A Calmodulin-binding/CACG Box DNA-binding Protein Family Involved in Multiple Signaling Pathways in Plants*

Received for publication, August 5, 2002, and in revised form, September 3, 2002
Published, JBC Papers in Press, September 5, 2002, DOI 10.1074/jbc.M207941200

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We reported earlier that the tobacco early ethylene-responsive gene \textit{NtER1} encodes a calmodulin-binding protein (Yang, T., and Poovaiah, B. W. (2000) \textit{J. Biol. Chem.} 275, 38467–38475). Here we demonstrate that there is one \textit{NtER1} homolog as well as five related genes in \textit{Arabidopsis}. These three genes are rapidly and differentially induced by environmental signals such as temperature extremes, UVB, salt, and wounding; hormones such as ethylene and abscisic acid; and signal molecules such as methyl jasmonate, H$_2$O$_2$, and salicylic acid. Hence, they were designated as \textit{AtSR}1–6 (\textit{Arabidopsis thaliana} signal-responsive genes). Ca$^{2+}$/calmodulin binds to all AtSRs, and their calmodulin-binding regions are located on a conserved basic amphiphilic α-helical motif in the C terminus. \textit{AtSR1} targets the nucleus and specifically recognizes a novel 6-bp CACG box (A/C/G/CACG/G/T/C). The multiple CACG cis-elements are found in promoters of genes such as those involved in ethylene signaling, abscisic acid signaling, and light signal perception. The DNA-binding domain in AtSRs is located on the N-terminal 146 bp where all AtSR-related proteins share high similarity but have no similarity to other known DNA-binding proteins. The calmodulin-binding nuclear proteins isolated from wounded leaves exhibit specific CACG box DNA binding activities. These results suggest that the \textit{AtSR} gene family encodes a family of calmodulin-binding/DNA-binding proteins involved in multiple signal transduction pathways in plants.

Plants are constantly exposed to a variety of adverse environmental conditions such as temperature extremes, UV light, salt, and pathogen attacks. Thus, plants have to endure these stresses by modulating the expression of specific genes. Regulated gene expression is one of the most complex activities in cells. It involves many transcription factors that contribute to the basal transcription machinery or mediate gene regulation in response to developmental, environmental, or metabolic cues. Based on data from the \textit{Arabidopsis} genome project, it was predicted that there would be more than 1709 transcription factor genes (about 6.7% of total 25,498 genes) that encode proteins with significant similarity to known classes of plant transcription factors classified by conserving DNA-binding domains. However, less than 10% of these factors have been genetically characterized (1).

Accumulating evidence indicates that Ca$^{2+}$/mediated signaling is involved in the transduction of physical signals such as temperature, wind, touch, light, and gravity; oxidative signals such as those arising from pathogen attacks; and hormone signals such as ethylene, abscisic acid (ABA), gibberellins, and auxin (2–7). All these signals have been shown to trigger changes in amplitude or oscillation in cytosolic free Ca$^{2+}$ level. Recently, the signal-induced nuclear free calcium changes were also observed (8). Free Ca$^{2+}$ changes are sensed by a number of Ca$^{2+}$-binding proteins that usually contain a common structural motif, the “EF-hand,” a helix-loop-helix structure (9). One of the best characterized Ca$^{2+}$-binding proteins is calmodulin (CaM), a highly conserved and multifunctional regulatory protein in eukaryotes. Its regulatory activities are triggered by its ability to modulate the activity of a certain set of CaM-binding proteins after binding to Ca$^{2+}$, and thereby generating physiological responses to various stimuli (10–15).

The CaM-regulated basic helix-loop-helix family of transcription factors was reported in mammals, where CaM inhibits the protein-DNA interaction by competing with the DNA-binding domain in certain proteins (16). In plants, TGA3, a member of a family of basic leucine zipper transcription factors, showed the Ca$^{2+}$/CaM enhanced-binding activities to CACG box (17). However, the CaM-binding property of TGA3 was not defined. We cloned and characterized an early ethylene-responsive gene (\textit{NiER1}) in tobacco that encodes for a CaM-binding protein (18). Bouche \textit{et al.} (19) reported that a \textit{Brassica} homolog \textit{Bn-CAMTA} is a CaM-binding protein with nonspecific DNA-binding activity. They also showed that one of the \textit{Arabidopsis} homologs (\textit{AtCAMTA1}) encodes a CaM-binding protein with a transcription activation domain.

The \textit{Arabidopsis} genome has one \textit{NiER1} homolog (\textit{AtSR1}) and five related genes (\textit{AtSR2}–6). Here we report that these six genes exhibit rapid and differential response to environmental stimuli such as UV, extreme temperatures, high salt concentration, and physical wounding; hormones such as ethylene and ABA; and signal elicitors such as methyl jasmonate (MJ), H$_2$O$_2$, and salicylic acid (SA). Furthermore, we demonstrate that calcium/CaM binds to a 23-mer peptide in all AtSRs that corresponds to the CaM-binding region of \textit{NiER1}. We also show

* This work was supported by United States Department of Agriculture Grant 2002-00741, National Science Foundation Grant MCB 96-3033, and National Aeronautics and Space Administration Grant NAG-10-0061. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{TM} / EBI Data Bank with accession number(s) AF506897.

