp region (*pBI 4delA*) and a mutant with two mutated residues in the GT-1 cis-element (*pBI 4delA M2*). These promoters were fused to the minimal TATA promoter-*GUS* constructs. pDel.151-8 indicates the TATA-minimal promoter GUS used as control. B, The effect of mutation of the GT-1 cis-element within *SCaM-4* promoter on *GUS* expression. Transfected Arabidopsis protoplasts were treated with water, 150 mM NaCl, or *PsD* for 12 h. For normalization of transfection efficiency, the CaMV 35S promoter-LUC plasmid was cotransfected in each experiment. The data are presented as mean ± SE of three independent samples.

The AtGT-3b protein was further analyzed for interactions with the GT-1 cis-element in the *SCaM-4* promoter in vitro and in a yeast selection system. To test the binding activity of AtGT-3b to the GT-1 cis-element within the E4 fragment of the *SCaM-4* promoter, we produced a recombinant AtGT-3b protein fused to glutathione S-transferase (GST) in *Escherichia coli*. As shown in Figure 8A, the ability of the recombinant GST-AtGT-3b fusion protein to bind to the E4 oligonucleotide was validated by EMSA. The DNA binding specificity of GST-AtGT-3b was also confirmed by competition experiments (Fig. 8A). A 200-fold molar excess of unlabeled E4 oligonucleotide completely blocked E4 binding to GST-AtGT-3b (Fig. 8A, lane 9). In contrast, neither AtGT-1 nor AtGT-4 formed protein-DNA complexes under these conditions (data not shown).

The interaction of the AtGT-3b protein with the GT-1 cis-element was reconfirmed with a yeast selection system. We constructed a YM4271 yeast strain carrying integrated copies of *HIS3* and *lacZ* as dual reporter genes with four tandem repeats of the E4 fragment upstream of the TATA element. The yeast cells were then transformed with *AtGT-3b* cDNA fused to the transcriptional activation domain of yeast GAL4 (Fig. 8B). As predicted by the in vitro binding assay, the AtGT-3b protein and the E4 fragment conferred *HIS* selection in the presence of 45 mM 3-AT, a competitive inhibitor of the *HIS3* gene product (HIS3p). This result provides strong evidence for an interaction in the yeast system. In contrast, yeast cells carrying plasmids with cDNA inserts of *AtGT-1* or *AtGT-4* did not grow

