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RAPID COMMUNICATION

Molecular characterization of a pepper C2 domain-containing SRC2 protein implicated in resistance against host and non-host pathogens and abiotic stresses

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Abstract Plants guard themselves against pathogen attack using multi-layered defense mechanism. Calcium represents an important secondary messenger during such defense responses. Upon examination of a pepper cDNA library, we observed that the gene *CaSRC2-1* (*Capsicum annum* SRC2-1) was upregulated significantly in response to infection with the type II non-host pathogen *Xanthomonas axonopodis* pv. glycines 8 ra, which elicits a hypersensitive response. *CaSRC2-1* encodes a protein that contains a C2 domain and it exhibits a high degree of homology to the protein Soybean genes regulated by cold 2 (SRC2). However, little is known about how *SRC2* expression is elicited by biotic stresses such as pathogen challenge. Further sequence analysis indicated that the CaSRC2-1 C2 domain is unique and contain certain amino acids that are conserved within the C2 domains of other plants and animals. *CaSRC2-1* transcription was up-regulated under both biotic and abiotic stress conditions, including bacterial and viral pathogen infection, $CaCl_2$ and cold treatment, but unaffected by treatment with plant defense-related chemicals such as salicylic acid, methyl jasmonic acid, ethephone, and abscisic acid. Intriguingly, under steady state conditions, *CaSRC2-1* was expressed only in the root system. A CaSRC2-1-GFP fusion protein was used to determine localization to the plasma membrane. A fusion protein lacking the C2 domain failed to target the membrane but remained in the cytoplasm, indicating that the C2 domain plays a critical role in localization. Thus, *CaSRC2-1* encodes a novel C2 domain-containing protein that targets the plasma membrane and plays a critical role in the abiotic stress and defense responses of pepper plants.

Keywords Hypersensitive response · Type II non-host resistance · Pathogenesis-related genes

Abbreviations

HR	Hypersensitive response
CaSRC2	*Capsicum annum* soybean genes regulated by cold 2

Introduction

Plants possess multi-layered defense mechanisms against biotic and abiotic stresses. A plant species (or its cultivars) that is susceptible to a given pathogen is referred to as the "host" for that pathogen (Baker et al. 1997). Most pathogens exhibit narrow host specificity and do infect "non-host" species; the resistance of plants to the vast majority of potential pathogens is termed "non-host resistance" (Heath 2000; Kamoun 2001; Thordal-Christensen 2003; Nürnberger et al. 2004). Recently, Mysore and Ryu (2004) subdivided non-host resistance into two types, i.e., type I, which does not result in visible cell death and type II, in which a hypersensitive response (HR) occurs and results in cell death at the site of infection. This proposal was proven in

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tobacco, in which type-specific transcription patterns of defense-related genes were elicited by viral and bacterial types I and II non-host pathogens (Oh et al. 2006). In general, the mechanisms and signaling components that relate to non-host resistance have not been studied extensively. The mechanisms underlying HR (type II non-host resistance) have been investigated (Dangl et al. 1996). Following recognition of molecules derived from pathogens, plant receptors trigger signaling cascades for defense. Endogenous signaling molecules such as salicylic acid (SA), ethylene, methyl jasmonic acid (MeJA) and abscisic acid (ABA), as well as secondary messengers such as calcium ions (Ca^{2+}), cyclic AMP and inositol-3-phosphate, act as transducers of plant defense responses (Dong 1998; Reymond and Farmer 1998; Mauch-Mani and Mauch 2005; Zhao et al. 2005).

Intracellularly, Ca^{2+} is an important secondary messenger and it affects a diverse range of cellular responses. Various stimuli such as pathogen attack, cause an increase in cytosolic Ca^{2+} concentrations, which are coupled to downstream signal transduction pathways via Ca^{2+}-binding proteins (Kopka et al. 1998; Hetherington and Brownlee 2004). Most of these Ca^{2+}-binding proteins contain C2 domains (Ca^{2+}-regulatory domains) which function together with EF-hands (Kretsinger 1980) and annexin folds (Gerke and Moss 2002). The C2 domain was first identified in Ca^{2+}-dependent isoforms of protein kinase C. These C2 domains bind phospholipids in a Ca^{2+}-dependent manner and they are ca. 130 amino acid residues in length (Nalefski and Falke 1996). The C2 domain contains five aspartic acid residues, which form a Ca^{2+}-binding site that is conserved among the Ca^{2+}-dependent C2 domain-containing proteins of both plants and animals (Rizo and Südhof 1998; Kim et al. 2003). In mammals, C2 domain-containing proteins play roles in various cellular mechanisms. For example, the C2 domain of cytosolic phospholipase A2 (PLA2) mediates translocation of the soluble protein from the cytosol to the membrane in a Ca^{2+}-dependent manner (Clark et al. 1991). The C2 domain of protein kinase C (PKC) is involved in Ca^{2+}- and phospholipid-dependent activation of proteins (Kaibuchi et al. 1989; Wang 2002). Other C2 domains have been reported to act as modules for protein-protein interactions (Miyazaki et al. 1995). Recently, the C2 domain of PKC_{δ} was identified as a phosphotyrosine-binding domain (Benes et al. 2005). In addition to animals, several C2 domain-containing proteins have been reported in plants. In rice, the small protein OsERG1 contains a single C2 domain and is induced by treatment with a fungal elicitor resulted in the protein binding to phospholipid vesicles in Ca^{2+}-dependent manner (Kim et al. 2003). In mung beans (*Vigna radiate* L.), the C2 domain of V3-PLC3 (a putative plasma membrane-localized phosphoinositide-specific phospholipase C) plays an important role in translocation of the protein to the membrane in response to abiotic stress (Kim et al. 2004; Wang 2002). In addition, Xoconostle-Cazares et al. (1999) demonstrated that Cmpp16-1, which is a C2 domain-containing protein from pumpkin, may be involved in RNA delivery. However, only a few C2 domain-containing proteins have been identified or investigated in plants.