The abbreviations used are: ABA, abscisic acid; CaM, calmodulin; MCP, 1-methylcyclopropene; SA, salicylic acid; GFP, green fluorescent protein; RT, reverse transcriptase; oligo, oligonucleotide; SF, subfamilies; MJ, methyl jasmonate.
that AtSR1 targets the nucleus and has the specific binding activity to a novel DNA element (A/C/G)CGCG(G/T/C), referred to as “CCGG box.”

EXPERIMENTAL PROCEDURES

Plant Materials and Treatments—Arabidopsis thaliana ecotype Columbia were grown in a 1:1 mixture of soil mix and vermiculite under a 12-h photoperiod/10-h dark at 20–22 °C in a greenhouse or growth room. The 3-week-old seedlings were subjected to various treatments.

RNA Extraction and RT-PCR

RNA isolation was performed as described (21, 22). The nuclear proteins were further purified with CaM-Sepharose column (Amersham Biosciences) according to Yang and Poo (23).

Transient Transient Assays with GFP Fusion Constructs—The full-length AtSR1 or ΔC (147–1032) or ΔN (1–146) were amplified by PCR amplification with Pfu DNA polymerase using the gene-specific primers listed in Table I. The primers were AtSR1-F5/H6 for AtSR1, AtSR1-F5/7or ΔC, and AtSR1-P8/6 for ΔN. In the 5′ of each primer, an adapter sequence of GTCTAGCGGATCC was added to create an artificial BamHI HI site. The 3′ was created in-frame fusion with the GFP reading frame. The amplified fragments were subcloned into the pBlue-script KS vector, and the DNA was sequenced from both sides for verification. These plasmids were digested with BamHI and ligated with the BamHI digested psgMPG, which has a BamHI site between cauliflower mosaic virus 35S promoter and GFP (20). psgMPG was used as a control. GFP expression was monitored by a transient assay using leaves of 3-week-old Arabidopsis seedlings. Plasmid DNA was introduced by particle bombardment using the method described by Christou (21). Seven-μm gold spheres were coated with plasmid DNA and accelerated by a 7-kV discharge toward the leaves placed on agar Petri plates. After bombardment, the leaves were kept in the dark for 24 h prior to automation. The sprayed leaves were observed under a Bio-Rad MRC 1024 confocal laser scanning system with a Nikon microscope. The leaves were directly examined on a glass slide using argon laser (488 nm) for green fluorescence.

Preparation of Nuclear Proteins—Nuclear protein extracts were prepared from 3-week-old Arabidopsis plants after the wounding treatment for 4 h or control plants that grow in normal conditions. Nuclear protein extracts were extracted from harvested samples (30 g) following protocols described by Green et al. (22). The nuclear proteins were further purified with CaM-Sepharose column (Amersham Biosciences) according to Yang and Poo (23).

The purified nuclear proteins as well as the recombinant proteins were dialyzed and concentrated with Centricon YM-50 or YM-10 (Millipore) against a nuclear extraction buffer (25 mM HEPES/KOH, 7.5, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 30 μg/ml phenylmethylsulfonyl fluoride) at 4 °C before gel retardation assays.

Gel Retardation Assays—The oligo selection procedure was performed as described in Figure 5C (24). Briefly, a pool of double-stranded random oligo molecules was labeled by primer synthesis of the random oligo OS-1 using the OS-2 primer (Table I). The labeled probe was purified by 8% non-denaturing PAGE. After gel retardation assays, the retarded DNA was eluted and labeled by PCR amplification using the OS-3 primer (Table I). The amplified probe was purified by electrophoresis and used as a probe. GFP expression was monitored by a transient assay using leaves of 3-week-old Arabidopsis seedlings. Plasmid DNA was introduced by particle bombardment using the method described by Christou (21). Seven-μm gold spheres were coated with plasmid DNA and accelerated by a 7-kV discharge toward the leaves placed on agar Petri plates. After bombardment, the leaves were kept in the dark for 24 h prior to automation. The sprayed leaves were observed under a Bio-Rad MRC 1024 confocal laser scanning system with a Nikon microscope. The leaves were directly examined on a glass slide using argon laser (488 nm) for green fluorescence.
except AtSR1. Instead, the gene-specific primers for AtSR1 were designed from the 5'-region where the differences were observed between this study and the GenBank™ prediction. The forward/reverse primers are as follows: AtSR1-A/B, AtSR2-A/B, AtSR3-A/B, AtSR4-A/B, AtSR5-A/B, and AtSR6-A/B. The actin 8 gene (AtACT8) was used as a positive internal control. The PCR primers for detection of AtACT8 mRNAs were AtACT8-A/B. All primers are listed in Table 1. Two μg of total RNA were treated with 1 unit of RNase-free DNase (Invitrogen) for 10 min at 37 °C followed by 5 min at 90 °C to inactivate the DNase. The reverse transcription was carried out using 0.5 μg of oligo(dT)8 as a primer in a 20-μl reaction mixture as described by the manufacturer (Invitrogen). The PCR was performed in a 25-μl reaction mixture containing 1 μl of reverse-transcribed cDNA as the template, two gene-specific primers (0.5 μM each), and 1.5 mM MgCl2. To maintain the amplification of the internal control and AtSRs within the exponential phase, the number of PCR cycles was adjusted to 25 cycles for each primer. The amplified products were subcloned into pCR2.1 (Invitrogen). The experiments were repeated three times. The amplified PCR products (9 μl) were electrophoresed on a 1.5% (w/v) agarose gel, stained with ethidium bromide, and scanned using an image analyzer.

