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Molecules and Cells

Functional Analysis of the Stress-Inducible Soybean Calmodulin Isoform-4 (*GmCaM-4*) Promoter in Transgenic Tobacco Plants

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The transcription of soybean (*Glycine max*) calmodulin isoform-4 (*GmCaM-4*) is dramatically induced within 0.5 h of exposure to pathogen or NaCl. Core *cis*-acting elements that regulate the expression of the *GmCaM-4* gene in response to pathogen and salt stress were previously identified, between -1,207 and -1,128 bp, and between -858 and -728 bp, in the *GmCaM-4* promoter. Here, we characterized the properties of the DNA-binding complexes that form at the two core *cis*-acting elements of the *GmCaM-4* promoter in pathogen-treated nuclear extracts. We generated GUS reporter constructs harboring various deletions of approximately 1.3-kb *GmCaM-4* promoter, and analyzed GUS expression in tobacco plants transformed with these constructs. The GUS expression analysis suggested that the two previously identified core regions are involved in inducing *GmCaM-4* expression in the heterologous system. Finally, a transient expression assay of *Arabidopsis* protoplasts showed that the *GmCaM-4* promoter produced greater levels of GUS activity than did the *CaMV35S* promoter after pathogen or NaCl treatments, suggesting that the *GmCaM-4* promoter may be useful in the production of conditional gene expression systems.

INTRODUCTION

Calcium (Ca^{2+}) is a second messenger for a variety of fundamental and specialized cellular activities in plants, mediating responses to both abiotic (e.g., red light, low temperature, touch, hypoxia and gravity) and biotic (e.g., phytohormones and pathogens) stress factors (Bush, 1995). Changes in $[Ca^{2+}]_{cyt}$ (i.e., free cytoplasmic calcium concentration) in plant cells are deciphered by diverse intracellular Ca^{2+}-binding proteins that convert the signal into a wide range of biochemical responses (Chin and Means, 2000). The best-characterized family of Ca^{2+}-binding proteins in plants is calmodulin (CaM), and other families of Ca^{2+}-binding proteins in plants include the Ca^{2+}-dependent protein kinases (CDPKs) (Harmon et al., 2000) and the annexins (Hofmann et al., 2000; Lim et al., 1998).

The ubiquitously expressed CaM is highly conserved among all eukaryotes, and is an acidic protein containing a characteristic EF-hand. Its 148 amino acids are arranged in two globular domains that are connected by a long flexible helix, which participates in signal transduction (Cheung, 1980; O'Neil and DeGardo, 1990). In plants, multiple CaM genes have been identified in *Arabidopsis* (Ling et al., 1991), potato (Takezawa et al., 1995), wheat (Yang et al., 1996), tobacco (Yamakawa et al., 2001), and soybean (Lee et al., 1995a). Expression of each CaM isoform is differentially induced in response to external stimuli of both abiotic and biotic origin (Snedden and Fromm, 2001). In addition, CaMs are the primary intracellular Ca^{2+}-sensors or -adaptors that transduce Ca^{2+} signals by modulating the activities of their target protein effectors. More than 30 CaM-binding proteins have been identified as enzymes, including metabolic enzymes, or as transcription factors, ion channels, pumps, and structural proteins (Jeong et al., 2007; Kao et al., 2000; Lee et al., 1999; Liao et al., 1996; Zymanski et al., 1996).

We have reported five genes encoding CaM isoforms (Gm CaM-1 through -5) from soybean and divided these CaM isoforms into two groups, namely a conserved group (GmCaM-1 through -3) and a divergent group (GmCaM-4 and -5), on the basis of their primary structures (Lee et al., 1995a). The two isoforms in the divergent group exhibit only 78% identity with GmCaM-1, and are the most divergent isoforms of CaM reported in the plant or animal kingdoms thus far (Lee et al., 1995a; Yamakawa et al., 2001). The divergent GmCaM isoforms are known to be involved in the plant disease resistance response, and were shown to increase the expression of systemic acquired resistance (SAR) genes via a salicylic acid-independent manner in tobacco and *Arabidopsis* transgenic plants (Heo et al., 1999; Park et al., 2004a). *Arabidopsis* plants heterologously expressing GmCaM-4 exhibited salt tolerance by up-regulating a MYB transcription factor that regulated the

