Expression and Promoter Analyses of Pepper CaCDPK4 (*Capsicum annuum* calcium dependent protein kinase 4) during Plant Defense Response to Incompatible Pathogen

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CaCDPK4, a full-length cDNA clone encoding *Capsicum annuum* calcium-dependent protein kinase 4, was isolated from chili pepper (*Capsicum annuum* L.). Deduced amino acid sequence of CaCDPK4 shares the highest homology with tobacco NpCDPK8 and chickpea CaCDPK2 with 79% identity. Genomic blot analyses revealed that CaCDPK4 is present as a single copy in pepper genome, but it belongs to a multigene family. CaCDPK4 was highly induced when pepper plants were inoculated with an incompatible bacterial pathogen. Induced levels of CaCDPK4 transcripts were also detected in pepper leaves by the treatment of ethephon, an ethylene-inducing agent, and high-salt stress condition. The bacterial-expressed GST-CaCDPK4 protein showed to retain the autophosphorylation activity in vitro. GUS expression driven by CaCDPK4 promoter was examined in transgenic *Arabidopsis* containing transcriptional fusion of CaCDPK4 promoter. GUS expression under CaCDPK4 promoter was strong in the root and veins of the seedlings. GW (-1965) and D3 (-1377) promoters conferred on GUS expression in response to inoculation of an incompatible bacterial pathogen, but D4-GUS (-913) and D5-GUS (-833) did not. Taken together, our results suggest that CaCDPK4 can be implicated on signal transduction pathway of defense response against an incompatible bacterial pathogen in pepper.

**Keywords**: CDPK, chili pepper, non-host resistance, promoter

Plants encounter various environmental stresses and also developmental changes during their life cycle. Calcium is an essential element in physiological responses and changes in cytosolic calcium concentration are involved in plant responses to various stimuli including light, environmental stresses, pathogen attack, and hormones (Evans et al., 2001; Knight et al., 1997; Sanders et al., 2002). Calcium fluxes are decoded by calcium-related genes such as calmodulin (calcium-binding protein) or calcium-regulated kinases. Among them, CDPKs are distinguished from other calcium-regulated genes since they exist only in plants, in green algae, and protozoans, but not in animals (Hrabak et al., 2003; Satterlee and Sussman, 1998).

CDPKs are a unique class of Ser/Thr protein kinases consisting of an N-terminal variable domain, a conserved kinase catalytic domain, a junction domain, and a calmodulin-like domain (CLD) with conserved calcium binding motifs (Roberts and Harmon, 1992). Calmodulin domain consists of 4 EF-hands Ca2+ binding motifs and plays a regulatory role on stimulating kinase activity in presence of Ca2+ (Harmon et al., 1987; Harper et al., 1994). Their calcium-stimulated kinase activities are independent of calmodulins (CaM), unlike calcium/calmodulin-dependent protein kinases (CaMKs; Roberts and Harmon, 1992).

CDPKs belong to a multigene family. There are 34 annotated CDPK genes in *Arabidopsis* and 29 in rice genomes, respectively (The Arabidopsis Genome Initiative, 2000; Asano et al., 2005). It is indicative that each CDPK isoform may play distinct role in plant according to its signaling cues of developmental or external stresses (Lee et al., 2003; Romeis et al., 2001). Identification of various in vivo downstream targets can implicate their diverse roles in signal cascade via Ca2+ during developmental and various extracellular signals (Cheng et al., 2002). The subcellular localization study of CDPK indicated that they are targeted to the membrane (plasma membrane or ER) (Dammann et al., 2003; Lu and Hrabak, 2002), nuclei (Patharkar and Cushman 2000), and the cytoskeleton (Putnam-Evans et al., 1989) or cytosol (Chung et al., 2004). These results indicated that the localization of each CDPK is in accordance with its necessity of phosphorylating specific target proteins (Chehab et al., 2004; Harper et al., 2004).

Increasing evidence has been provided for CDPKs being involved in biotic stress responses in plants. Elevation of CDPK transcripts was reported after exposure of tobacco
(Yoon et al., 1999), maize (Murillo et al., 2001) and tomato (Chico et al., 2002) to fungal elicitors and pepper to non-host bacterial pathogen (Chung et al., 2004). Virus-induced gene silencing (VIGS) of \textit{NicCDPK2} and \textit{NicCDPK3} resulted in delayed HR after race-specific elicitation (Romeis et al., 2001). These results suggested that \textit{NicCDPK2} and closely related subfamily members are indeed required in a defence-related signaling cascade. The \textit{Arabidopsis} \textit{AtCPK1} homologous to \textit{NicCDPK2} has also been implicated in the plant defense response (Xing et al., 2001).

In this study, we present molecular cloning of \textit{CaCDPK4} from chili pepper and expression of \textit{CaCDPK4} mRNA following inoculation of \textit{Xanthomonas axonopodis} pv. \textit{glycines} 8ra (Xag), several defense-related chemicals and osmotic stresses. In vitro kinase assay of GST-\textit{CaCDPK4} fusion protein demonstrated that \textit{CaCDPK4} gene encode a functional protein kinase. In addition, we were also able to demonstrate the \textit{CaCDPK4} expression by incompatible pathogen inoculation is correlated with GUS gene expression by monitoring expression of GUS driven by \textit{CaCDPK4} promoter deletions in transgenic \textit{Arabidopsis} plants. This evidence supports that the function of \textit{CaCDPK4} gene is closely related with signal pathway of defense response in pepper.