In this study, we have identified a pepper gene encoding CaSRC2-1, which is a single C2 domain-containing protein induced by type II non-host pathogen infiltration. Interestingly, transcripts of this gene accumulated only in the root system. To investigate its function in CaSRC2-1, we deleted the C2 domain in protein localization test and found that it performs an important role in plasma membrane-targeting in pepper. Taken together, our results indicate that CaSRC2-1 represents a novel biotic and abiotic stress-elicited protein that is root-specific and which contains an essential C2 domain.

Materials and methods

Plants and pathogen inoculation

Peppers (*Capsicum annuum* L. cv. Bukang) were cultivated in a growth chamber at 25°C under a 16 h/8 h light/dark photo-cycle. Roots, stems, leaves, flowers, seeds, germinating seeds and seedlings were prepared from healthy pepper plants and frozen immediately in liquid nitrogen for RNA blot analysis. For pathogen challenge, the incompatible bacterial pathogens *Xanthomonas axonopodis* pv. *glycines* (*Xag*) 8 ra (OD_{600} = 0.4 in 10 mM $MgCl_2$) were pressure-infiltrated into pepper leaves using a needle-less syringe, as described previously (Oh et al. 2005, 2006; Yi et al. 2004). Healthy leaves from ca. 1-month-old plants were used for treatment and nucleic-acid extraction (Oh et al. 2005; Suh et al. 2003). All bacterial pathogens were cultured overnight at 28°C in LB medium supplemented with the appropriate antibiotics. Ten-week-old leaves were inoculated with sap (pH 6.8) prepared from tobacco plants infected with *Tobacco mosaic virus* (TMV) strain T0 and P1.2, and following inoculation, the treatment area was rubbed gently with carborundum. Chemical treatments of pepper leaves were performed as described previously (Oh et al. 2005, 2006; Yi et al. 2004). Leaves were harvested at the times indicated and frozen immediately in liquid nitrogen for total RNA extraction. Intact pepper leaves were used for non-stress treatments (Suh et al. 2003). Following inoculation with each pathogen, plants were returned to the growth chamber and leaf tissues were harvested at 1.5, 3, 6, 9, 12, 24, 48 and 72 h post-inoculation and then used for isolation of total RNA.

Genomic DNA blot analysis

Genomic DNA was prepared from pepper plants as described previously (Yi et al. 2004). Total DNA (20 μg) was digested with *Eco*RI or *Xba* I and separated by 0.8% (w/v) agarose gel electrophoresis. Southern blotting analysis was performed as described previously (Sambrook and Russell 2001), as were probe labeling, blot hybridization and washing conditions (Choi et al. 1996; Yi et al. 2004). Membranes were exposed to an imaging plate and scanned using BAS-1800 (Fujifilm, Japan).

PCR amplification and probes

Total RNA was extracted from the leaves of infected pepper plants using TRIZOL solution (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using 2 μg total RNA, oligo d(T) primer and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Semi-quantitative reverse transcription (RT)-PCR was performed as described previously (Chung et al. 2006; Ryu et al. 2004). Samples from each reaction (4 μl) were used in a 20 μl premix PCR mixture containing Taq polymerase (Bioneer, Daejeon, Republic of Korea). RT-PCR amplification was performed for 15, 18, 21, 25 and 30 cycles at 94°C for 50 s, 55°C for 50 s, and 72°C for 30 s. Amplification of the pepper actin gene was performed as a control. *CaPR1, CaPin-II* and *Cadhn* transcripts were detected by PCR as described previously (Oh et al. 2005, 2006; Yi et al. 2004). The intensities of PCR-generated fragments were analyzed using the Digi Doc-It Version 1.1.25 (Ultra-Violet Products, Upland, CA).

Subcellular localization of *CaPIF1*

Subcellular localization of the 35S-CaSRC2-1-smGFP fusion protein was determined as described previously (Oh et al. 2005). The full-length *CaSRC2-1* ORF without the termination codon was amplified from cDNA by PCR using primers containing a *Bam*HI site at 5′ end (5′-GGATCC ATGGCACTTGAAGCTTTG-3′ and 5′-GGATCCAAT GTTGAAATAATTC-3′). A *CaSRC2-1* (*ΔC2-CaSRC2-1*) cDNA fragment withoug the C2 domain, and the full-length *CaSRC2-1* fragment were inserted into a GFP expression vector (35S-smGFP) respectively for adding C-terminal fusion tags (David and Vierstra 1996). The empty 35S-smGFP and H+-ATPase-RFP vectors were used as controls. For transient expression analysis, pepper protoplasts were placed on basic MS agar. The plasmid DNA (4 μg each of p*35S- CaSRC2-1-smGFP*, p*35S- ΔC2-CaSRC2-1 -smGFP* or p*35S-smGFP*) was introduced into BY-2 tobacco protoplasts via polyethylene glycol (PEG)-mediated transformation (Abel and Theologis 1994). Fluorescence photographs of protoplasts were taken using a Zeiss Axiophot (Jena, Germany) fluorescence microscope fitted with fluorescein isothiocyanate filters (excitation filter, 450–490 nm; emission filter, 520 nm; dichroic mirror, 510 nm) and Fuji 400 color film.