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accumulation of high levels of proline (Yoo et al., 2005). In addition, GmCaM-1 and GmCaM-4 differ in their ability to activate target enzymes, and have strikingly different Ca^{2+} concentration requirements for activating target enzymes (Cho et al.,1998; Kondo et al., 1999; Lee et al., 2000).

Although CaM has been well characterized in a variety of eukaryotes, the *cis*- and *trans*-acting elements that modulate CaM gene expression are poorly defined. We recently reported that two regions of the *GmCaM-4* promoter (i.e., the A2 region, spanning -1207 to -1128 bp; and the C1 region, spanning -858 to -728 bp) contain *cis*-elements that are key for the expression of the *GmCaM-4* gene following pathogen or salt stress. The -1207 to -1128 bp region of the *GmCaM-4* promoter has a 30-nt A/T-rich *cis*-acting element that specifically binds to two transcriptional regulators, GmZF-HD1 and GmZF-HD2 (Park et al., 2007), whereas the -858 to -728 bp region has a GT-1 *cis*-acting element that interacts with a GT-1 like transcription factor (Park et al., 2004b).

Here, we report the properties of the DNA-binding complexes that form at the two promoter regions described above in pathogen-treated nuclear extracts. We also provide evidence, in a heterologous expression system, that these two regions of the *GmCaM-4* promoter contain *cis*-elements that are required for the expression of *GmCaM-4* in response to pathogen and salt stress. In addition, analyses of promoter:*GUS* fusions transiently expressed in *Arabidopsis* showed that the *GmCaM-4* promoter caused greater levels of GUS expression in response to pathogen and salt stress as did the *CaMV35S* promoter. Taken together, our results suggest that the *GmCaM-4* promoter may be used to develop transgenic plants with an increased tolerance to environmental stresses.

MATERIALS AND METHODS

Plant materials and bacterial strains

Soybean suspension cells (*Glycine max* L. cv. Williams 82; W82) were cultured in MS medium supplemented with 0.75 $mg.L^{-1}$ benzyl adenine and maintained at 25°C in the dark with shaking at 130 rpm. Tobacco (*Nicotiana tabacum* cv. Xanthi) was used in the stable transformation experiments, and *Arabidopsis thaliana* (ecotype Columbia) protoplasts were used in the transient assays. The strains of bacterial pathogen were used according to the plant species: soybean suspension culture cells were inoculated with *Pseudomonas syringae* pv. *glycinea* carrying *avrA* (*Psg*), *Arabidopsis* plants were inoculated with *Pseudomonas syringae* pv. *tomato DC3000* (*PsD*), and tobacco plants were inoculated with *Pseudomonas syringae* pv. *tabaci* (*Pst*). These bacteria were grown in liquid King's medium B supplemented with the appropriate antibiotics (King et al., 1954). Bacterial cultures were washed and resuspended in 10 mM $MgCl_2$, and bacterial density was determined by absorbance at OD_{600} nm. Plant leaves were inoculated with bacteria, at a final concentration of 10^8 colony-forming units/ml, via vacuum infiltration (Katagiri et al., 2002). For DNA cloning, the *Escherichia coli* XL1-Blue *MRF'* and DH5α bacterial strains were used (Stratagene, USA).

Genomic PCR

Transgenic tobacco plants were identified by genomic PCR using the following oligonucleotide primers designed to amplify the 5′-*UTR* region of the *GmCaM-4* gene: upstream primer, 5′-AAGCAACCAGCTAGCGTGGTCTTTGGG-3′; and downstream primer, 5′-GGTGTATTTGTCTTTCAAACTTCAACC-3′. After a standard PCR of 30 cycles, aliquots were fractionated on an agarose gel.