**Materials and Methods**

**Plant material and treatments.** Chili pepper (\textit{C. annuum} L. cv. Bukang) seeds were germinated and grown under fluorescent illumination (approximately 50 \mu mol m^{-2} s^{-1}) in a growth chamber at 24°C with 16 h light and 8 h dark photoperiod cycle. \textit{A. thaliana} (ecotype Columbia) plants were grown in the same condition as described above. Healthy leaves from about 2-month old plants were used for the treatment and following nucleic acid extraction. For RNA extraction of various parts of plant, leaves, roots, open and closed flowers and young fruits were taken from the healthy plants.

For the pathogen treatments, bacterial suspensions originated from the single colony of the freshly grown \textit{X. axonopodis} pv. \textit{glycines} 8ra (Xag) were pressure-infiltrated into pepper leaves with a needleless syringe (\textit{A}_{\text{osm}}: 1.0 in 10 mM of MgCl\textsubscript{2} buffer) (Lee et al., 2004; Park and Hwang 1999). As a mock treatment, 10 mM of MgCl\textsubscript{2} was infiltrated into pepper leaves. \textit{Arabidopsis} plant was inoculated with a non host pathogen \textit{PstT1}. \textit{PstT1} was grown in King’s medium \textit{B} (King et al., 1954) at 30°C for 2 days and then the bacterial suspension was pressure-infiltrated into the \textit{Arabidopsis} leaves with a needleless syringe (\textit{A}_{\text{osm}}: 0.05 in 10 mM of MgCl\textsubscript{2}).

To analyze the gene expression by chemical challenge, various chemicals were treated to intact pepper plants as previously described (Chung et al., 2004). SA (5 mM) was dissolved in distilled water and 100 \mu M of MeJA, or 5 mM of ethephon were dissolved in 10% acetone was sprayed onto pepper leaves. ABA solutions of 100 mM were applied by spraying to chili pepper leaves. For the osmotic stress treatments, the stems of whole plants were excised and the excised plants were placed in either 0.4 M of NaCl stock solution or 0.4 M of mannitol solution up to 24 h at room temperature. As a control experiment, the excised pepper leaves were placed in distilled water for 24 h. Sample tissues were frozen in liquid nitrogen immediately after treatment and stored at -80°C for total RNA extraction.

**Cloning \textit{CaCDPK4} cDNA using 5’ RACE (Rapid Amplification of 5’ cDNA Ends).** Chili pepper cDNA library was constructed by the standard protocol and each cDNA was amplified in \textit{E. coli} after \textit{in vivo} excision as previously described (Choi et al., 1996). Each 5’ end partial nucleotide and deduced amino acid sequences obtained were analyzed (URL: http://genomepool.kribb.re.kr; Lee et al., 2004). For all the DNA work, \textit{E. coli} strains DH5\textalpha was used. In order to obtain 5’ region of \textit{CaCDPK4}, 5’ RACE kit was used (Roche, USA). The intact poly (\textit{A})’ was used for the generation of the 1st strand cDNA and the tailed cDNA template was used for PCR-amplification with gene-specific SP1-CDPK4 primer (5’-ATGCAGAGCTCCGTCCTTATGGCAC-3’) and oligo dT-anchor primer (5’-GACCACCGGTATCGATGCAGACTC-3’). Thirty cycles of 1st PCR were carried out: annealing at 55°C, 1 min; extension at 72°C, 2 min, denaturation at 94°C, 1 min; final extension, 7 min. Second PCR was further conducted using the 1/100-diluted first-PCR reaction as a template with nested SP2-CDPK4 primer (5’-TTGCGGTTAATGCTTGTTGCTC-3’) and PCR anchor primer (5’-GACCCACTCGGTAGCTGCA-3’). The purified PCR products were ligated into pGEM T-Easy cloning vector (Promega, USA) and subsequently transformed into \textit{E. coli}. The identity of cloned PCR product to the attempted gene was confirmed by DNA sequencing analysis.

**RNA blot analyses and genomic DNA blot analyses.** Total RNAs were extracted from pepper leaves using the LiCl-phenol extraction method described by Prescott and Martin (1987). Total RNAs (30 \mu g/lane) were separated by size on denaturing formaldehyde 1.0% (w/v) agarose gels and transferred onto nylon membranes according to Sambrook et al. (1989). For the detection of \textit{CaCDPK4} RNA, PCR product containing the 3‘ UTR region (from 1,810 to 2,264) of the \textit{CaCDPK4} cDNA clone was amplified with 5-CaCDPK4 primer (5’-AGATGACCGAG-AITGAAACACACAGCG-3’) and 3-CaCDPK4 primer
Over-expression and purification of GST-CaCDPK4 fusion protein in E. coli. The entire coding sequence of CaCDPK4 cDNA was amplified using Pfu DNA polymerase with EcoRI-5′CDPK4 primer (5′-AAGATCGAATTCACATATGTTG-3′) and Sall-3′CDPK4 primer (5′-TACCTCTCAGCAATCGATGCAGCAG-3′). The PCR products were digested with SalI and EcoRI enzymes and the resulting DNA fragments were ligated into the pGEX-4T-3 expression vector (Amersham Pharmacia Biotech, USA). The fusion regions of both recombinant constructs were sequenced for confirmation of its in-frame fusion with the C-terminus of GST sequence using pGEX-F primer (5′-GGCCGATCCAGAGCCGCTTTG-3′).