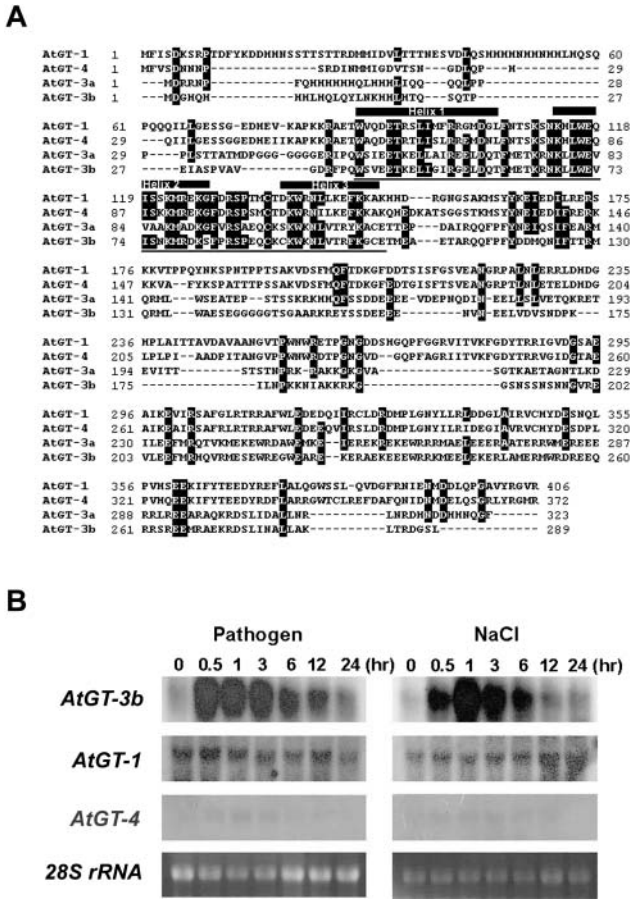

Figure 7. Alignment of the deduced amino acid sequence and northern-blot analysis of GT-1 related transcription factor genes isolated from Arabidopsis. A, Alignment of the deduced amino acid sequences of four Arabidopsis genes encoding GT-1 related factors; *AtGT-1* (At1g13450), *AtGT-4* (At3g25990), *AtGT-3a* (At5g01380), and *AtGT-3b* (At2g38250; Ayadi et al., 2004). Identical residues are indicated with black boxes. Dashes indicate the absence of residues. The trihelix-DNA binding domain is underlined. B, Expression levels of *AtGT-3b, AtGT-1*, and *AtGT-4* mRNA were examined by northern-blot analysis. Each lane was loaded with 20 μg of total RNA isolated from 4-week-old Arabidopsis plants treated with pathogen (*PsD*) or 150 mM NaCl for the indicated time periods. The blots were hybridized with ^{32}P-labeled cDNA as probes. Ethidium bromide staining of the *28S rRNA* was used to show equal loading.

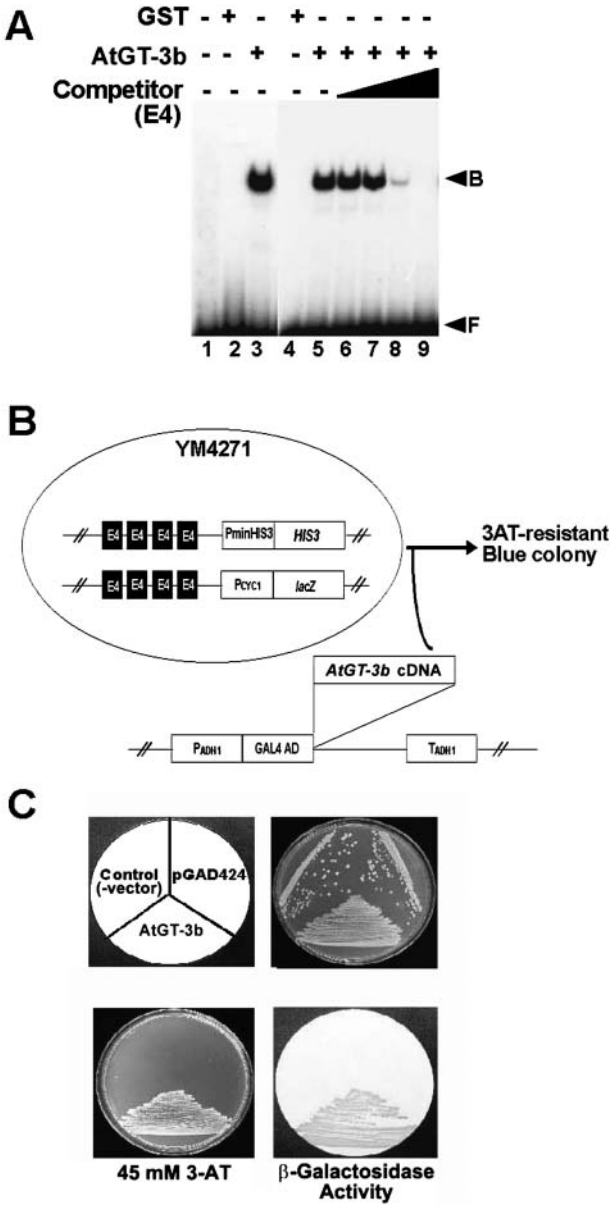

Figure 8. Interaction of AtGT-3b with the GT-1 cis-element within the E4 fragment of the *SCaM-4* promoter in vitro and in a yeast selection system. A, EMSA using bacterially produced recombinant AtGT-3b protein and E4 oligonucleotide. The ^{32}P-labeled E4 oligonucleotide was incubated in the presence or absence of the recombinant AtGT-3b protein. Lane 1 contained only the free probe. Lanes 2 and 4 contained 10 μg of bacterial extracts containing the GST protein. Lanes 3 and 5 contained 10 μg of bacterial extracts containing the GST-AtGT-3b protein. For the competitive EMSA, 10 μg of bacterial extract containing the GST-AtGT-3b protein was preincubated with 10- (lane 6), 50- (lane 7), 100- (lane 8), or 200- (lane 9) fold molar excess of unlabeled E4 oligonucleotide before the addition of the ^{32}P-labeled E4 oligonucleotide as probe. Free (F) and protein-complexed (B) E4 probes were separated on an 8% polyacrylamide gel and visualized by autoradiography. B, Strategy for detecting the interaction between the AtGT-3b protein and the GT-1 cis-element within the E4 fragment of the *SCaM-4* promoter in the yeast selection system. C, Interaction of AtGT-3b with the GT-1 cis-element within the E4 fragment as determined by the yeast selection system. The yeast strain YM4271 was transformed with the constructs indicated in the top left plate and grown in the presence of 45 mM 3-AT (a competitive inhibitor of the *HIS3* gene product). The expression of *β-galactosidase* in the colonies grown in YPD medium was determined by a filter-lift assay.

on medium lacking His in the presence of 45 mM 3-AT (data not shown).