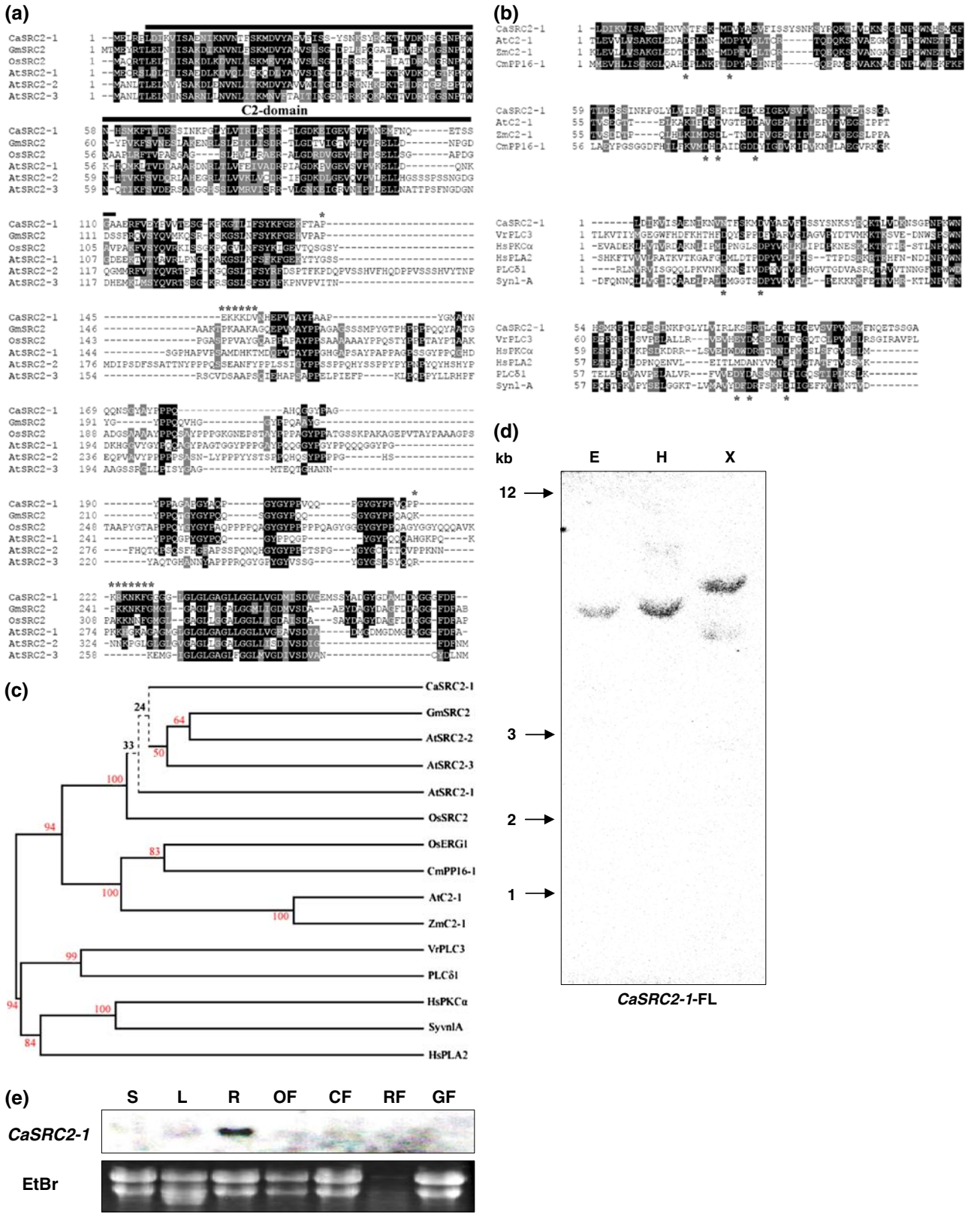
Isolation of total RNA and Northern blot analysis

Total RNA was isolated from inoculated leaf tissues, according to the protocol described by Choi et al. (1996). Total RNA (20 μg) from each sample was separated by formaldehyde-containing agarose gel electrophoresis and transferred onto a Nytran® N membrane (Amersham, Piscataway, NJ). Relative loading was checked by ethidium bromide staining of total RNA (0.1 μg/ml). Each cDNA clone was labeled with ^{32}[P]-dCTP using the Prime-a Gene System (Promega Corp., Madison, WI) and subjected to Northern blot hybridization. Both pre-hybridization and hybridization of the membrane were carried out in 5 × SSC, 5 × Denhardt's solution, 0.5% SDS and 100 μg/ml salmon sperm DNA. Hybridization was conducted at 60°C overnight and then the membrane was rinsed in 1 × SSC and 0.1 × SDS at room temperature for 15 min, followed by a wash in 0.5 × SSC and 0.1 × SDS at 60°C for 2 h. Prior to development, the membrane was exposed to X-ray film (Eastman Kodak Co., Rochester, NY) for 24 h using intensifying screens at −70°C. Experiments were repeated three times with similar results.

Results and discussion

Isolation and sequence analysis of *CaSRC2-1*

In order to characterize non-host resistance-related genes in pepper plants, we applied the soybean pustule pathogen *Xanthomonas axonopodis* pv. glycines 8 ra (*Xag*; a type II non-host pathogen) to pepper leaves and performed expression analysis using a cDNA chip array with 4865 unique EST clones (http://plant.pdrc.re.kr, Lee et al. 2004). We identified 88 EST clones whose expression was induced or repressed more than two-fold in the presence of this pathogen. Northern blot analysis was performed with 29 of these clones and one gene was found to exhibit significant up-regulation in response to non-host pathogen challenge (data not shown). Since this gene showed significant homology to *SRC2* (soybean genes regulated by cold 2), which is induced by cold treatment in soybean (Takahashi and Shimosaka, 1997), it was named *CaSRC2-1* (*Capsicum annuum SRC2-1*). The full-length cDNA of *CaSRC2-1* (1,080 bp) contains a single open reading frame that encodes a 276 amino acid protein (Fig. 1a, b, GenBank accession no.: DQ465394), with a predicted protein molecular



mass of 29.9 kDa and pI value of 5.99. Analyses of the deduced amino acid sequences of *CaSRC2-1* using InterProScan (http://www.ebi.ac.uk/InterProScan/) and SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) revealed the presence of an N-terminal calcium-dependent phospholipid-binding module, i.e. a C2