Real Time PCR—The Real Time quantitative PCR was performed in the PE Biosystems GeneAmp 5700 sequence detection system using the SYBR green detection as recommended by the manufacturer. Each reaction contained 2.5 μl of the reverse transcription products, 10 μl of SYBR green dye, 200 nM dATP, dGTP, and dCTP, 400 nM dUTP, 2 mM MgCl2, 0.625 units of AmpliTaq Gold DNA polymerase; 250 nM forward and reverse primers (listed in Table 1), and 1 μl of the cDNA from reverse transcription. AtACT8 was used as an internal control. The reactions were performed in a MicroAmp 96-well plate capped with MicroAmp optical caps. The reaction mixtures were incubated at 96 °C for 5 min, and followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The data generated from the SYBR green detection were analyzed as described by Schmittgen et al. (25). Briefly, we used ΔΔCt method, in which the end point Ct is defined as the PCR cycle number that crosses an arbitrarily placed signal threshold. The fold change of the gene expression was calculated by 2-ΔΔCt, where ΔΔCt = (Ct (AtSRs–Ct (AtACT8))−(Ct (AtSRs–Ct (AtACT8))).

Accession Numbers—GenBank™ accession numbers for the genes are as follows: AF506897 (AtSR1), AF253511 (NiER1), AF096260 (LeER66), AF303397 (EICBP), S48041 (CaM-binding), AB174776 (rice EST), BF278598 (cotton EST), AVS33910 (barley EST), BE343151 (Sorghum EST), BE344315 (potato EST), BAA74836 (human, KIAA0969), and AAM133969 (Brassica BnCAMTA).

RESULTS

Structure of AtSRs—The Arabidopsis data base was searched using NiER1 amino acid sequences. One NiER1 homolog (AtSR1) and five related proteins (AtSR2–6) were found in the Arabidopsis genome, with gene identification numbers At2g22300, At3g04110, At3g22990, At5g64220, At1g67310, and At4g16150, respectively. Based on the alignment of the amino acid sequences of AtSR1–6, some variations were observed in the N-terminal portions of AtSR1 and AtSR6, whereas AtSR2–5 showed higher similarity to each other (see Fig. 1 in the Supplemental Material). To verify the cDNA sequence of AtSR1, a gene-specific primer (AtSR1-P1) and T3 primer in the vector were used to clone the 5′ region of AtSR1. The longest DNA fragment (~490 bp) was subcloned, and DNA sequencing revealed that the first 54 amino acids in AtSR1 were different from the prediction in GenBank™ but showed a linear similarity with other AtSRs N-terminal regions (see Fig. 1 in the Supplemental Material). Comparison of AtSR1 cDNA sequences with the genomic sequence indicated the differences resulted from the different RNA splicing sites in introns 1 and 2 (data not shown). Further cloning of the full cDNA showed that sequences in other region were the same as predicted. Reddy et al. (26) reported the isolation of a clone (EICBP, a partial cDNA sequence was reported in GenBank™) which had the same cDNA sequence as the GenBank™ prediction, suggesting this gene could have two types of transcripts. AtSR1 has 3302 nucleotides, and its largest open reading frame encodes a protein composed of 1032 amino acids with a predicted molecular mass of approximately 116 kDa. The predicted AtSR1 is an acidic and hydrophilic protein (pI 5.3) with no obvious membrane-spanning domains, and an overall secondary structure of α-helices. AtSR1 contains several noteworthy structural features as follows: 1) N-terminal 62–79 has a typical bipartite nuclear targeting signal (27); 2) C-terminal 900–922 has an almost identical amino acid sequence as the characterized CaM-binding region in NtER1 (18); 3) the central portion 661–726 has two ankyrin-like repeats which is a motif known to be responsible for mediating protein-protein interactions (28); 4) the C-terminal 893–896 has two IQ motifs, which is a CaM-binding motif in many proteins (29); and 5) the C-terminal 1003–1019 has an acidic domain with 11 acidic amino acids.