Construction of *GmCaM-4* promoter deletions fused to the GUS reporter gene

For the promoter analysis in transgenic tobacco plants, constructs containing the *GmCaM-4* promoter-*GUS-nos* cassette were used. The constructs were produced according to a previously described method (Park et al., 2004b), and they were transformed into and propagated in *E. coli*, XL1-Blue *MRF'* (Stratagene). The plasmid constructs were isolated by equilibrium centrifugation on CsCl gradients containing ethidium bromide (Ausubel et al., 1987). All constructs were confirmed by sequencing or enzyme mapping.

Preparation of soybean nuclear extracts and electrophoretic mobility shift assay (EMSA)

Soybean nuclear extracts were prepared from soybean suspension culture cells (W82) that had been treated with 10 mM $MgCl_2$ (mock inoculation) or *Pseudomonas syringae* pv. *glycinea* (pathogen treatment) for about 1 h, using a procedure described previously (Nagao et al., 1993). Protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad), and the extracts were stored at -70°C until used. The nuclear extracts (4 μg of protein) were pre-incubated in a binding buffer [20 mM Hepes (pH 7.9), 0.5 mM DTT, 0.1 mM EDTA, 2 μg of poly [dI/dC], 50 mM KCl, and 15% glycerol] for 10 min at room temperature, and then incubated with 40 Kcpm of end-labeled probes for 20 min at room temperature, before being subjected to electrophoresis on non-denaturing 5% polyacrylamide gels in 0.5× TBE buffer. The gels were subsequently dried and exposed to X-ray film.

Plant transformation and histochemical GUS assays

To generate transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc), constructs containing various deletions of the *GmCaM-4* promoter fused to the *GUS* reporter gene were introduced into *Agrobacterium tumefaciens* GV3101, and transgenic tobacco plants were generated by the leaf disc transformation method (Horsch et al., 1988). Kanamycin-resistant transgenic tobacco plants were selected on medium containing 100 μg/ml kanamycin and 250 μg/ml cefotaxime, and shoots were rooted in medium containing 100 μg/ml kanamycin. Approximately 20 independent plants transformed with each promoter deletion were selected. Rooted plantlets were transferred to soil and maintained at 25°C during the day and 22°C during the night, on a 16 h photoperiod, and with 65% relative humidity. The R_2 progeny of the transgenic tobacco plants were used in these experiments. Histochemical GUS staining was performed on the transgenic plants using a method described by Jefferson et al. (1987). After 12 h of pathogen (*Pst*) or NaCl (150 mM) treatment, leaf disks of transgenic tobacco plants were incubated overnight at 37°C in GUS staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) and 50 mM phosphate (pH 7.0) (Lee et al., 1995b).

Transient expression assay with *Arabidopsis* protoplasts

Arabidopsis protoplasts were prepared from whole seedlings, and polyethylene glycol-mediated DNA transformation was performed, as described previously (Abel and Theologis, 1994). The transient expression assay was performed as described (Park et al., 2004b).