◀ **Fig. 1** Amino acid sequence comparison between CaSRC2-1 and its homologues, as well as *CaSRC2-1* copy number and localization. **a** Amino acid sequence comparison between pepper CaSRC2-1 and homologues from soybean GmSRC2 (AB000130), rice OsSRC2 (AP003246) and *A. thaliana* AtSRC2-1 (AJ007586), AtSRC2-2 (NM_112522) and AtSRC2-3 (NM_148335). A *black line* indicates the Ca^{2+}-dependent phospholipid-binding module (C2 domain). *Black and shaded boxes* indicate sequence identity and similarity, respectively. **b** Comparison between CaSRC2-1 and single C2 domain-containing proteins from other plants (*upper alignment*), including *A. thaliana* AtC2-1 (AAG52148), maize, ZmC2-1 (U64437) and pumpkin, CmPP16-1 (AF079170). Comparison between CaSRC2-1 and single C2 domain-containing proteins from plants and animals (*lower alignment*), including mung bean VrPLC3 (AY394078), human hsPKCα (P17252), human hsPLA2 (M72393), bovine PLCδ1 (P10895) and rat synaptotagmin I C2A, Synl-A (P21707). Amino acid residues identical to C2 domain sequences are shown in *black* and putative Ca^{2+}-binding aspartate residues are indicated by an *asterisk*. **c** Phylogenic tree of C2 domain amino acid sequences. Sequences include CaSRC2-1 and the plant and animal orthologs compared above. **d** Genomic DNA gel blot analysis of *CaSRC2-1*. Genomic DNA was digested with *Eco*RI (E), *Hind*III (H) and *Xba*I (H), separated by 0.8% agarose gel electrophoresis and blotted onto a nylon membrane. The membrane was hybridized with a full-length *CaSRC2-1* cDNA probe. The sizes of DNA molecular weight standards are indicated at the left. **e** Tissue-specific expression of *CaSRC2-1* in pepper plants. Total RNA (20 μg) was extracted from the stem (S), Leaf (L), root (R), open flower (OF), closed flower (CF), red fruit (RF) and green fruit (GF) of pepper. RNA was separated by electrophoresis on a 1% agarose gel containing formaldehyde, transferred onto a nylon membrane and hybridized with a full-length *CaSRC2-1* cDNA probe (*upper panel*). Relative loading was confirmed by ethidium bromide staining of tRNA (*lower panel*)

domain (6-Leu to 111-Ala; Rizo and Südhof 1998). In addition, the CaSRC2-1 amino acid sequence contained two putative nuclear localization signals (NLSs), as well as a C-terminal Gly-Tyr-Gly-Tyr-Pro-Pro-Val-Gln repeat region of unknown function. BLASTX analysis indicated that the CaSRC2-1 amino acid sequence shares 43% identity with *Glycine max* SRC2 (GmSRC2; Takahashi and Shimosaka 1997), 36% identity with *Oryza sativa* SRC2 (OcSRC2) and 41, 35, and 39% similarity with the *Arabidopsis thaliana* orthologs AtSRC2-1, AtSRC2-2, and AtSRC2-3 (Aubourg et al. 1999), respectively (Fig. 1a).

The C2 domain is a Ca^{2+}-dependent phospholipid-binding module with five conserved aspartic acid (Asp) residues that interact with Ca^{2+} ions (Rizo and Südhof 1998). These C2 domain-containing proteins play critical roles in the cellular signaling of plants and animals (Kopka et al. 1998; Nalefski and Falke 1996). However, these five Asp residues are not conserved in CaSRC2-1 (Fig. 1b) and thus, it was not classified with other small single C2-domain-containing proteins from plants and animals. In fact, phylogenetic comparison with these proteins revealed that CaSRC2-1 protein was unique (Fig. 1c). Furthermore, to our knowledge this is the first report of *CaSRC2-1* induction in response to a non-host pathogen challenge in pepper.

Southern blot analysis indicated that there is only a single copy of *CaSRC2-1* in the pepper genome (Fig. 1d). To examine steady-state without any treatment expression of *CaSRC2-1* in various tissues of pepper plants, we performed Northern blot analysis of total RNA extracted from the stems, leaves, roots, open flowers, closed flowers, red fruits and green fruits. High *CaSRC2-1* transcript levels were found in the roots, but not in other tissues (Fig. 1e). At 25°C, Takahashi and Shimosaka (1997) found that the expression of *SRC1* and *SRC2* was difficult to detect in soybean leaves, stems or roots, but that transcription was up-regulated significantly in leaves and stems following cold treatment at 5°C. However, those authors only observed a slight accumulation of *SRC2* mRNA in soybean root, whereas we observed a high level of *CaSRC2-1* expression in the pepper root. In addition, soybean *SRC2* was not induced by high temperature, drought stress treatments, or inoculation of virus. Collectively, differences between *SRC2* and a pepper *CaSRC2-1* in copy number and expression patterns were existed, clearly suggesting that soybean *SRC2* and pepper *CaSRC2-1* may have a different function depending on plant species, despite considerable similarity in their amino acid sequences.

$CaCl_2$ induction of CaSRC2-1 and its subcellular localization

CaSRC2-1 transcripts accumulated after 10 min of 10 mM $CaCl_2$ treatment; maximum transcript levels were observed at 30 min and decreased gradually thereafter with a burst of expression at 3 h (Fig. 2a). As expected, these results indicate that *CaSRC2-1* expression is strongly related to calcium-mediated signal transduction. Sequence analysis of *CaSRC2-1* indicated that it encoded a C2 domain (Fig. 1a) and it is known that C2 domains bind phospholipids in a Ca^{2+}-dependent manner (Nalefski and Falke 1996). Rice OsERG1 contains a single C2 domain that binds specifically to phospholipids in a Ca^{2+}-dependent manner. It binds predominantly to anionic phospholipids such as phosphatidylserine and phosphatidylinositol, rather than to cationic phospholipids such as phosphatidylcholine or phosphatidylethanolamine (Kim et al. 2003). In mung bean, the C2 domain of Vr-PLC3 is important for localization of this protein to the plasma membrane (Kim et al. 2004).