DISCUSSION

The expression of plant *CaM* and *CaM*-like genes from a number of species is differentially regulated in response to external stimuli of both abiotic (e.g. light, gravity, heat, touch, cold, salinity, and drought) and biotic (e.g. phytohormones and pathogens) origins (Snedden and Fromm, 1998, 2001). We have previously shown that SCaM-4 and SCaM-5 are more rapidly induced by fungal elicitors or pathogens than are the SCaM-1, -2, and -3 isoforms (Heo et al., 1999). Here we have shown that the mRNA of SCaM-4 is also significantly induced in response to salt stress (Fig. 3A). Furthermore, our group has already shown that SCaM-4 activates a different pattern of CaM-dependent enzymes than SCaM-1 (Lee et al., 2000). These findings suggest that particular Ca^{2+}/CaM signaling pathways can be mediated by different CaM isoforms, which, in turn, may give plant cells the ability to have diverse cellular responses to Ca^{2+} signals. To elucidate the differences between SCaM-1 and SCaM-4 at the level of transcriptional regulation, we isolated the 5′-flanking regions of the *SCaM-1* gene (2.4 kb) and the *SCaM-4* gene (2 kb).

To further understand the upstream signaling mechanisms of *SCaM-4* gene expression in response to pathogen and NaCl signals, we analyzed the cis- and trans-acting elements involved in *SCaM-4* gene expression. Based on a report that Arabidopsis also contains *SCaM-4/-5* gene homologs (Zielinski, 2002), we prepared transgenic Arabidopsis carrying soybean *SCaM-4* 2-kb promoter-*GUS* constructs and found that GUS was expressed in the apical meristem and hypocotyl regions but not in root tissues. This is consistent with the results of a northern-blot analysis of soybean seedlings (Lee et al., 1995a). This result strongly suggests that the expression patterns of the *SCaM-4* promoter are maintained in heterologous transgenic Arabidopsis plants. The GUS activity of the *SCaM-4* promoter was enhanced about 3- to 7-fold when treated with *PsD*, glycol chitin, NaCl or the Ca^{2+}-ionophore A23187 (Fig. 3D), strongly suggesting that the *SCaM-4* promoter responds to transcriptional regulators under various environmental stress conditions.

Using EMSAs, we examined protein-DNA interactions with the *SCaM-4* promoter and the soybean nuclear extracts and precisely identified the cis-acting elements involved in plant responses to pathogen attack and NaCl stress. Two promoter regions, −1,286 to −1,065 (A) and −858 to −566 (C), were critical for the *SCaM-4* promoter binding of nuclear extracts prepared from pathogen- or NaCl-treated soybean suspension

culture cells (W82). This result is in good agreement with the data obtained from the in vivo transient expression assay using Arabidopsis protoplasts. From a DNase I footprinting analysis and EMSA using synthetic oligonucleotides, we identified a GT-1 cis-element within a subfragment of the C region, the E4 fragment, as an important element involved in *SCaM-4* gene expression (Figs. 5 and 6). Additionally, a base substitution analysis demonstrated that GA in the GT-1 cis-element (5′-GAAAAA-3′) is required for binding to nuclear factor(s) in response to pathogen- or salt-induced stress. Together, these data imply that a GT-1-related transcription factor positively regulates *SCaM-4* gene expression under the conditions of pathogen attack or NaCl stress.

The GT-1 cis-element, one of many cis-acting DNA elements found in plants, was first identified in pea (*Pisum sativum*) as the Box II element (5′GTGTGGTTAATATG3′) in the promoter of the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit gene (*RBCS-3A*; Green et al., 1987). Depending on the promoter structures, GT-1 cis-elements can have a positive or a negative effect on transcription. One common feature found in all GT-1 cis-elements is a core sequence of four or five nucleotides, which consists of T or A preceded by one or two G nucleotides at the 5′ end. The deduced consensus core sequence is currently defined as 5′-G-Pu-(T/A)-A-A-(T/A; Zhou, 1999). It is thought that the high degeneracy of the GT-1 cis-element partly explains its diverse functions as well as its light-specific regulatory functions.