GST-CaCDPK4 fusion proteins were expressed in E. coli BL21(DE3) pLyS (Stratagene, USA). Cells were grown at 37°C to an A660 of 0.7-0.8 and then induced with 1 mM of IPTG at 30°C for 2 h. The induced cells were harvested and lysed by incubation for 15 min with lysozyme (2 mg mL−1) in lysis buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride]. Dithiothreitol was added to final concentration of 5 mM and Triton X-100 to 0.1% to the lysate followed by mild sonication. After centrifugation for 20 min at 12,000 g, the supernatant was loaded to the column containing Glutathione Sepharose 4B slurry (Amersham Pharmacia Biotech, USA). The resin was washed extensively with washing buffer (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3, 1% Triton X-100). Fusion proteins were eluted with GST binding buffer (10 mM glutathione, 50 mM Tris-HCl, 1 mM EDTA, 0.02% Triton X-100). Proteins were concentrated by ultrafiltration with Centricon-50 units (Amicon, USA). Concentration of the fusion protein was determined by comparing Coomassie staining intensity of standard known BSA with the fusion protein on SDS-PAGE analysis.

In vitro autophosphorylation assay. Protein autophosphorylation assay was carried out as previously described by Putnam-Evans et al. (1990). Twenty μL reaction volume contains 1 μg sample of enzyme, 5 μCi [γ−32P] ATP (100 μM final ATP concentration) in a kinase buffer [25 mM Hepes (pH 7.0), 10 mM MgCl2]. To determine the effect of calcium on the kinase reaction, CaCl2, or EGTA was added to final concentration of 1 mM. Shrimp alkaline phosphatase (SAP; final concentration of 0.1 unit) (Roche USA) was added into the reaction to see if SAP has the ability to remove kinase activity. Reaction mixtures were incubated for 30 min at 30°C and the reactions were terminated by boiling for 5 min in 5 X Laemmli sample buffer (Laemmli, 1970). The samples were fractionated on 10% SDS-PAGE and the gels were stained with Coomassie staining solution to confirm the equal loading of protein. The gel was dried in gel drier for autoradiography.

Histological analysis of CaCDPK4 promoter-GUS fusion constructs in transgenic Arabidopsis. About 2.0-kb upstream DNA of CaCDPK4 gene was isolated using GenomeWalker™ kit (Clontech USA). Gene-specific primers of CaCDPK4 gene were used as follows; w3-CDPK4 (5′-ATCCACAAACAAAAACCTCACTTTCCACAGC-3′) and w4-CDPK4 (5′-AAGGGIATCTCAAGATTTGCAGTACTTTACACTA-3′). The resulting PCR products were cloned into pGEM-T vector (Promega USA) and they were sequenced for confirmation.

To generate the full-length promoter GUS fusion construct, the entire upstream DNA sequences of CaCDPK4 was amplified using Pfu DNA polymerase with SP6 25-mer forward primer (5′-ATTAGGTCGACACTATAGATTACT-3′) and Sall-GWCDPK4 primer (5′-ACCTCAAAACTGTCGACTAAAGGGTGCTC-3′). For the promoter deletion constructs, the promoter of CaCDPK4 was amplified using Pfu DNA polymerase with SP6 25-mer forward primer (5′-ATTAGGTCGACACTATAGATTACT-3′) and D3-GWCDPK4 primer (5′-ATGACGCAAGTCGACACAATAATTCCAG-3′), D4-GWCDPK4 primer (5′-TTGGTTTCTTTAGTGTGCAGCTCGATTTG-3′) or D5-GWCDPK4 primer (5′-AAAGCAAAACAGTCGACAGAGTTGGGG-3′). The PCR products were digested with SalI restriction enzyme and the resulting DNA fragment was ligated into the SalI sites of pb101 binary vector to generate transcriptional fusion of GUS genes. The plasmid containing each CaCDPK4 promoter-GUS fusion, GW- (full-length: 1965), D3- (1377), D4- (913) or D5-GUS (833) was transformed
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into Agrobacterium tumefaciens strain C58c1 competent cells via freeze-thaw method (An et al., 1988). Arabidopsis plants were transformed with the promoter-GUS constructs via floral-dip method using A. tumefaciens C58c1 (Clough and Bent, 1998). The transformed T1 seeds were selected after germination on kanamycin for further study. Fixation, staining, and clearing of the transgenic Arabidopsis plants with X-Gluc were performed as described previously (Jefferson et al., 1987). The mature leaves were syringe-infiltrated with PstTI (λag: 0.05) and leaf samples were harvested for GUS histochemical and fluorometric assays at 0, 3, 6 and 24 h after inoculation.