The amino acid sequences of AtSR6 were corrected based on the reported EST (GenBank™ accession number T04795) (see Fig. 1 in the Supplemental Material). AtSR2–6 had predicted lengths ranging from 852 (AtSR3) to 1035 amino acids (AtSR5) with pI values of 5.2–8. Overall similarity among six homologs ranges between 43 and 78%. They shared very high similarity in the N-terminal portion and in the C-terminal portions but not in the central portions (see Fig. 1 in the Supplemental Material). In the C terminus, all six AtSRs and NtER1 showed over 75% similarity and 65% identity, especially in the CaM-binding region with greater than 90% similarity and 79% identity. In the N-terminal portions, AtSR1–6 had over 66% similarity and 50% identity. Similar to AtSR1, AtSR2–5 have a predicted nuclear targeting signal sequence in the N terminus, one or two ankyrin repeat(s) in the center portion (except AtSR6), and more than two IQ motifs around the CaM-binding region.

All six AtSRs have just one copy each in the Arabidopsis genome. Five Arabidopsis chromosomes have one AtSR gene each, except chromosome 5 which has two, separated by about 20,000 kb. Phylogenetic analysis revealed that AtSR1–6 could be grouped into four subfamilies (SF): SF1 (AtSR1), SF2 (AtSR2, 4), SF3 (AtSR 3, 6), and SF4 (AtSR5) (data not shown). The overall conserved structure of all the AtSRs suggests that they may diverge from a single ancestral origin. AtSR2 and -4 share the highest similarity and are both located on chromosome 5, which suggests that they evolved by gene duplication most recently.

AtSRs Are CaM-binding Proteins—Fig. 1A shows the alignment of the CaM-binding region of AtSR1 with other AtSRs, tobacco NtER1 and tomato LeER66. The predicted CaM-binding domains (23 amino acids) corresponding to the NtER1 CaM-binding region are highly conserved. For example, AtSR1 has only two conserved amino acid sequence substitutions as compared with the counterpart of NtER1 in this portion (amino acids 900–922). Helical wheel projection in GCG 10 (version 10 of the GCG program) revealed that AtSR1–6 had the basic amphiphilic α-helix structure (data not shown), a typical secondary structure for most characterized CaM-binding proteins (18, 30, 31). To determine that the AtSRs are CaM-binding proteins, the full-length AtSR1 and two truncated constructs were expressed in E. coli. The ΔC (147–1032) was fused to a His tag in the C terminus and was purified by His-Trap column chromatography. The full-length and ΔN1−147 recombinant proteins were purified by a CaM-Sepharose column. The recombinant proteins were then subjected to a CaM binding assay. The results revealed that CaM binds to both AtSR1 and ΔN, but not ΔC, in the presence of 0.1 μM CaCl2 (Fig. 1B). No CaM binding was observed for all proteins when 0.1 μM CaCl2 was replaced by either 0.5 mM calcium chelator EGTA (Fig. 1B).
or other divalent ions such as 0.5 mM MgCl₂ (data not shown). Therefore, CaM binding to AtSR1 was Ca²⁺/H11001-dependent, and the CaM-binding region was within the C-terminal residues 147–1032. Furthermore, four peptides (representing four SFs) corresponding to the putative CaM-binding domains of AtSR1,2,3,5 were synthesized. Gel mobility shift assays revealed that CaM bound to all of them in a Ca²⁺/H11001-dependent manner. Two examples (AtSR1 and AtSR3) are shown in Fig. 1C. These results indicated that AtSR1–6 were all Ca²⁺/H11001-dependent CaM-binding proteins. Because many IQ motifs were CaM-binding domains, the peptides corresponding to the two IQ motifs of AtSR1 were used for the mobility shift assay. CaM did not bind to these two peptides, either in the presence of CaCl₂ or EGTA (data not shown).

**AtSR1 Targets the Nucleus**—A search of GenBank™ revealed that several partial clones from both dicots and monocots had over 66% similarity and 50% identity with AtSRs N-terminal portion (AtSR1, amino acids 13–134). These plants included parsley, potato, cotton, rice, barley and sorghum. This portion also showed over 56.6% similarity and 42.4% identity with two predicted proteins, KIAA0833 and KIAA0909, based on cDNA isolated from adult human brains. Alignment of these sequences indicated that they had the similar secondary structure with several predicted α-helices and two β-sheets, as well as several
positive charged amino acids (more than 10 net positive charges) in this portion. In particular, they all have conserved bipartite nuclear localization signals (AtSR1, amino acids 62–79).