To determine the subcellular localization of CaSRC2-1, we prepared a CaSRC2-1-GFP fusion using soluble modified GFP (smGFP) as a C-terminal fluorescent marker. *GFP* was fused in-frame to the 3′ end of the *CaSRC2-1* coding region (Fig. 2b) and PEG-mediated transformation was used to introduce the expression constructs into pepper protoplasts (Park et al. 2001). Control GFP (smGFP) from vector alone was distributed throughout the cytoplasm, while the CaSRC2-1-GFP fusion protein localized to the plasma membrane (Fig. 2c). As a positive control for plasma membrane targeting, we co-transfected protoplasts with vector

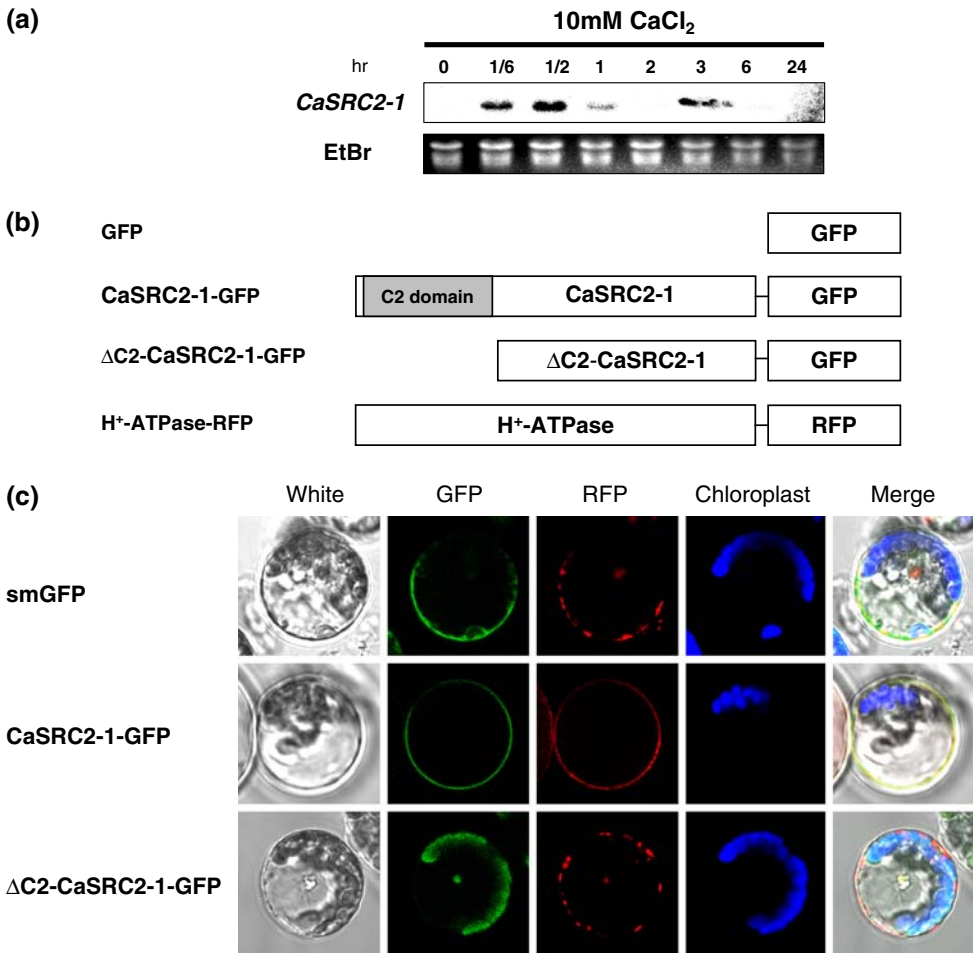


Fig. 2 *CaSRC2-1* induction by $CaCl_2$ and subcellular localization of CaSRC2-1 in pepper. **a** Temporal expression pattern of *CaSRC2-1* in response to treatment with $CaCl_2$. Detached pepper leaves were submerged into $CaCl_2$. *CaSRC2-1* expression was evaluated at 10 and 30 min, as well as 0, 1, 2, 3, 6 and 24 h after treatment. **b** Construction of CaSRC2-1-GFP and the truncated ΔC2-CaSRC2-1-GFP fusion protein. The GFP coding region was fused in-frame to the full-length CaSRC2-1 coding region or to the truncated ΔC2-CaSRC2-1 coding region. H+-ATPase-RFP was used as a marker for plasma membrane protein. The constructs were introduced into pepper protoplasts by PEG-mediated transformation. **c** Subcellular localization of each construct under different light sources. Fluorescent photographs of protoplasts were taken using a confocal laser-scanning microscope (Carl Zeiss LSM510, Germany) at 24 h after transformation

expressing a H^+ATPase-red fluorescent protein (RFP) fusion protein, which also localized to the plasma membrane (Fig. 2b, c; Kim et al. 2004). A close overlap was observed between the green and red fluorescent signals of CaSRC2-1-GFP and H^+-ATPase-RFP, respectively. However, in protoplasts transfected with the ΔC2-CaSRC2-1-GFP fusion construct (Fig. 2b), which lacks an N-terminal C2 domain, the fluorescent signals were distributed predominantly in the cytoplasm (Fig. 2c). This result suggests that the C2 domain is critical for localization of CaSRC2-1 to the plasma membrane.

Interestingly, CaSRC2-1 was predicted to contain two nuclear localization signal (NLS) in its C-terminal region (Fig. 1a). Membrane translocation of protein kinase C (PKC) Apl II from *Aplysia* was recently reported to be regulated by phosphorylation of the C2 domain (Pepio and Sossin 2001). Despite the presence of an N-terminal C2 domain, translocation of this PKC was Ca^{2+}-independent. In fact, it is phosphorylation of a serine (Ser) residue in the C2 domain other than mediates the membrane translocation of the protein. In plants, translocation of these proteins may be regulated by phosphorylation of the C2 domain, in the absence of Ca^{2+}-binding. Although CaSRC2-1 did not contain the five conserved Asp residues that are known to participate in Ca^{2+}-binding, its C2 domain was rich in Ser residues (Fig. 1b) and it is possible that it exhibits phosphorylation-mediated regulation. However, this possibility requires further study.