Determination of GUS activity. Leaf samples infiltrated with PstTI were collected in 1.5 ml eppendorf tube and ground in 500 μl GUS extraction buffer (50 mM NaHPO4, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na2EDTA, 0.1% Triton X-100). After centrifugation for 20 min (12,000 g) at 4°C, 5 μl of supernatant was mixed with 45 μl of GUS assay solution (1 mM 4-methylumbelliferyl-d-glucuronide in extraction buffer). Stop buffer (150 μl; 0.2 M sodium carbonate) was added to a 50 μl aliquot to be used as 0 min. The rest of the mixture was incubated at 37°C for 30 min. GUS activity was determined using the VICTOR® II fluorometer (PerkinElmer, USA) and protein concentration of tissue homogenates was determined with the Bradford reagent (BioRad, USA).

Results

Cloning CaCDPK4 induced by non-host bacterial pathogen inoculation. To study the early molecular events that occur during the defense response raised by the challenge of the non-host pathogen Xag in chili pepper plants, we examined 5 pepper CDPKs present in the pepper EST sequence database. One of those genes, referred to CaCDPK4 (Capsicum annum calcium-dependent protein kinase 4), was induced specifically by pathogen inoculation, but not by water-deficit stress (data not shown). A full-length CaCDPK4 cDNA was obtained by 5' RACE (AY904339). The entire cDNA sequence of CaCDPK4 gene is 2,437-bp long with a 448-bp long 5' UTR and a 417-bp long 3' UTR.

The evolutionary relationship among the entire deduced amino acid sequences of seven CDPKs from other plants showing high similarity with CaCDPK4 were analyzed (Fig. 1A). The most similar CDPKs to CaCDPK4 are chickpea CaCDPK2 and Nicotiana plumbaginifolia NpCDPK8 with 79% identities. Strawberry FaCDPK shares 78% identity with CaCDPK4. The most similar Arabidopsis CDPKs to CaCDPK4 were CPK8 (76% identity), CPK32 (74% identity) and CPK14 (74% identity). The identity between CaCDPK4 and rice OsCDPK is 73%. The functions of these CDPK genes including CaCDPK4 have not been identified yet.

CaCDPK4 protein is composed of 524 amino acids (estimated M.W. 59.4 kDa; pI 5.87). The peptide structure of deduced amino acid of CaCDPK4 represents a typical CDPK protein: N-terminal variable domain (1-52), the conserved kinase domain (53-311), a junction domain (312-357) and 4 calcium-binding motifs (EF-hand) (358-386; 394-422; 430-458; 466-494) (Fig. 1B). The N-terminus of CaCDPK4 has a putative myristoylation consensus sequence in Gly residue (MGNCCA...) predicted by NMT site (http://mendel.imp.univie.ac.at/myristate/SUPLpredicter.htm) (Fig. 1B). PSORT II (http://www.psort.org) predicted a putative bipartite nuclear targeting sequence (KKNNPV-SLGETV/KARKL) between 315 and 331, located within junction domain (Fig. 1B).

Genomic blot analyses and tissue-specific expression of CaCDPK4. Pepper genomic DNA blot analyses were performed for CaCDPK4 (Fig. 2A). First, blot of genomic DNA was hybridized with a probe made from PCR product covering the full-length cDNA of CaCDPK4. The full-length probe blot revealed multiple hybridizing bands, which indicated that several closely related genes of CaCDPK4 are present in chili pepper genome (Fig. 2A). Secondly, the same blot was stripped off and then hybridized with a probe made from PCR products covering 3'UTR of CaCDPK4 cDNA. As a result, one single hybridizing band was detected in the blot (Fig. 2A). These results indicated that CaCDPK4 exists as a single copy, but belongs to multigene family in chili pepper genome. Since it is known that protein kinases are developmentally regulated, Northern blot analysis was carried out for tissue-specific RNA expression (Fig. 2B). CaCDPK4 RNA was not detectable in leaf and fruit, but present in floral tissues at the basal level. Interestingly, CaCDPK4 transcript was mostly abundant in non-stressed roots.