RESULTS

Construction of deletion fragments of the *GmCaM-4* promoter

As a first step to studying the regulatory mechanism controlling

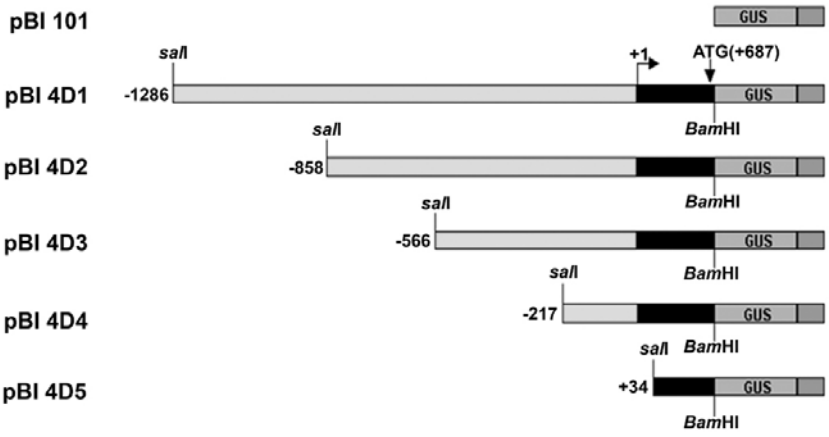

Fig. 1. Diagram of the deletion derivatives of the *GmCaM-4* promoter used to transform tobacco plants. Deletion end points are indicated in bp from the transcription start site, which was determined by the primer extension experiment. All promoter derivatives were fused to the 5′ end of *GUS* in the reporter vector, *pBI 101*.

the expression of the *GmCaM-4* gene, we mapped the transcription start site of the gene using a primer extension analysis. Two long extension products, which were positioned 689 bp and 683 bp upstream of the first ATG site, were detected, suggesting heterogeneity in the mRNA 5′-ends or a premature stop in one of the reverse transcription products (data not shown). As shown in Fig. 1, the G residue corresponding to the longer extension product was taken to be the transcription start site and numbered +1. In addition, we constructed a series of 5′ deletions of the upstream region of the *GmCaM-4* promoter (Fig. 1). Each construct was stably introduced into the heterologous tobacco system, via *Agrobacterium tumefaciens* (Horsch et al., 1988).

Characterization of DNA-binding complexes

To demonstrate that the complexes originated from the interactions of A2 (i.e., -1207 to -1128 bp) and C1 (i.e., -858 to -728 bp) probes with proteins, and not with contaminants in the pathogen-treated nuclear extracts, we performed EMSAs using the A2 region and C1 region as probes. The A2 complex is heat-unstable and sensitive to proteinase K (Fig. 2A), whereas the C1 complex is heat-stable, but sensitive to proteinase K (Fig. 2B), suggesting that the associations observed in pathogentreated nuclear extracts are DNA-protein complexes.

Histochemical GUS expression in transgenic tobacco plants using 5′ *GmCaM-4* promoter deletion derivatives

To identify the regions of the *GmCaM-4* promoter required for activation of the *GmCaM-4* gene in response to pathogen or NaCl stress, we monitored GUS activity in transgenic tobacco plants transformed with a series of 5′ deletion derivatives of the *GmCaM-4* promoter fused to *GUS*, in response to pathogen or NaCl treatment. Transformation of the tobacco plants was confirmed by genomic PCR, using oligonucleotides designed to detect a specific 5′-*UTR* region of the *GmCaM-4* promoter (Fig. 3C-II). The R_2 progeny of the transgenic tobacco plants were treated with pathogen (*Pst*) or NaCl for 12 h and then subjected to GUS histochemical staining (Figs. 3A, 3B, and 3C-I). The leaves of tobacco plants transformed with a construct harboring approximately 1.3-kb *GmCaM-4* promoter fused to *GUS* (i.e., *pBI 4D1*) expressed GUS in all regions in response to pathogen or NaCl treatment. In contrast, the leaves of transgenic tobacco plants carrying the promoterless-*GUS* construct (i.e., *pBI 101*) did not show GUS expression. In addition, only the *pBI 4D1* (up to -1286 bp) and *pBI 4D2* (up to -858 bp) constructs resulted in GUS activity upon NaCl treatment (Fig. 3C-I) or exposure to the *Pst* pathogen (data not shown). These data