We further selected AtSR1 for detailed studies on its subcellular localization by making the full-length of AtSR1, ΔN-(1–146) and ΔC-(147–1032) with GFP fusion constructs. Transient transformation into Arabidopsis leaves was performed by DNA bombardment, and the image was analyzed 24 h after transformation using a confocal microscope. The green fluorescence was throughout the cytoplasm for the GFP control construct. However, both AtSR1-GFP fusion and ΔC-GFP fusion predominantly were localized to the nucleus. In contrast, the ΔN-GFP fusion was visualized as patches in the cytoplasm (Fig. 2). Thus AtSR1 targets nuclei, and the nuclear localization signals are within N-terminal 146 amino acids. Furthermore, the fact that all other AtSRs and related proteins have conserved bipartite nuclear localization signals suggests that they all are nuclear proteins.

**AtSR1 Recognizes Specific DNA Elements**—Parsley CG-1 (147 amino acids) is a partial clone with high similarity with N-terminal portion of AtSRs. da Costa e Silva (32) reported that parsley CG-1 bound to a DNA fragment CCCTTTAATCTCCAACAAACCCCTTCTAG in which CGCG was crucial for DNA binding. The gel retardation assay showed that neither full-length AtSR1 nor deletion mutants bound to this DNA fragment (data not shown). In order to test whether the nuclear protein AtSR1 had specific interacting DNA elements, an oligo selection procedure was used with a pool of 30 completely random sequences of oligonucleotides. Because the putative DNA-binding domain was located in the N terminus, the recombinant AtSR1ΔC-(147–1032) was used for gel retardation assays to avoid the potential negative effects of other domains on DNA binding. The poly(dl-dC) was used as a nonspecific competitor. After three rounds of selection, gel-retarded oligo DNA molecules were amplified, and then a library enriched in DNA inserts containing specific sequences recognized by ΔC was established.

The DNA sequencing revealed that half of the positive clones had a common DNA element of 6-bp ACGCGG (or CCGCGT). However, AtSR1 also bound to other fragments with ACGCGT (30%), CCGCGG (10%), ACGGCC (or CGCGCGT) (5%), CCGCGC (or GCGCGG) (5%). They share a consensus sequence CGCG in the middle. Mutations of any nucleotide of CGCG abolished the DNA binding (Fig. 3A). Nonetheless, CGCG alone is not sufficient for DNA binding. The minimum DNA-binding elements are 6-bp CGCG box, (A/C/G)CGCGC(G/T). Some variations were observed in 1st and 6th nucleotides of CGCG boxes. No T was observed in position 1, and no A was observed in position 6. Substitutions with T in position 1 or with A in position 6 abolished the DNA binding (Fig. 3A). Note that a faint band with a faster mobility was visualized besides a strong protein-DNA complex band with a slower mobility (Fig. 3A). One possible explanation is that AtSR1ÈC binds to the target DNA fragment with both monomer and dimer (or oligomer) but mainly with dimer (or oligomer).

To determine whether full-length AtSR1 had the same binding specificity as ΔC, the recombinant AtSR1 and ΔN-(1–146) were subjected to gel retardation assays. The results show that AtSR1 had a DNA binding activity to probe 1 similar to ΔC. However, ΔN did not interact with probe 1 (Fig. 3B), indicating that AtSR1 had only one specific DNA-binding region that was within the N-terminal 146 amino acids. To demonstrate that AtSR1 was in fact the protein present in the mobility-shifted band, the different amounts of ΔC protein were added in reactions with the same amount of probe 1 for gel retardation assay. The DNA-protein complex intensity was correlated with the increasing protein amount. No DNA-protein complex was observed without addition of the protein, confirming the presence of ΔC protein in the complex (Fig. 3C). The ΔC binding activity to the CGCG box was further demonstrated in competitive gel retardation assays (Fig. 3D). Formation of the DNA-protein complexes between labeled probe 1 and ΔC was subjected to specific competition by wild type CGCG fragments (unlabeled probe 1). However, the mutated CGCG fragments (unlabeled probe 6) were not capable of competing for binding to ΔC (Fig. 3D). To examine the effect of CaM binding on DNA binding activity, CaCl2, EGTA, CaCl2/CaM, and EGTA/CaM was added in the reactions with full-length AtSR1 and probe 1, respectively. No obvious effect was observed on DNA binding activity by any of them, which suggested that CaM binding had no effect on DNA binding (data not shown).

**Genes with CGCG cis-Elements**—A search of the data base revealed that cis-acting elements ACGCGG/CCGCGT were present in the promoter regions of about 130 genes (more than two copies) in Arabidopsis genome. Some of these genes are listed in Table II. The promoter regions are assumed to be within ~1 kb upstream of the starting transcription site (for the known genes) or the first ATG (for the predicted genes). These genes are related to ethylene signaling (EIN3) and ABA signaling (a putative ABA responsive protein), light perception (phytochrome A, phyA), stress responsive such as the DNA repairing protein, heat shock protein, touch protein (TCH 4), and CaM-regulated ion channel. CaM genes (CaM2 and CaM3) and AtSR6 also contains CGCG cis-elements in their promoter regions.