Upregulation of CaCDPK4 in response to Xag and ethephon in pepper plants. CaCDPK4 expression was monitored in mock-treated samples compared to that in Xag inoculated samples (Fig. 3A). Steady-state mRNA CaCDPK4 was detectable at 3 h after buffer treatment, but remained at low level over time (Fig. 3A). In case of Xag inoculation, CaCDPK4 was induced as early as 1 h after inoculation and induced-level of CaCDPK4 RNA was detected until 24 h after inoculation (Fig. 3A). PR-1a expression was also monitored in the duplicated RNA blot to check when Xag inoculation was properly treated to pepper plants (Fig. 3A). In the same condition, PR-1a expression was specifically induced after 6 h in Xag-
Fig. 1. Phylogenetic analysis and structural features of CaCDPK4 and other CDPKs. (A) Evolutionary relationship of CaCDPK4 and other related CDPKs (GenBank accession numbers are given in parentheses). The tree was constructed using TreeViewX version 0.5.0 program (http://jade.cs.pusan.ac.kr/phyldraw/). The scale of bar indicates 0.1 change per amino acid. Peptide sequences are CDPKs from rice Oryza sativa OsCDPK (BAC83205), Nicotiana plumbaginifolia NpCDPK8 (CAG27839), strawberry Fragaria x ananassa FaCDPK (AAB88537), chickpea Cicer arietinum CaCDPK2 (AAP72282) and Arabidopsis thaliana CPK8 (S71778), CPK14 (T46189) and CPK32 (BT012274). (B) Alignment of deduced amino acid sequences of CaCDPK4, OsCDPK, NpCDPK8, FaCDPK, CaCDPK2, CPK8, CPK14 and CPK32. Overlines indicate each consensus domain: an N-terminus variable domain, a Ser/Thr kinase domain, a junction domain and a calmodulin domain containing four EF-hands. The putative myristoylation site in the Gly residue of the 2nd amino acid was marked as asterisk (*) and the putative bipartite NLS sequence was marked as dots (....) below the sequences in the middle of junction domain. Black boxes denote the identical amino acids and grey boxes denote highly conserved amino acids. Dashes indicate gaps in the sequence to allow for the maximal alignment. Multiple alignment of the protein sequence was carried out using the T-Coffee program (Notredame et al., 2000).
Fig. 2. Genomic DNA gel blot analyses and tissue-specific RNA expression of the CaCDPK4 gene. (A) Southern blot analyses of CaCDPK4. Genomic DNA (20 µg) isolate from chili pepper leaves was digested with EcoRI (E), HindIII (H) and XbaI (X), separated on 0.8% (w/v) agarose gel, and hybridized with the 32P-labeled probe corresponding to the full-length and to the 3’ UTR gene-specific region under high-stringency conditions as previously described (Church and Gilbert, 1984). DNA molecular mass markers are indicated at left side. (B) Steady-state of CaCDPK4 RNA accumulation in leaf, root, closed flower, open flower and fruit. Northern blot analysis was carried out as described in experimental procedures. For each northern blot analysis, an equal loading of the RNA samples on the gel was checked by ethidium bromide-staining of the ribosomal RNA (rRNA).

 inoculated samples, but was absent in the mock-treated samples (Fig. 3A). This shows that CaCDPK4 expression is specifically regulated by Xag inoculation in pepper.

During incompatible interaction of plants against pathogens, a number of chemical compounds or hormones including SA (salicylic acid), ethylene, or JA (jasmonic acid) are generated and they are known to be involved in defense signaling pathway (Ecker, 1995; Reid and Farmer, 1998). Northern blot analyses were carried out to identify a key pathogen-related signal molecule that regulates CaCDPK4 expression (Fig. 3B, 3C and 3D). The exogenous application of 5 mM SA did not induce CaCDPK4 gene except for the transient RNA expression 6 h after the treatment (Fig. 3B). PR-1a RNA blot was prepared to confirm if SA was adequately applied to chili pepper plants (Fig. 3B). PR-1a was strongly induced after 12 h of SA treatment. It indicates that the plants were properly treated with SA. Application of MeJA (methyl jasmonic acid), a representative wound hormone, resulted in the transient induction of CaCDPK4 mRNA at 1 h of treatment, but the transient levels were undetectable after that (Fig. 3C). MeJA treatment appeared to be performed adequately because PIN II (proteinase inhibitor II) was strongly induced by the MeJA treatment (Fig. 3C). Compared to low expression level of CaCDPK4 by SA or MeJA, exogenous application of 5 mM ethephon, an ethylene-producing chemical significantly turned on CaCDPK4 expression after 30 min of treatment (Fig. 3D). CaCDPK4 RNA expression was peaked at 9 h and was decreased after 12 h of the ethephon treatment (Fig. 3D). An ethylene-inducible ACC oxidase (1-aminocyclopropane-1-carboxylate oxidase) RNA blot was prepared to confirm the proper ethephon treatment (Fig. 3D). Since ACC oxidase transcript level increased specifically after ethephon treatment and reached maximum at 24 h, ethephon treatment to chili pepper plants appeared to be adequate for ethylene experiment (Fig. 3D). Collectively, ethylene may be the major regulator in the induction of CaCDPK4 in chili pepper.