In vitro experiments have shown that the GT-1 transcription factor can interact with the TFIIA-TBP-TATA complex, suggesting that GT-1 may activate transcription through direct interactions with the minimal preinitiation complex (Le Gourrierec et al., 1999). It has also been reported that GT-1 interacts with important enhancer regions in the *Cpr* (NADPH; cytochrome P450 reductase; Lopes Cardoso et al., 1997) and *Str* (Pasquali et al., 1999) genes, which are induced by fungal elicitors and yeast extracts, respectively. In this research, we showed that the *AtGT-3b*, a GT-1-like transcription factor, was rapidly induced by pathogen and salt stress. In addition, the AtGT-3b protein specifically bound to the GT-1 cis-element within the E4 fragment of the *SCaM-4* promoter both in vitro and in a yeast selection system (Fig. 8). The induction by pathogen and NaCl stress along with specific binding to the GT-1 cis-element, both in the EMSAs and in the yeast selection system, strongly suggests that AtGT-3b is a transcription factor involved in *SCaM-4* gene expression. Our results using soybean suspension-culture cells demonstrate that an *AtGT-3b* homolog might also exist in soybean plants, where it may mediate the induction of the *SCaM-4* gene in response to pathogen or salt stress.

The data obtained from this study lead to a model in which environmental stresses induce *SCaM-4* gene expression by mediating the binding of a GT-1-like transcription factor to the GT-1 cis-element (GAAAAA) within the −858 to −728 region of the *SCaM-4* promoter. Additional binding event(s), mediated by yet to be defined trans-acting factor(s), may be required at the upstream −1,215 to −1,065 bp cis-element. Currently, we are characterizing the −1,286 to −1,065 bp region with respect to its contribution to the induction of *SCaM-4* gene transcription in response to pathogens and NaCl. Interestingly, we have found a 65 bp sequence within the −1,215 to −1,150 region that is retarded in EMSAs from pathogen- or NaCl-treated nuclear extracts (data not shown). Further investigation into the regulation of *SCaM-4* will involve characterization of other cis-elements and their cognate transcription factors. This will provide a better understanding of the roles played by DNA-protein interactions in *SCaM-4* gene expression during plant defense responses.

MATERIALS AND METHODS

Plant Materials and Bacterial and Yeast Strains

Soybean (*Glycine max*) cells (W82) were grown in suspension culture in Murashige and Skoog medium supplemented with 0.75 mg L^{-1} benzyl adenine, maintained at 25°C in the dark, and stirred at 130 rpm. Arabidopsis (ecotype Columbia) plants were used for the preparation of transgenic plants. For DNA cloning, *Escherichia coli* XL1-Blue *MRF′* and DH 5α (Stratagene, La Jolla, CA) were used as bacterial strains. The expression of the GST-fusion protein was performed in *E. coli*, BL21 (pLys S) DE3. The yeast strain YM4271 (*MATa*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *leu2-3*, *112*, *trp1-901*, *tyr1-501*, *gal4-Δ512*, *gal80-Δ538*, *ade5*::*hisG*) was used for reporter vector integration in the yeast selection system (Wilson et al., 1991; Liu et al., 1993).

Isolation of the 5′ Upstream Sequences of *SCaM-4* and *SCaM-1*

The 5′ upstream region of the *SCaM-4* gene was obtained using the Universal Genome Walker kit (CLONTECH, Palo Alto, CA). First, separate aliquots of soybean genomic DNA were digested with five blunt-end restriction enzymes (*Eco*RV, *Sca*I, *Dra*I, *Pvu*II, and *Stu*I), and ligated to Genome Walker adaptors. Primary PCR was performed using adaptor primer 1 (AP 1) and a *SCaM-4* cDNA specific primer (5′-GTCCTCGGTAAGAAACAGACTCATCC-3′). The second PCR was performed using adaptor primer 2 (AP 2) and the same *SCaM-4* cDNA specific primer. The amplified PCR products were examined on an agarose gel, and subcloned into the *pGEM T-Easy* vector. After sequencing of overlapping deletion products using the Erase-A-Base kit (Promega, Madison, WI), the 5′ upstream region of the 2-kb *SCaM-4* gene was connected by asymmetric PCR.