In order to confirm that AtSR1 bound to those promoters in vitro, we selected the promoter regions of EIN3 (from ~330 to ~295), CaM2 (from ~221 to ~199), and phyA (from ~162 to ~104) for gel retardation assays (Fig. 3E). The results revealed that AtSR1 had the specific binding activity to these DNA fragments. However, no binding activities were detected in all CGCG mutants (Fig. 3E).

**Expression Patterns of AtSRs**—The expression level of AtSR1–6 is relatively low in plants. By using RT-PCR, the signal was detected around 42 cycles under normal growth conditions, whereas the exponential stage for internal control ACT8 was detected around 25 cycles (Fig. 4). All AtSRs had higher expression in stem and flowers, as well as siliques (except AtSR6) at 6-week stages. This was consistent with the
expression pattern of tobacco NtER1 and tomato LeER66. NtER1 was highly expressed in senescent leaves and flowers (18), and LeER66 had more expression during fruit ripening (33). Several AtSRs (AtSR1,3,4) also had a relatively higher expression in 4-day- and 7-day-old seedlings, which suggested that they had some role in early stages of development. However, the expression patterns of AtSR1–6 differed between other tissues and developmental stages. Generally the roots exhibited a higher expression of AtSRs than leaves both at the 2-week stage and the 6-week stage, suggesting an important role for AtSRs in root growth and development. AtSR1,2,5,6 were highly expressed in roots at both the 2-week stage and the
6-week stage, whereas the AtSR4 expression was higher at the 6-week stage than the 2-week stage. AtSR5 exhibited the highest expression level in leaves, whereas AtSR6 showed very little expression at both the 2-week stage and the 6-week stage. AtSR4 was barely detected in 2-week-old leaves, but it was highly expressed in leaves at the 6-week stage.

Expressions of AtSRs Are Differentially Regulated by Multiple Signals—Tobacco NtER1 and tomato LeER66 were ethylene-responsive genes. To study the effects of ethylene on the expression of AtSRs, 3-week-old seedlings were either treated with ethylene or with 1-methylcyclopropene (MCP) for 2 h prior to ethylene treatment which is known to block ethylene action. Only AtSR1,2,5 responded to ethylene treatment. Treatment with MCP for 2 h prior to ethylene treatment blocked their induction (data not shown), indicating that AtSR1,2,5 were ethylene-responsive genes. The time course of the induction showed that all three AtSRs were induced within 15 min after treatment and reached their peaks after 30 min of treatment. Among these genes, AtSR1 was highly induced as compared with AtSR2,5. Application of MJ showed a similar effect on AtSR induction as ethylene (Fig. 5A).

To investigate whether AtSRs responded to other signals, plants were subjected to abiotic stresses such as heat, cold, high NaCl concentration, UVB, and wounding, hormone ABA, as well as the signal elicitors H$_2$O$_2$ and SA. In these experiments, plants were exposed to stress conditions for short periods of time (4 h) to avoid possible secondary effects. These stimuli differentially induced the expression of AtSR genes. ABA, H$_2$O$_2$, and SA showed similar patterns by inducing the expression of AtSR3,4,5,6 with no effect on AtSR1,2. Heat shock, cold, and UVB showed a separate pattern of expression by inducing AtSR1,2,3,5,6. However, none of these treatments showed any effect on the expression of AtSR4. Salt stress also had a similar effect but did not induce the expression of AtSR6 (Fig. 5A). No amplification were observed for AtSRs in all controls (data not shown).

Furthermore, the physical wounding induced the expression of all six AtSRs. The quantitative expression profiles of AtSR1–6 following wounding were studied using real time RT-PCR. The results showed that the induction level varied among genes significantly. AtSR1,2 showed the fastest and highest induction. Their expression levels were increased by over 80-fold within 2 h. However, the induction of AtSR3,4 were just around 12-fold (Fig. 5B).

CaM-binding Nuclear Proteins Bind to CGCG cis-Elements—To confirm the presence of CGCG box-binding proteins in vivo, nuclear proteins were isolated from wounded leaves. The specific DNA binding activity to the DNA fragment of EIN3 promoter was detected in the total nuclear proteins from wounded plants, but the nuclear proteins from untreated plants showed almost no DNA binding activity (data not shown). No protein-DNA complex was observed when the CGCG mutant was used as a probe. These results indicated...
that plant leaves contained nuclear CGCG-binding proteins which were induced by wounding. These are consistent with real time RT-PCR analysis which showed that wounding induced the expression of all AtSRs. To investigate whether the CGCG box-binding proteins were CaM-binding proteins, the total nuclear extract from wounded plants was further purified by CaM-Sepharose column. Out of 110 μg of nuclear proteins, about 4 μg of CaM-binding nuclear proteins were purified. The fraction of CaM-binding nuclear proteins was subjected to gel retardation assay. The specific DNA binding activities were dramatically increased for CaM-binding nuclear proteins (data not shown), indicating that CGCG box-binding nuclear proteins in vivo are CaM-binding proteins.