Induction of CaCDPK4 in response to high salt stress. CaCDPK4 was not induced by water-deficit stress at all (data not shown), but was weakly expressed in the buffer-infiltrated samples (Fig. 3A). Northern blot analysis was performed to investigate whether osmotic stresses or ABA treatments induce transcripts of CaCDPK4 in pepper. For the osmotic stresses, pepper plants were exposed to high concentration of NaCl (0.4 M) or mannitol solution (0.4 M). Salt (0.4 M NaCl) treatment rapidly induced CaCDPK4 gene expression as early as 30 min after the treatment and the induced level of CaCDPK4 mRNA was sustained up to 12 h of the treatment (Fig. 4A). Comparably, application of the high-concentration (0.4 M) of mannitol mildly induced CaCDPK4 from 1.5 h to 3 h and then its expression was diminished away after 6 h of the treatment (Fig. 4B). An osmotic-stress specific chili pepper Cadhn gene (Capsicum annuum dehydrin) (Chung et al., 2003; Yi et al., 2004) was strongly induced by both stresses (Fig. 4A and 4B). These results imply that both stresses resulted in serious osmotic stresses, but there are other factors that control CaCDPK4 gene expression by high salt stress. It is known that osmotic stress induces abscisic acid (ABA) biosynthesis and ABA-dependent signal transduction pathways in plants (Bray 1993). Northern blot analysis was performed to assess if ABA induces CaCDPK4 expression in relation to osmotic stresses in pepper plants (Fig. 4C). The exogenous appli-
Fig. 3. Expression pattern analyses of CaCDPK4 in chili pepper leaves treated with incompatible pathogen Xag, or various signal molecules. (A) Time-course RNA samples were prepared from the leaves syringe-infiltrated with 10 mM MgCl₂ (Mock) or Xag suspension cells. (B) RNA blots were carried out with RNA samples prepared from the leaf tissues treated with 5 mM of SA stock solution. RNA blots were hybridized with probes for CaCDPK4 and PR-1a (A and B). (C) RNA expression of CaCDPK4 and PIN II upon 100 μM of JA treatment to the pepper plants. Plants were sprayed with stock solution of 100 μM of MeJA. The RNA blot was hybridized with 32P-labeled CaCDPK4 and PIN II probe as a marker gene. (D) Northern blot analyses were performed with RNA samples prepared from the tissues treated with 5 mM of ethephon stock solution. The RNA blot was hybridized with 32P-labeled CaCDPK4 and ACC oxidase probe as a marker gene. RNA blot analyses were performed as described in experimental procedures.

Induction of 100 μM ABA induced CaCDPK4 transiently at 1.5 h after the treatment and CaCDPK4 mRNA expression was not induced after that point in chili pepper plants (Fig. 4C). ABA treatment was properly carried out in pepper plants because Cadh expression was strongly induced from 1.5 h to 6 h after ABA treatment. Based on these expression data, we concluded that the expression of CaCDPK4 gene is affected by high salt rather than triggered by ABA signaling pathway in chili pepper plants.

In vitro autophosphorylation assay of GST- CaCDPK4. To determine whether CaCDPK4 encodes a functional protein kinase, we conducted kinase assays with the purified GST-fused CaCDPK4 expressed in E. coli. The full-length coding sequence of CaCDPK4 cDNA was fused to the C-terminus of GST in-frame and the recombinant proteins were expressed in E. coli. The GST fusion protein was further purified and the size of the purified protein was slightly larger than 92 kDa of molecular weight marker (Fig. 5A). The purified GST fusion proteins were digested with thrombin and two bands were derived from the digestion reaction corresponding to GST (M.W.; 30 kDa) and CaCDPK4 (M.W.; 59 kDa) at their expected size (Fig. 5A).

To examine if the recombinant protein functions as a kinase, in vitro autophosphorylation assay was performed using GST-CaCDPK4 fusion protein as described in experimental procedures (Fig. 5B). The autophosphorylation activity remained at basal level without the addition of extra calcium ion. Kinase activity was slightly increased by the addition of calcium ion. The presence of calcium chelator, EGTA, abolished kinase activity. SAP is a general phosphatase enzyme that dephosphorylates 5′ phosphates from the phosphorylated molecules. The addition of SAP to the kinase reaction resulted in complete loss of autophosphorylation activity. In vitro autophosphorylation assay indicates that the recombinant CaCDPK4 protein functions as a kinase in vitro system.
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**Fig. 4.** Time courses of *CaCDPK4* expression in detached chili pepper leaves exposed to osmotic stresses or exogenous ABA feeding. (A) RNA accumulation of *CaCDPK4* and *Cadhn* in detached chili pepper leaves treated with high-salt stress of 0.4 M NaCl. (B) RNA levels of *CaCDPK4* and *Cadhn* in detached leaves placed in high-osmoticum solution of 0.4 M of mannitol. (C) RNA expressions of *CaCDPK4* and *Cadhn* in leaves exogenously fed by 100 μM of ABA stock solution. The blots were hybridized with either *CaCDPK4* or *Cadhn* probe. Northern blot analyses were performed as described in experimental procedures.

**Fig. 5.** Purification and *in vitro* autophosphorylation assay of the GST-*CaCDPK4* fusion protein. (A) Purification and digestion of GST-*CaCDPK4* fusion protein. GST-*CaCDPK4* fusion protein was expressed in *E. coli*, purified and concentrated as described in experimental procedures. Samples of a portion of the fusion protein, GST and the digestion reactions with thrombin protease were size-fractionated using 10% SDS-PAGE followed by Coomassie blue staining. Molecular weight marker is included as a size comparison. (B) *In vitro* kinase assay of GST-*CaCDPK4* recombinant protein. Autoradiogram of the autophosphorylated GST-*CaCDPK4* protein analysed on 10% SDS-PAGE. The recombinant protein (2 μg) was autophosphorylated in the kinase reaction buffer (10 mM MgCl₂, 25 mM Hepes pH 7.0) added by either free calcium (1 mM final concentration) or by EGTA (1 mM final concentration) or shrimp alkaline phosphatase (SAP) and electrophoresed on a 10% polyacrylamide gel in the presence of SDS. The gel was dried for the autoradiogram.