The upstream region of the *SCaM-1* gene was isolated by screening a soybean (*Glycine max* cv Williams 82) genomic DNA library constructed in bacteriophage λ Fix II (Stratagene, Heidelberg, Germany). A 2.4-kb internal *Eco*RI fragment that hybridized to the *SCaM-1* cDNA probe was subcloned into the multiple cloning site of the pBluescript II SK (−) vector (Stratagene, La Jolla, CA). A sequential series of overlapping deletions from both ends were made using the Erase-A-Base kit (Promega) according to the manufacturer's protocol and sequenced.

RNA Gel-Blot Analysis

Various tissues of transgenic Arabidopsis plants and W82 cells collected on filter papers (Whatman, Clifton, NJ) by vacuum filtration were used for isolation of total RNA as described (Park et al., 2002). RNA gel-blot analyses were carried out as described previously (Sambrook et al., 1989). Gene-specific probes were made from the 3′-untranslated regions of each cDNA, using a 276-bp *Hae*III/*Xho*I fragment of *SCaM-1* and a 347-bp *Eco*RI/*Xho*I fragment of *SCaM-4* cDNA. The ^{32}P-labeled 1.87-kb *GUS* cDNA and three *GT-1*–related cDNA clones of Arabidopsis were used for hybridization.

Construction of Promoter Deletion Derivatives of the *SCaM-4* Genes Fused to a *GUS*

For promoter analysis in transgenic plants and Arabidopsis protoplasts, *SCaM-4* promoter-*GUS-NOS* cassette constructs were used. Deleted promoters were cloned into the *Sal*I/*Bam*HI sites of the binary vector *pBI 101* (CLONTECH). The following deletion derivatives were cloned into the *Sal*I/*Bam*HI site of the binary vector, *pBI 101*: a *SCaM-1* promoter containing a fragment from −2,230 to +84, named *pBI 1D1*, and a *SCaM-4* promoter containing various fragments (−1,286 to +750, *pBI 4D1*; −858 to +750, *pBI 4D2*; −566 to +750, *pBI 4D3*; −217 to +750, *pBI 4D4*; and +34 to +750, *pBI 4D5*). For characterization of the promoter in more detail, the −1,286 to −728 bp region of the *SCaM-4* promoter was subdivided into six different fragments and ligated into the region upstream of the TATA-containing minimal promoter of the *pDel*. *151-8* vector (Sundaresan et al., 1995). These fragments were as follows: −1,286 to −728, named *pBI 4delA*; −1,286 to −1,065, *pBI 4delB*; −1,065 to −858, *pBI 4delC*; −858 to −728, *pBI 4delD*; −1,065 to −728, *pBI 4delE*; −1,286 to −1,065, and −858 to −728, *pBI 4delF*; and −1,286 to −858, *pBI 4delG*. The *SCaM-1/-4* promoter-*GUS* and *SCaM-4* promoter deletion-*GUS* constructs were propagated in *E. coli*, XL1-Blue *MRF′* (Stratagene). The plasmid constructs were isolated using CsCl gradients (Ausubel et al., 1987). The structures of all constructs were confirmed by sequencing or restriction digest mapping.

Arabidopsis Protoplast Transfection and Fluorometric GUS Assays

Isolation of Arabidopsis protoplasts and polyethylene glycol-mediated DNA transfection were performed as described previously (Abel and Theologis, 1994). Typically, 5 mL of Arabidopsis protoplast suspension (5×10^6 per mL) was cotransfected with 15 μg of a test construct and 5 μg of a CaMV 35S promoter-LUC control vector, *pJD 300*. The transfected Arabidopsis protoplasts were incubated in W5 solution under various conditions for 12 h in the dark at room temperature. Using the transfected protoplasts, GUS assays were performed fluorometrically with the substrate 4-methyl umbelliferyl glucuronide as described (Jefferson et al., 1987). LUC assays were performed using the Promega LUC assay system according to the manufacturer's instructions. In order to normalize for transfection efficiency, the CaMV 35S promoter-*LUC* plasmid was cotransfected in each experiment.

Plant Transformation and Histochemical GUS Assays

To generate transgenic Arabidopsis plants (ecotype Columbia), *pBI 4D1* and *pBI 101* plasmids were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation, and Arabidopsis plants were transformed by vacuum infiltration (Clough and Bent, 1998). Histochemical GUS staining of transgenic Arabidopsis plants was performed according to a previously described method (Lee et al., 1995b).