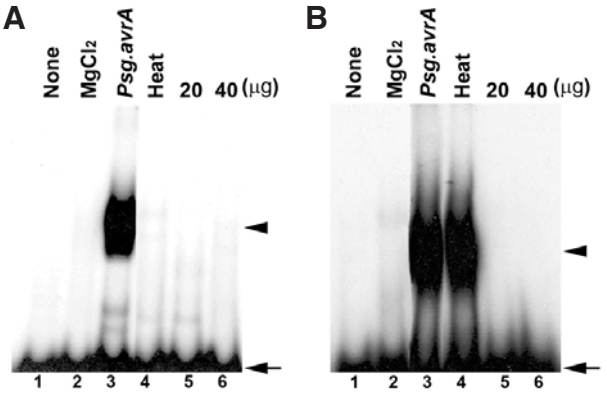

Fig. 2. Properties of binding complexes formed at the A2 and C1 regions of the *GmCaM-4* promoter in nuclear extracts treated with pathogen. The effect of pre-treating the nuclear extract with incubation at 65°C for 5 min (lane 4) or with 20 μg (lane 5) or 40 μg (lane 6) of proteinase K, on the mobility of the labeled A2 (-1207 to -1128 bp, A) and C1 (-858 to -728 bp, B) fragments, as compared with the mobility of reference fragments in the absence of nuclear extract (lane 1) or in the presence of nuclear extracts treated with $MgCl_2$ (lane 2) or *Psg. avrA* (*Pseudomonas syringae* pv. *glycinea carrying avrA*) (lane 3), as controls. (A) Nuclear extract binding pattern of the A2 fragment. (B) Nuclear extract binding pattern of the C1 fragment.

were consistent with the results of transient assays in *Arabidopsis* protoplasts (Park et al., 2004b).

Comparison of *GmCaM-4* and *CaMV35S* promoter activities in response to stress

After demonstrating that the full-length *GmCaM-4* promoter-GUS construct produces a high level of GUS activity in transgenic tobacco plants, we compared the ability of approximately 1.3-kb *GmCaM-4* promoter to drive expression of GUS in response to pathogen or salt stress with that of the cauliflower mosaic virus (*CaMV*) *35S* promoter. To do this, we produced *Arabidopsis* leaf mesophyll protoplasts harboring the GUS reporter plasmid alone (*pBI 101*), or the GUS reporter plasmid containing the *CaMV35S* promoter (*pBI 121*), or the *GmCaM-4* promoter (*pBI 4D1*) fused upstream of and in-frame with the *GUS* gene (Fig. 4A). To compare the GUS activity produced by the *GmCaM-4* promoter with that produced by the *CaMV35S* promoter in response to pathogen or salt stress, each construct was co-transfected with the *CaMV35S* promoter-Luciferase vector, *pJD300*, into protoplasts