**GUS analysis of transgenic *Arabidopsis* plants containing the *CaCDPK4* promoter fused to GUS reporter gene.** About 2.0 kbp upstream DNA fragment of *CaCDPK4* was isolated using GenomeWalker™ kit (Clontech) from chili pepper genome (Fig. 6A). The entire upstream DNA sequences (GW-GUS: from -1965 to -48) and each deleted promoter (D3-GUS: from -1377 to -48, D4-GUS: from -913 to -48 and D5-GUS: from -833 to -48) were fused to GUS reporter gene (Jefferson et al., 1987) as a transcriptional fusion (Fig. 6A).

GUS histochemical staining was analyzed in *CaCDPK4* GW-GUS transgenic *Arabidopsis* plants (Fig. 6B). Histochemical staining of untreated transgenic *Arabidopsis* revealed that GUS expression was strongly detected in roots, stems, and cotyledons from the seedlings (Fig. 6B). GUS staining was strong in the veins of the cotyledons and shoots of the seedlings. GUS expression was weaker in upper leaves than cotyledons and older leaves (Fig. 6B).
GUS expression was restricted to the stigma of the pistil and to the junction part of siliquae from the reproductive organ (Fig. 6B).

Differential expression of CaCDPK4 in response to incompatible pathogen infection prompted us to examine the GUS expression driven by CaCDPK4 promoter after
Expression and Promoter Analyses of Pepper CaCDPK4

Fig. 7. GUS histochemical and fluorometric assays in response to incompatible pathogen infection in CaMV 35S-GUS, CaCDPK4 promoter GW-, D3-, D4- and D5-GUS transgenic Arabidopsis. (A) GUS expression pattern in mature leaves from CaMV 35S-GUS and CaCDPK4 GW-, D3- and D4-GUS transgenic plants in response to incompatible pathogen infection. Bacterial suspension cells (P. syringae pv. T1) were syringe-infiltrated into the leaves and the leaf samples were harvested at 0, 3, 6 or 24 h of inoculation, respectively. (B) GUS activity in mature leaves from CaMV 35S-GUS and CaCDPK4 GW-, D3-, D4- and D5-GUS transgenic plants in response to incompatible pathogen infection. GUS fluorometric assays were carried out as described in experimental procedures using each transgenic Arabidopsis line infiltrated with PstT1.

incompatible pathogen inoculation (Fig. 7). Pseudomonas syringae pv. tomato T1 (PstT1) is an incompatible pathogen in Arabidopsis (ecotype Columbia) (Whalen et al., 1991). Bacterial suspension cells of PstT1 were infiltrated on to the leaves and time-course GUS expression was monitored in CaMV 35S-GUS and CaCDPK4 GW-, D3-, D4-GUS transgenic Arabidopsis plants (Fig. 7A). At the sites of inoculation, GUS expression started to be evident between 3 h and 6h after inoculation in both CaMV 35S-GUS and CaCDPK4 GW-, D3- and D4-GUS transgenic Arabidopsis plants (Fig. 7A). D5-GUS did not have any GUS expressions by any treatment (data not shown). In CaMV 35S-GUS plants, GUS staining at the site of infection weakened at 6 h and disappeared at 24 h of inoculation (Fig. 7A). However, GUS staining became stronger in the neighboring tissues of the infected sites at 6 h and at 24 h of inoculation in CaCDPK4 GW- and D3-GUS transgenic plants (Fig. 7A). GUS staining pattern was correlated with GUS activity measured in CaMV 35S-GUS, CaCDPK4 GW- and D3-GUS transgenic plants (Fig. 7B). Weak GUS expression disappeared at 24 h after inoculation in D4-GUS transgenic line (Fig. 7A). However, GUS activity was negligible in
D4-GUS transgenic line after PstI infection (Fig. 7B). These results lead us to conclude that DNA region between -1377 and -913 including GW and D3 contains an important DNA sequences for GUS expression following incompatible pathogen inoculation.

**Discussion**

Previously we proposed that pepper CaCDPK3 gene is implicated on common signaling pathway of water-deficit stress and incompatible pathogen interaction (Chung et al., 2004). In this study, we were able to demonstrate that CaCDPK4 is transcriptionally regulated by incompatible pathogen, ethylene treatments and salt stress in pepper. This provides new insights of CaCDPK4 gene playing an important role in signaling pathways of defense response against pathogen and salt stress in pepper.

Inoculation of incompatible pathogen elicits rapid programmed cell death known as hypersensitive response (HR) in plants (Heath, 2000). CDPK has been reported one of the signal components in the early response of signal transduction pathway in HR response. A number of CDPK genes were transcriptionally regulated in response to pathogen inoculation or elicitor treatments (Chung et al., 2004; Murillo et al., 2001; reviewed by Ludwig et al., 2004). Rapid transcriptional induction of CaCDPK3 and CaCDPK4 by incompatible pathogen inoculation tends to be similar (Fig. 3A; Chung et al., 2004). Transcriptional regulation of CaCDPK4 appears to be specific to pathogen since the expression of CaCDPK4 mRNA was much weaker in the buffer-infiltrated leaves than that of CaCDPK3 (Fig. 3A). Weak expression of CaCDPK4 mRNA expression is probably caused by hypo-osmotic stress or wounding effect. It was reported that the activation of tobacco NtCDPK2 was shorter and weaker by the osmotic stress than by fungal elicitor treatments (Romeis et al., 2001). Ludwig et al. (2004) suggested that overactivation of AtCDPK2 leads to a functional cross-talk between abiotic and biotic signaling pathways. Both CaCDPK4 and CaCDPK3 are induced by incompatible pathogen, but the responses of CaCDPK4 to chemical signals are different from those of CaCDPK3 gene (Fig. 3B, 3C and 3D; Chung et al., 2004). Previously, CaCDPK3 mRNA accumulation was induced by treatments of SA, JA or ethephon, an ethylene-inducing agent (Chung et al., 2004). In contrast, SA or JA treatment induced transiently expression of CaCDPK4 (Fig. 3B and 3C), but ethephon did. Possibly, ethylene may play a role in the stress induction of CaCDPK4 during pathogen infection (Fig. 3D).