The full-length CaSRC2-1 primarily localized primarily to the plasma membrane while the fusion without a C2 domain localized to the cytoplasm. This finding suggests that the two NLSs do not function in plasma membrane targeting. However, our experiments were performed under normal conditions and since *CaSRC2-1* is induced under various stress conditions, it may be that CaSRC2-1 is translocated from the plasma membrane or cytoplasm to the nucleus in response to various stimuli. Recently, the *Arabidopsis* membrane protein AtSRC2 was found to use a Pro-Ile-Glu-Pro-Pro-Pro-His-His-His motif in its cytoplasmic tail to enable trafficking from the endoplasmic reticulum (ER) to protein storage vacuoles (Oufattole et al. 2005). Therefore, it will be interesting to determine the subcellular localization of CaSRC2-1 following treatment with various stimuli. To our knowledge, the subcellular localization of SRC2 proteins following treatment with various stimuli has not been reported for plants such as soybean and

Arabidopsis. As expected, CaSRC2-1 exhibited localization to the plasma membrane, a finding that is in agreement with our hypothesis that it is a member of the C2 domain-containing SRC2 family. In *Arabidopsis*, BAP1 containing a C2 domain was detected in the membrane fraction (Yang et al. 2006a). The authors reported that the BAP1 protein possessed the capacity to bind phospholipids in calcium-dependent manners and detected association with *Arabidopsis* membranes in vivo. However, the authors did not assess direct subcellular localization of BAP1 in a plant cell in situ.

CaSRC2-1 expression in response to host and non-host bacterial and viral pathogens

Initially, we examined *CaSRC2-1* expression in response to non-host pathogens over 48 h. Following infiltration of leaves with *Xag* 8 ra, *CaSRC2-1* expression increased steadily and reached its maximum accumulation at 9 h after inoculation (Fig. 3a). In contrast, *CaSRC2-1* expression was not induced in leaves infiltrated with a 1 mM $MgCl_2$ buffer control (Fig. 3a). As a positive control, *CaPR1* transcripts were induced at 6 h post-inoculation and continued to accumulate thereafter. The *CaSRC2-1* transcript decreased gradually after 9 h while *CaPR1* expression significantly increased until 48 h after leaf infiltration of a non-host pathogen *Xag* 8 ra indicating that *CaSRC2-1* gene can be earlier respond to non-host pathogen infection than general plant defense genes such as *CaPR-1* was elicited (Fig. 3a). Similar induction of *CaSRC2-1* was exhibited with other bacterial pathogens including *Pseudomonas syringae* pv. syringae 61, suggesting that *CaSRC2-1* may be involved in the molecular events underlying non-host resistance prior to the appearance of a visible HR (data now shown). Intriguingly, at 6 h *CaSRC2-1* expression was stronger than that of *CaPR-1*, an indicator of non-host resistance that mediates systemic acquired resistance in response to pathogen challenge.

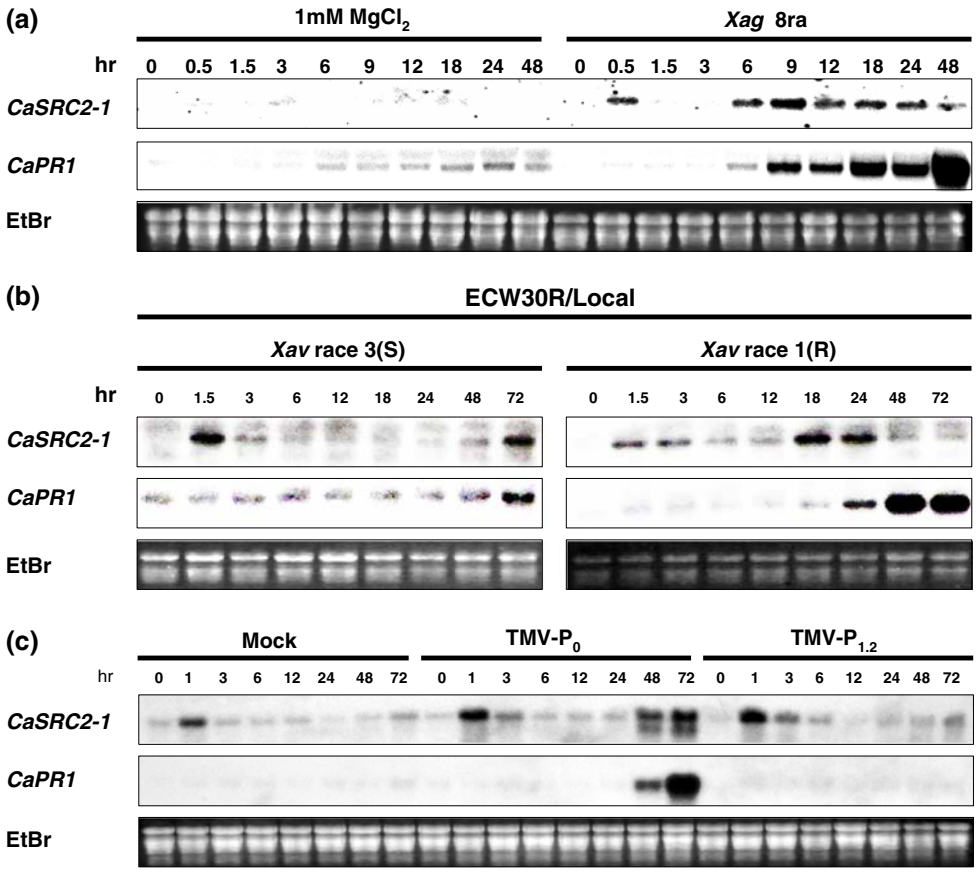


Fig. 3 *CaSRC2-1* expression in response to host and non-host pathogens. **a** Pepper leaves were infiltrated with 1 mM $MgCl_2$ (Buffer control) or a solution containing the non-host bacterial pathogen *Xag* 8 ra. **b** ECW30R pepper plants were inoculated with the pepper leaf spot pathogen, *Xav* race 1 (resistance, R) or race 3 (susceptible, S). Total RNAs were isolated from the leaves of infected pepper plants and examined by Northern hybridization using full-length ^{32}P-labeled *CaSRC2-1* or *CaPR1* cDNA probes. C) Expression patterns of *CaSRC2-1* or *CaPR*, following TMV inoculation of pepper leaves. Pepper leaves were inoculated with TMV-P_0 and TMV-$P_{1.2}$ and mock-inoculation was performed using buffer and carborundum. Total RNA was extracted at the time points indicated. Samples containing 20 μg of total RNA were blotted onto Nytran membranes and hybridization was performed with ^{32}P-labeled *CaSRC2-1*, *CaPR1*, or *CaPIN-II* cDNA probes