**DISCUSSION**

Earlier we reported that an early ethylene-responsive gene *NtER1* in tobacco encodes a CaM-binding protein and suggested that this type of gene exists in other plants, such as *Arabidopsis* and tomato (18). Later, an *Arabidopsis* homolog (*EICBP*) was identified as an ethylene-responsive gene, and calcium/CaM was shown to bind to the recombinant protein (26). Also, a *Brassica* homolog BrCAMTA and one of the *Arabidopsis* homologs (AtCAMTA1) were classified as CaM-binding proteins with nonspecific DNA binding activity (19). The results described herein show that the six *NtER1* homologs in *Arabidopsis* (AtSR1–6) are responsive to a variety of stimuli (Fig. 5). Each AtSR has a conserved structural feature with a DNA-binding region (CGCG domain) in the N terminus and a CaM-binding domain in the C terminus. The detailed study of AtSR1 revealed that the CGCG domain (amino acids 1–146 in AtSR1) targets the nucleus and specifically interacts with a novel CGCG box, a 6-bp double-strand DNA element (A/C/G)CGCG(G/T/C) (Figs. 2 and 3). The optimal target sequence for AtSR1 is an ACGCGG (or CCGCGT) element. Further comparisons revealed that the CGCG domain protein is present in other plants, both dicots and monocots. They all have conserved CGCG domains similar to AtSR1, suggesting that they all bind to the CGCG box. However, they may have different target sequence preferences on the nucleotides of positions 1 and 6 in the 6-bp DNA element. For example, the five members of AtERF proteins (*Arabidopsis* ethylene-responsive binding factors) have distinct DNA-binding preferences on the GCC box (34). AtERF1, -2, and -5 appear to be most sensitive to the
The regulation of phyA expression has been intensively studied. Chrome A is one of the best characterized photoreceptors, and CGCG tor, that functions downstream of CTR1 and EIN2 (51). Three
ways in plants. Ethylene and ABA are two major plant hormones that affect almost all stages of plant development, such as seed germination, cell fate, fruit ripening, senescence, and abscission. They are also key regulators that mediate the response of a plant to biotic and abiotic stresses (35–37). Recently, it has been documented that MJ, H2O2, and SA play a role as defense signal molecules. The responses of plants to the environmental stresses such as heat shock, cold, UV, wounding, and high salt or pathogen and insect attacks are often mediated by these hormones and/or signal elicitors (5, 38–44). Ca2+ and Ca2+-
expression (Fig. 5). All of the genes were induced by physical wounding (Fig. 5B). It is well established that jasmonic acid plays a central role in plant responses to wounding. Hormones such as ABA and ethylene have also been proposed to play a role in wound signaling (43). Our results support that both jasmonic acid-dependent and -independent wound signaling pathways were rapidly activated to regulate AtSR expression following wounding. Furthermore, these genes respond to signals very rapidly (within minutes), which indicates that they are all early signal responsive genes. Early signal responsive genes are believed to play a prominent role in regulating late-responsive genes (33, 50). These results indicate that the multiple signal transduction pathways regulate the expression of the Ca2+/CaM-binding AtSR gene family, suggesting that AtSR is one of the early hubs for cross-talk among signaling pathways in plants.

Identification of the AtSR1-specific DNA-binding elements led us to investigate further the potential downstream regulatory genes. Several of these genes are involved in ethylene and ABA signaling, DNA repairs (UV damage), signal perception, and stress response (Table II), which is consistent with the AtSR gene expression profiles in response to signals (Fig. 5). For example, EIN3 is an ethylene-responsive transcription factor, that functions downstream of CTR1 and EIN2 (51). Three CGCG cis-elements are present in its promoter region. Phytochrome A is one of the best characterized photoreceptors, and the regulation of phyA expression has been intensively studied by characterizing several cis-acting elements, such as GT, PE3, and RE1 elements (52). Four CGCG cis-elements appear in Arabidopsis phyA. A CGCG cis-element was found inside the positive element (PE3) of phyA from monocots oat, rice, and maize, and another CGCG element also appeared in a negative element (RE1) in oat (52). It has been suggested that calcium/ CaM may be one of signaling intermediates in phototransduction (53). Our data provide additional clues for a role of calcium/CaM in phototransduction. Coincidentally, several light-responsive cis-elements, such as I boxes (GATA), CCAAT elements, and GT-1 sites (54), were found in the promoters of AtSR1,4,5,6 (data not shown). Gel retardation assays show that the recombinant AtSR1 specifically recognizes the wild type EIN3 and phyA promoter fragments but not the CGCG mutants (Fig. 3E). Furthermore, CaM-binding nuclear proteins purified from wounded plants had the specific DNA-binding activity to CGCG boxes. In contrast, DNA binding activities were barely detected for the nuclear proteins from control plants that were grown under normal conditions. Our data suggest that AtSR1 may regulate the expression of these genes in vivo. Isolation and characterization of the knockout mutants of AtSRs will provide direct evidence of downstream regulatory genes.