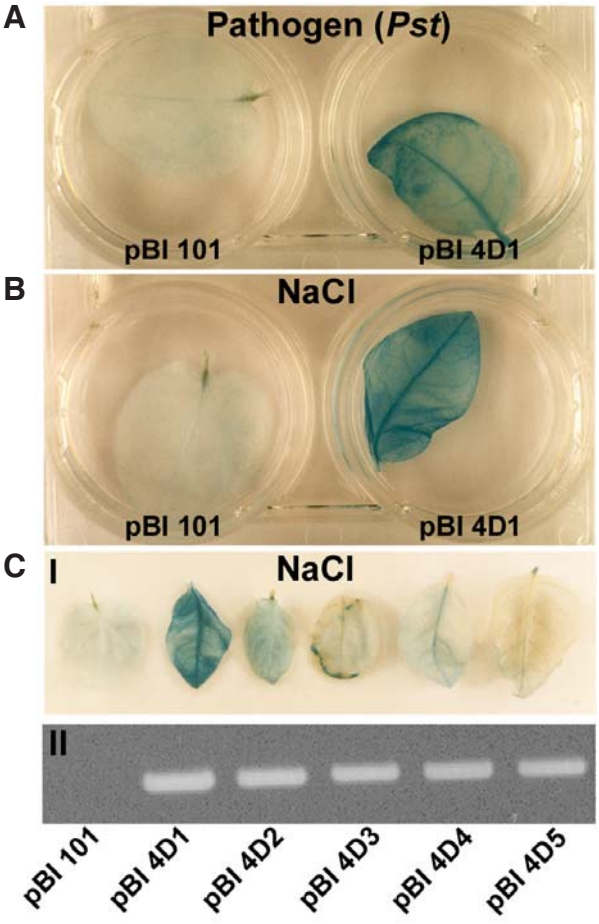

Fig. 3. Stress response in leaves of transgenic tobacco plants carrying a series of 5′-deletions of the *GmCaM-4* promoter in a GUS reporter construct after pathogen or NaCl treatment. GUS activity in the leaves of transgenic tobacco plants carrying the *pBI 101* (vector control) or *pBI 4D1* (up to -1286 bp of *GmCaM-4* promoter) construct after 12 h of pathogen (*Pseudomonas syringae* pv. *tabaci*; *Pst*) (A) or 150 mM NaCl (B) treatments. (C) I, GUS activity in the leaves of transgenic tobacco plants carrying the vector control, *pBI 101,* or a series of 5′ deletions of the *GmCaM-4* promoter, after 12 h of NaCl treatment. II, Identification of transgenic tobacco plants containing specific regions of the *GmCaM-4* 5′-untranslated region, using genomic PCR.

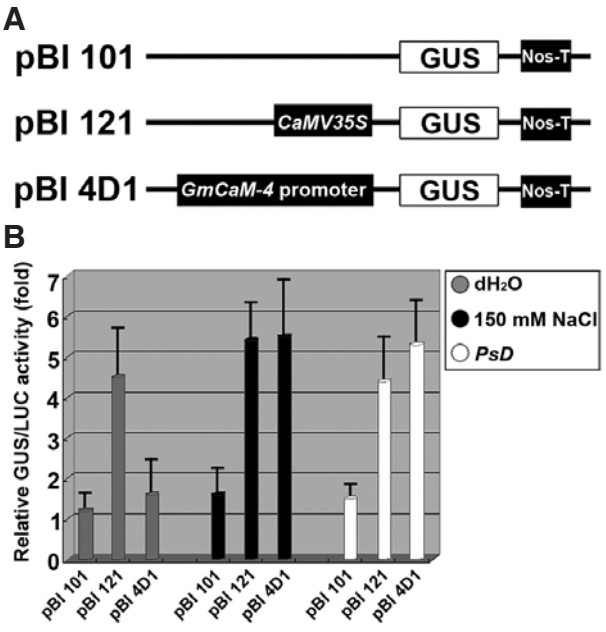

Fig. 4. Comparison of the *GmCaM-4* promoter activity with that of the *CaMV35S* promoter after pathogen or NaCl treatment. (A) Schematic representation of the constructs used for the transient expression assays in *Arabidopsis* protoplasts. (B) Fluorometric analysis of GUS activity in *Arabidopsis* protoplasts transiently expressing the DNA constructs presented in (A) along with the *CaMV35S* promoter-Luciferase vector, *pJD300*. The transfected *Arabidopsis* protoplasts were treated with distilled water (control), 150 mM NaCl, or *PsD* for 12 h. GUS activity was calculated relative to LUC activity. The data are presented as the mean ± SE of three biological replicates.

prepared from *Arabidopsis* leaves. The transfected protoplasts were treated with pathogen or 150 mM NaCl for 12 h, and then the induced GUS and LUC activities were compared. Whereas GUS activity driven by the *GmCaM-4* promoter was enhanced approximately four-fold in response to pathogen or NaCl (150 mM) treatment (Fig. 4B), the GUS activity driven by the *CaMV35S* promoter (*pBI 121*) was about the same as that in the control treatment, where *Arabidopsis* protoplasts were treated with distilled water instead of NaCl or the pathogen. These results demonstrate that the *GmCaM-4* promoter may potentially be used to develop transgenic plants with an increased tolerance to environmental stresses.