In addition to characterizing non-host resistance expression patterns, we examined *CaSRC2-1* expression in R gene-mediated resistance against bacterial and viral pathogens (Fig. 3b). Pepper plants of the ECW30R cultivar, which carry the *Bs3* resistance gene, were inoculated with avirulent and virulent races of the pepper leaf spot pathogen *Xanthomonas axonopodis* pv. vesicatoria (*Xav*; race 1 and 3, respectively). Following inoculation with *Xav* race 3 (susceptible condition), *CaSRC2-1* transcripts were detected at 1.5 h post-inoculation and were detected again at 48 h, with the highest levels observed at 72 h. We cannot exclude a possibility that the induction of the *CaSRC2-1* at 1.5 h can be a non-specific wound response. A similar expression pattern at 48 and 72 h was observed for *CaPR1* gene under the same conditions. In the incompatible interaction against *Xav* race1, we observed an increase in *CaSRC2-1* transcript levels at the earliest time-point (1.5 h), similar to our observation in the susceptible condition. However, expression increased significantly at 18 and 24 h under resistance conditions. As a positive control for pathogen inoculation, we monitored *CaPR1* expression; transcripts were first detected at 18 h and increased gradually to 72 h. In contrast with the susceptible interaction, *CaPR1* induction occurred at time points where *CaSRC2-1* expression had begun to decrease. Overall, the results indicated that *CaSRC2-1* expression was specifically induced as part of the pepper defense mechanism against bacterial pathogens.

To examine *CaSRC2-1* expression pattern in gene for gene resistance, pepper leaves were inoculated with *Tobacco mosaic virus* (TMV). The pepper cultivar Bukang exhibits resistance to the P_0 pathotype but is susceptible to the $P_{1.2}$ pathotype of TMV. Pepper leaves inoculated with TMV-P_0 have been shown to elicit a visible HR at 3 days post inoculation (Shin et al. 2001). Since *CaSRC2-1* transcripts were found to accumulate 1 h after inoculation with TMV-P_0 or $P_{1.2}$, as well as with the mock treatment, it is likely that expression indicates a non-specific wound response. However, *CaSRC2-1* expression was detected 48 h after inoculation with TMV-P_0 and it increased significantly by 72 h, which is when a visible HR appeared. Furthermore, *CaPR1* (positive control) exhibited a similar expression pattern in response to TMV-P_0.

We have shown that *CaSRC2-1* transcripts accumulate specifically during incompatible interactions between pepper and bacterial and virus pathogens (Fig. 3b, c) raises the possibility that CaSRC2-1 is functional in pathogen-induced defense responses in pepper plants. Recently, few results regarding the function of C2-domain containing protein in plant disease resistance have been published (Yang et al. 2006a, b, 2007). *Arabidopsis* C2-like domain containing protein BAP1 (BON1 ASSOCIATED PROTEIN1) which binds phospholipids in a calcium-dependent manner is considered to be negative regulators of defense responses against pathogenic bacteria and oomycetes because a loss of function mutant exhibited increased disease resistance (Yang et al. 2006a). BAP1 expression is also induced various stimuli such as inoculation of virulent pathogen *Pseudomonas syringae* pv. tomato DC3000, temperature stress, and SA treatments. From these results, BAP1 are speculated that is probably components of a complex pathway that negatively regulate defense responses but could be positively modulated by environmental conditions. Unlike *AtBAP1*, *CaSRC2-1* transcripts were specifically and strongly expressed under avirulent bacterial pathogen inoculation and also its expression was not affected by SA treatment (data not shown). In addition, expression patterns of *CsSRC2-1* under abiotic stress treatments quite different with that of *AtBAP1*. Taken together, these results indicate that functions of C2 domain proteins such as BAP1 and CsSRC2-1 against biotic and abiotic stresses are complex.

CaSRC2-1 expressions following treatment with defense-related signal molecules

In addition, we examined the expression patterns of *CaSRC2-1* in response to treatment with stress-related chemicals such as SA, ethephone, MeJA, and ABA (data now shown; Fig. 4). However, *CaSRC2-1* transcription was not detected following treatment with any of these chemicals or with water alone (data now shown). Pepper leaves were treated with water as negative control, since all of these chemicals were dissolved in water. In contrast, *CaPR1* expression was induced at 24 h and increased further by 48 h post-treatment with SA and ethephone, as observed previously (Yi et al. 2004; Oh et al. 2005). In addition, we monitored the expression patterns of *CaPIN-II* and *Cadhn*, as positive controls for treatment with MeJA and ABA, respectively (Shin et al. 2001; Chung et al. 2003). These results clearly indicate *CaSRC2-1* expression was not elicited by the defense-related chemicals tested in this experiment. In plants, chemicals such as SA, ethylene, MeJA and ABA, are closely related with the plant defense response (Reymond and Farmer 1998; Mauchi-Mani and Mauch 2005); they represent important signaling compounds for defense against biotic and abiotic stimuli and they act independently or coordinately in many resistance mechanisms (Thomma et al. 2001; Mauchi-Mani and Mauch 2005). However, our results show that *CaSRC2-1* expression was more affected by the calcium-signaling pathway than defense related-hormone signaling. In plants, a few C2 domain-containing proteins are known to be induced by biotic and abiotic stresses (Kim et al. 2003, 2004). In rice, *OsERG1* expression is induced strongly by treatment with a fungal elicitor or calcium, but its expression is not affected by other stresses such as H_2O_2, NaCl and SA (Kim et al.