All AtSRs are shown to be calcium-dependent CaM-binding proteins (Fig. 1). However, the specific role of CaM-binding to AtSRs is not yet clear. It has been reported that Ca2+/CaM inhibits the protein-DNA binding by competing with the basic helix-loop-helix DNA-binding domain in certain transcription factors (16). The DNA-binding protein TAG3 also shows Ca2+/CaM enhanced-binding activities to C/G box (17). However, its CaM-binding domain has not been identified. In this study, we found that the CaM-binding region is far away from the DNA-binding region in all AtSRs. The possible role of Ca2+/CaM may be manifested in the control of interactions with other proteins or altering transcription activation of others. Recently, a trans-
scription activation domain was mapped to a region just up-

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**Fig. 6.** The schematic illustration of the proposed model for the involvement of Ca2+/CaM/AtSR in multiple signal transduction pathways. The environmental signals are mediated by hormones such as ethylene and ABA, signal elicitors such as MJ, H2O2, SA, Ca2+, and CaM. These signals are interacted each other and form a complex signal network. AtSRs differentially perceive and respond to a variety of signals and regulate the downstream genes by recognizing the CGCG cis-elements. The ankyrin repeats and the acidic domain may interact with transcription factors or others. In the end, the downstream gene expression is up-regulated or down-regulated. The question marks indicate some open questions. ET, ethylene; DNABD, DNA-binding domain; AR, ankyrin repeats; CaMBD, CaM-binding domain; AD, acidic domain; TF, transcription factors.
stream of ankyrin repeats in AtCAMTA1 by fusing with the DNA-binding domain of LexA transcription factor and testing in a yeast system (19). Identification of the AtSR1 recognition sequence as a CGCG box should help to define the importance of Ca<sup>2+</sup>/CaM-binding in transcription activation in plants.

In conclusion, multiple signals rapidly and differentially induce AtSR expression; AtSR-encoded proteins are Ca<sup>2+</sup>-dependent CaM-binding proteins; AtSR1 shows specific DNA binding activity to the CGCG box; and the CaM-binding nuclear proteins are able to specifically interact with the CGCG cis-elements. Based on these results and other studies, we propose that CaM-regulated AtSRs may serve as one of the early hubs in multiple signal transduction cascades by differentially responding to the multiple upstream signals. Furthermore, AtSRs recognize CGCG cis-elements and may regulate the downstream gene expression, which ultimately leads to the physiological responses to varieties of stresses (Fig. 6). Further characterization of the functional significance of AtSRs should provide a better understanding of the mechanisms of plant defense to biotic and abiotic stresses.

Acknowledgments—We thank Dr. Philip Berger, Margaret Dibble, Christina Moore, and Jenny Hansen for help with the bombardment experiment; Dr. Liqun Du for help with the gel retardation assays; Yanping Chen and J. Kandakumar for technical support; Dr. Thomas D. Schmittgen for help with the real time PCR; and Dr. John Fellman, Dr. Thomas Schmittgen, J. Kandakumar for technical support; Dr. Liqun Du for help with the gel retardation assays; Christina Moore, and Jenny Hansen for help with the bombardment experiments.

REFERENCES

1. The Arabidopsis Genome Initiative (2000) Nature 408, 823–826
2. Bush, D. S. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 95–122
3. Trewavas, A. J., and Malho, R. (1997) Plant Cell 9, 1181–1195
4. Bowler, C., and Flurin, R. (2000) Trends Plant Sci. 5, 241–246
5. Zhu, J. K. (2001) Curr. Opin. Plant Biol. 4, 401–406
6. Evans, N. H., McAlpin, M. R., and Hetherington, A. M. (2001) Curr. Opin. Plant Biol. 4, 415–420
7. Poovaiah, B. W., Yang, T., and Reddy, A. S. N. (2002) in Plant Roots: The Hidden Half (Waisel, Y., Kafkafi, U., and Eshel, A., eds) 3rd Ed., pp. 505–520, Marcel Dekker Inc., New York
8. Pauli, N., Knight, M. R., Thiele, P., van der Luit, A. H., Moreau, M., Trewavas, A. J., Ranjeva, R., and Mazars, C. (2000) Nature 405, 754–755
9. Natalie, C., Strynadaka, J., and Jams, M. N. G. (1989) Annu. Rev. Biochem. 58, 951–988
10. Poovaiah, B. W., and Reddy, A. S. N. (1987) CRC Crit. Rev. Plant Sci. 6, 47–103
11. Roberts, D. M., and Harmon, A. C. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 375–414
12. Poovaiah, B. W., and Reddy, A. S. N. (1993) CRC Crit. Rev. Plant Sci. 12, 183–211
13. Zielinski, R. E. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 697–725