Pathogen and Various Chemical Treatments

Different pathogenic bacteria (10^8 cfu/mL) were used for infection of the two plant species. *Pseudomonas syringae* pv *glycinea carrying avrA* (*Psg*) was used for infection of soybean suspension culture cells (W82), and *P. syringae* pv *tomato DC3000* (*PsD*) was used for Arabidopsis plants. Bacteria grown in liquid King's medium were washed and resuspended in 10 mM $MgCl_2$ (King et al., 1954). For northern-blot and GUS fluorometric assays, W82 cells and Arabidopsis protoplasts were treated with 0.05% glycol chitin, 150 mM KCl, 300 mM mannitol, 150 mM NaCl, Ca^{2+}+A23187 (25 μM Ca^{2+} ionophore A23187 plus 5 mM $CaCl_2$), 2 mM hydrogen peroxide (H_2O_2), 2 mM salicylic acid, 100 μM jasmonic acid, or 100 μM ABA.

Preparation of Soybean Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from W82 cells that had been treated with $MgCl_2$ (mock inoculation), pathogen, or 150 mM NaCl for about 1 h using a procedure described previously (Nagao et al., 1993). EMSAs were performed as described (Hong et al., 1995) using [^{32}P]-labeled double-stranded DNA probes. The assay mixtures contained soybean nuclear extracts (4 μg of protein) or *E. coli* extracts (10 μg of protein), 4×10^4 cpm of each binding probe, 2 μg of poly[dI/dC], 20 mM HEPES-KOH (pH 7.9), 0.5 mM DTT, 0.1 mM EDTA, 50 mM KCl, and 15% glycerol in a 20 μL reaction volume. The mixtures were incubated at room temperature for 15 min and electrophoresed on 5% or 8% polyacrylamide gels in 0.5× TBE buffer. Subsequently, the gels were dried and exposed to x-ray films.

RT-PCR

Total RNA was extracted from the pathogen- or 150 mM NaCl-treated samples of 4-week-old Arabidopsis seedlings. Total RNA (5 μg) was reverse-transcribed in a 50-μL reaction volume with 10 ng of oligo$(dT)_{17}$ primer using Superscript RTase according to the manufacturer's protocols (BRL Life Technologies, Grand Island, NY). The following oligonucleotides were synthesized for amplification of *GT-1*–related cDNAs in Arabidopsis (Ayadi et al., 2004): *AtGT-1* (At1g13450; upstream primer: 5′-GCGTCGACAATGTTCATTTCCGACAAATCTCGT-3′, downstream primer: 5′-CCGCTCGAGTCATCTCACACCTCGATACACAGC-3′), *AtGT-3b* (At2g38250; upstream primer: 5′-CGCGGATCCATGGATGGACATCAGCATCATCAC-3′, downstream primer: 5′-CCGCTCGAGTTAGAGGGAACCATCTCTAGTAAG-3′), and *AtGT-4* (At3g25990; upstream primer: 5′-CGCGGATCCATGTTTGTTTCCGATA ACAACAAT-3′, downstream primer: 5′-CCGCTCGAGTCATCTCATTCCTCTGTA TAAGCG-3′). After a standard PCR of 30 cycles, aliquots were run on an agarose gel. Each fragment of accurate size was cloned into a *pGEM-T Easy* vector (Promega) and identified by DNA sequencing.

Expression of AtGT-3b in *E. coli* and Yeast Selection Analysis

An *AtGT-3b* cDNA fragment was prepared by PCR and cloned into the *Bam*HI and *Xho*I sites of the *pGEX-2T-linker* I vector (Amersham Pharmacia Biotech, Uppsala). Using *E. coli* strain BL21 (pLys S) DE3, GST::AtGT-3b was overexpressed, and the bacterial supernatant was used for gel mobility shift assays. For analysis in the yeast selection system, construction of reporter plasmids and selection of the yeast reporter strain were performed according to the manufacturer's protocol (CLONTECH). To generate the AD-fused *AtGT-3b* cDNA construct, a *Bam*HI/*Pst*I fragment of *AtGT-3b* cDNA was ligated into the *pGAD424* vector. Positive interactions were verified by judging yeast growth on SD medium containing 45 mM 3-AT and assaying for β-galactosidase.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY052528 for *SCaM-1* promoter and AY052527 for *SCaM-4* promoter.

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