CDPK genes are also known to be involved in signaling pathway of osmotic stresses in plants (Botella et al., 1996; Patharkar and Cushman, 2000; Saijo et al., 2000). Salt stress appears to have the stronger effect on the expression of CaCDPK3, CaCDPK4 and Cadlin than mannitol stress (Fig. 4A and 4B; Chung et al., 2004). The levels of CaCDPK3 and Cadlin mRNA lasted up to 24 h after mannitol stress, but CaCDPK4 mRNA abundance was dramatically reduced 6 h after the stress. CaCDPK4 mRNA abundance was barely detected in the ABA-applied pepper leaves compared to that of CaCDPK3 and Cadlin (Fig. 4C; Chung et al., 2004). This result indicated that expression of CaCDPK4 is not regulated by ABA, but CaCDPK3 is. This result may imply different roles of CaCDPK4 and CaCDPK3 in stressed conditions of plant.

The structure of CaCDPK4 represents a typical CDPK containing the N-terminal variable domain, the Ser/Thr kinase domain, the calcium-binding domain (calmodulin) and junction domain known as an inhibitory domain (Fig. 1B). Heterologously expressed CDPK proteins have been shown to contain calcium-dependent autophosphorylation activity in vitro (Harper et al., 1994; Urao et al., 1994; Yoon et al., 1999). Purified GST-CaCDPK4 also shows autophosphorylation activity in vitro (Fig. 5B). However, kinase activity of GST-CaCDPK4 did not increase in the presence of extra calcium ion, but that of this protein was dramatically reduced by the addition of EGTA or SAP. These results indicated that CaCDPK4 encodes a functional kinase indicated by autophosphorylation assay using E. coli expressed GST-CaCDPK4 in vitro.

CaCDPK4 RNA was most abundant in untreated root tissues (Fig. 2B) and subsequently, the GUS expression was very strong in the roots in CaCDPK4 full-length promoter (GW-GUS) transgenic Arabidopsis (Fig. 6B). Promoter deletion analysis could be used as a way for studying the regulatory elements and factors that affect the functions of specific gene. In this study, we used CaCDPK4 deletion promoter analysis to narrow down the promoter region responsible for GUS expression during HR response. GUS expression in the GW- and D3-GUS transgenic plant was specifically responsive to PstI treatments (Fig. 7A and 7B). This result may indicate that DNA elements responsive to HR reside in the D3 (from -913 to -1377) of CaCDPK4 promoter.

Using PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE), we were able to find putative cis-acting DNA elements, G-box (ACGT core sequences) and W-box (TGAC core sequences) in CaCDPK4 promoter (Fig. 6A). The G-box is the cis-acting DNA-elements found in the upstream region of a number of genes regulated by various signals including ethylene (Whitelaw et al., 1997) and ABA (Kang et al., 2002; Marcotte et al., 1989). GW and D3 have two G-boxes (ACGT) while D4 has one G-box (Fig. 6A). Weak GUS expression in D4-GUS line may be indicated by the fact that D4 promoter has only one G-box, while GW
and D3 promoters have both G-boxes (Fig. 6A). Another putative cis-acting element, W-box (TGAC) was located within D5 promoter (Fig. 6A). W-box has been to be found in a number of pathogen-related gene promoters and WRKY transcription factors have been shown to bind to the W-box (Niggeweg et al., 2000; Yu et al., 2001). It is notable that D5 promoter does not direct any GUS expression by any treatments although it has one W-box consensus cis-acting DNA element (TGAC). These results indicate that the W-box in D5 did not confer any GUS expression during incompatible pathogen treatments. It can be inferred that GUS expression driven by the CaCDPK4 promoter may be due to the presence of the G-box, but not that of the W-box during non-host pathogen responses. However, at this point it is impossible to speculate which signal directs the G-box controlling CaCDPK4 gene expression during the stresses. Further characterization of the defined CaCDPK4 promoter could provide more insight on the regulatory elements and factors that affect roles of CaCDPK4 during stress conditions.

Based on CaCDPK4 RNA expression and GUS expression driven by CaCDPK4 promoter, we were able to conclude that CaCDPK4 is mainly regulated by HR response, ethylene and salt stress in plants and also highly expressed in the unstressed root tissues. According to CaCDPK4 promoter deletion analysis, the promoter region between -913 and -1377 was necessary for GUS expression by incompatible pathogen treatments. Taken together, these results may suggest that CaCDPK4 have a role in defense response of pepper plant against pathogen and salt stress and also developmental response especially in the roots.

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References