DISCUSSION

Plants possess excellent flexibility in modulating their metabolic processes and growth patterns in response to environmental stimuli. Both Ca^{2+} and Ca^{2+} sensors are key components of the signal transduction pathways that are triggered in response to stimuli. Moreover, the complexity and multiplicity of the Ca^{2+}/CaM messenger system are probably key factors in producing the variety of morphogenic and developmental responses exhibited by plants. Although CaM is one of the most highly conserved proteins in higher eukaryotes, studies in plants revealed the presence of multiple CaM isoforms in single organisms (Gawienowski et al., 1993; Ling et al., 1991; Roberts and Harmon, 1992).

We previously cloned five CaM isoforms (GmCaM1-5) from soybean (*Glycine max*) (Lee et al., 1995a). In addition, we found that the *GmCaM-4* gene is specifically induced upon pathogen or NaCl treatment (Park et al., 2004b). In previous study, we used primer extension analysis to show that the *GmCaM-4* cDNA contained an unusually long untranslated leader sequence of 600 bp (Park et al., 2004b). The majority of eukaryotic 5′-untranslated sequences are less than 100 nucleotides long, and only a quarter of mRNAs are between 100 and 300-bp long (Kozak, 1987). Long leader sequences in mammalian systems have been reported to enhance translation in response to various stresses (Kozak, 1988). Interestingly, the leader sequence of *GmCaM-1,* which is not induced by pathogen and salt stress, is only 70-bp long (data not shown). The long leader sequence of *GmCaM-4* may thus facilitate the translation of the *GmCaM-4* gene, resulting in higher expression levels in response to various stresses.

Tobacco plants transformed with the *GmCaM-4* promoter-*GUS* construct demonstrated GUS activity upon pathogen or NaCl treatment (Fig. 3). A previous report showed that a *GmCaM-4*-specific antibody immunologically cross-reacted with a protein (~17-kDa) from *Arabidopsis* and tobacco extracts suggests the presence of divergent CaMs in a wide variety of plant species (Lee et al., 1995a). In addition, it has been reported that *Arabidopsis* contains *GmCaM-4/-5* gene homologs (Zielinski, 2002). These results strongly suggest that the expression patterns of the *GmCaM-4* promoter can be maintained in transgenic tobacco plants.

In addition, both the A2 (i.e., -1207 to -1128 bp) and C1 (i.e., -858 to -728 bp) regions of the *GmCaM-4* promoter formed tight complexes when soybean nuclear extracts were challenged with pathogen or NaCl treatment (Fig. 2; Park et al., 2004b). By analyzing GUS activity in tobacco plants transformed with a series of GUS reporter plasmids harboring various 5′ deletions of the *GmCaM-4* promoter, we demonstrated that only promoter fragments that contained both the A2 and C1 regions were able to drive *GUS* expression (Fig. 3). This result is in good agreement with EMSA data obtained from soybean nuclear extracts exposed to pathogen or NaCl treatment (Fig. 2).

The *35S* promoter of Cauliflower Mosaic Virus (*CaMV*) is the most widely used as promoter for driving strong, constitutive expression of a target gene in transgenic dicotyledonous plants (Potenza et al., 2004). The promoter generally exhibits high levels of activity in the absence of stress. Although a wide range of constitutive plant promoters has been isolated and used for the generation of transgenic plants, there is still a big demand for plant-derived promoters that strongly drive the expression of transgenes. In addition, there is a need for conditional gene expression systems that are based on inducible promoters (Aoyama et al., 1997; Kasuga et al., 1999). Tobacco and *Arabidopsis* plants transformed with *GmCaM-4* had an enhanced tolerance against pathogen and salt stress (Heo et al., 1999; Yoo et al., 2005). Therefore, we compared the ability of the *GmCaM-4* promoter with that of the *CaMV35S* promoter to drive GUS expression in *Arabidopsis* mesophyll protoplasts after pathogen or NaCl treatment (Fig. 4). In contrast to the *CaMV35S* promoter, the *GmCaM-4* promoter was markedly induced by pathogen and NaCl treatments. The *GmCaM-4* promoter is therefore another useful tool for the development of stress-tolerant transgenic plants.

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