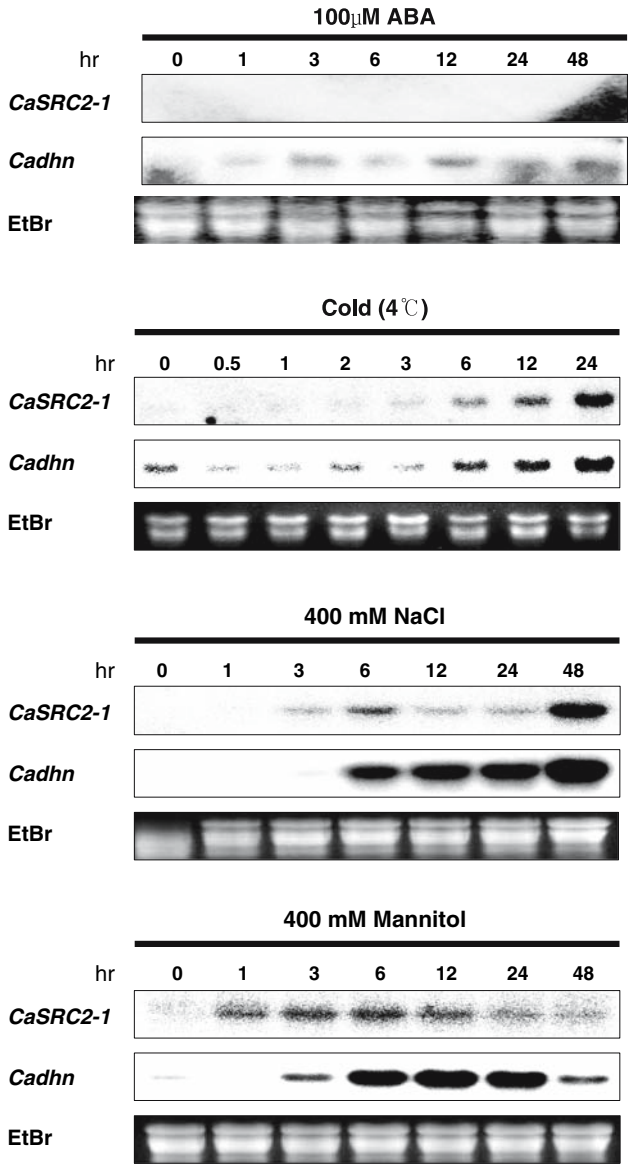


Fig. 4 *CaSRC2-1* expression following treatment with abiotic stress. *CaSRC2-1* expression in response to abiotic stresses. Detached pepper leaves were submerged in NaCl or mannitol solution. The transcriptional expression was examined at 0, 1, 3, 6, 12, 24, and 48 h after treatment. For cold stress, pepper plants were transferred to a 4°C chamber and the pepper leaves were sampled at the time points indicated. Total RNAs were isolated from the treated leaves and Northern blot was performed using ^{32}P-labeled *CaSRC2-1* or *Cadhn* cDNAs as probes

2003). Next, we examined a variety of abiotic stresses such as chilling, NaCl and mannitol, and found all three induced *CaSRC2-1* expression (Fig. 4).

CaSRC2-1 expression is elicited by cold and dehydration stresses but not by ABA treatment

Since SRC2 was first identified as a cold stress-induced gene in soybean (Takahashi and Shimosaka 1997), we examined *CaSRC2-1* expression gene in response to cold stress, as well as to water stresses such as drought and high salinity, and to ABA treatments (Fig. 4). Cold stress induced *CaSRC2-1* expression within 3 h of treatment and transcript accumulated to 24 h. *Cadhn* induction was used as a positive control; transcripts were first detected even at 0 h and increased to 24 h. In addition, *CaSRC2-1* expression was observed in response to other abiotic stresses, such as salt and drought (400 mM NaCl or mannitol, respectively). Under salt stress, *CaSRC2-1* expression was induced early (3–6 h), but its pattern appeared biphasic, with maxima at 6 and 48 h after treatment. In contrast, *CaSRC2-1* transcripts were first detected at 1 h post-treatment with mannitol, they then peaked at 6 h and decreased gradually thereafter. In mung bean, the induction of *VrPLC3* expression by abiotic stresses such as drought and salt was ABA-independent (Kim et al. 2004). In this study, we observed that although various abiotic stress treatments induced *CaSRC2-1* expression, its transcription was not induced by ABA treatment. Thus, *CaSRC2-1* exhibits similar expression patterns to known plant C2 proteins such as VrPLC3 that are controlled by ABA-independent signaling pathways (Kim et al. 2004; Wang 2002).

Conclusion

Here, we have reported that *CaSRC2-1* encodes a novel C2 domain-containing protein that is expressed during non-host resistance against the bacterial pathogen *Xag* 8 ra and in *R* gene-mediated host resistance against *Xav*. *CaSRC2-1* expression was also closely related to abiotic stress responses. CaSRC2-1 contains a single C2 domain, which plays an important role in localization at the plasma membrane, and similar C2 domains have been implicated in signal transduction and membrane trafficking processes in both plants and animals (Nalefski and Falke 1996; Kopka et al. 1998). Interestingly, the CaSRC2-1 C2 domain does not contain the five Asp residues that are predicted to participate in Ca^{2+}-dependent binding of other C2 domain-containing proteins (Fig. 1bc; Kim et al. 2003). However, *CaSRC2-1* exhibits Ca^{2+}-dependent expression and under steady state conditions its expression is root-specific. *CaSRC2-1* transcripts accumulated under abiotic stress conditions such as $CaCl_2$ and cold treatment, but they were not induced by abscisic acid, suggesting that CsSRC2-1 was involved in ABA-independent signaling during tolerance response. Taken together, our results indicate that *CaSRC2-1* encodes a novel C2 domain-containing protein, which localizes to the plasma membrane and plays a critical role in the abiotic stress and defense responses of pepper.

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