Plant Pathol. J. 23(2) : 76-89 (2007)

The Plant Pathology Journal

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

Eunsook Chung[1], Sang-Keun Oh[2], Jeong Mee Park[2] and Doil Choi[3,*]

[1]*BK21 Center for Silver-Bio Industrialization, Dong-A University, Busan 604-714, Korea*

[2]*Plant Genome Research Center, Korea Research Institute of Bioscience & Biotechnology, Yusung, Daejeon 305-333, Korea*

[3]*Department of Plant Sciences, Seoul National University, Seoul 151-921, Korea*

(Received on March 5, 2007; Accepted on May 7, 2007)

***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 Ca^{2+} binding motifs and plays a regulatory role on stimulating kinase activity in presence of Ca^{2+} (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 exterior 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 Ca^{2+} during developmental and various extracelluar 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

*Corresponding author.
Phone) +82-2-880-4568, FAX) +82-2-873-2056
E-mail) doil@snu.ac.kr

(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 *NtCDPK2* and *NtCDPK3* resulted in delayed HR after race-specific elicitation (Romeis et al., 2001). These results suggested that *NtCDPK2* and closely related subfamily members are indeed required in a defence-related signaling cascade. The *Arabidopsis AtCPK1* homologous to *NtCDPK2*, has also been implicated in the plant defense response (Xing et al., 2001).

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

Materials and Methods

Plant material and treatments. Chili pepper (*C. annuum* L. cv. Bukang) seeds were germinated and grown under fluorescent illumination (approximately 50 μmol m^{-2} s^{-1}) in a growth chamber at 24°C with 16 h light and 8 h dark photoperiod cycle. *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 *X. axonopodis* pv. *glycines* 8ra (*Xag*) were pressure-infiltrated into pepper leaves with a needleless syringe (A_{600}; 1.0 in 10 mM of $MgCl_2$ buffer) (Lee et al., 2004; Park and Hwang 1999). As a mock treatment, 10 mM of $MgCl_2$ was infiltrated into pepper leaves. *Arabidopsis* plant was inoculated with a non host pathogen *PstT1*. *PstT1* was grown in King's medium B (King et al., 1954) at 30°C for 2 days and then the bacterial suspension was pressure-infiltrated into the *Arabidopsis* leaves with a needleless syringe (A_{600}; 0.05 in 10 mM of $MgCl_2$).

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 μM 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 *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 *E. coli* after *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://genepool.kribb.re.kr; Lee et al., 2004). For all the DNA work, *E coli* strains DH5α was used. In order to obtain 5' region of *CaCDPK4*, 5' RACE kit was used (Roche, USA). The intact poly $(A)^+$ 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'-ATGCATGACTCCGTGCTTATGGCAC-3') and oligo dT-anchor primer (5'-GACCACGCGTATCGATGTCGACT_{16}-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'-TTGCCTTCAATGGTGCTGTCTC-3') and PCR anchor primer (5'-GACCACGCGTATCGATGTCGAC-3'). The purified PCR products were ligated into pGEM T-Easy cloning vector (Promega, USA) and subsequently transformed into *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 μg/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 *CaCDPK4* RNA, PCR product containing the 3' UTR region (from 1,810 to 2,264) of the *CaCDPK4* cDNA clone was amplified with 5-CaCDPK4 primer (5'-AGATGACGAGATTGAAACCAACAGCG-3') and 3-CaCDPK4 primer

(5'-TCCTTCACCATATTCCCTCAGATCGTCC-3') for the probe synthesis. PCR product containing the individual cDNA clone of *CaCDPK4*, *Cadhn*, *PR-1a*, *ACC oxidase* or *PIN II* genes was labeled with [α-^{32}P] dCTP by random priming (Promega, USA) as previously described (Oh et al., 2005). RNA hybridization, washing, and detection were carried out by a standard procedure as previously described (Chung et al., 2003).

Genomic DNA of chili pepper plants was prepared according to the method of Dellaporta et al. (1983). Twenty mg of total DNA was digested with *Hind* III, *Eco*R I, or *Xba* I. Digested DNAs were separated on 0.8% (w/v) agarose gel. Southern transfer was carried out by the standard method (Sambrook et al., 1989). For the genomic DNA detection, PCR products corresponding to the full-length or the 3' UTR region of *CaCDPK4* cDNA were used as probes. Probe labeling, blot hybridization and washing conditions were used as previously described (Chung et al., 2003). Dried blots were placed on X-ray film at -80°C for a week before developing.

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-5CDPK4 primer (5'-AAGATCGAATTCAATGGGTAATTG-3') and SalI-3CDPK4 primer (5'-TACTCTTCAAGTCGACATAAATCTGGC-3'). The PCR products were digested with *Sal* I and *Eco*R I enzymes and the resulting DNA fragments were ligated to 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'-GGGCTGACAAGCCACGTTTG-3').

GST-CaCDPK4 fusion proteins were expressed in *E. coli BL21(DE3) pLys* (Stratagene, USA). Cells were grown at 37°C to an A_{600} 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 phenylmethanesulfonyl 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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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)HHH . 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-^{32}P] ATP (100 uM final ATP concentration) in a kinase buffer [25 mM Hepes (pH 7.0), 10 mM $MgCl_2$]. To determine the effect of calcium on the kinase reaction, $CaCl_2$, 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.

Histochemical 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'-ATCCACACAACAAAACCTCAACTTTCACAGC-3') and w4-CDPK4 (5'-AAGGGTATCTCAAGATTGCACTTTACACTA-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'-ATTTAGGTGACACTATAGAATACTC-3') and SalI-GWCDPK4 primer (5'-ACCTCAAAACTGTCGACTAAAAGGGTCTC-3'). For the promoter deletion constructs, the promoter of *CaCDPK4* was amplified using *Pfu* DNA polymerase with SP6 25-mer forward primer (5'-ATTTAGGTGACACTATAGAATACTC-3') and D3-GWCDPK4 primer (5'-ATGACAAGTCGACACAATAAATCTTTCG-3'), D4-GWCDPK4 primer (5'-TTGGTTCTTGATGTCGACAATTTGGG-3') or D5-GWCDPK4 primer (5'-AAGCAACAACGTCGACAGTTAGGGAAC-3'). The PCR products were digested with *Sal* I restriction enzyme and the resulting DNA fragment was ligated into the *Sal* I sites of pBI101 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

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 media 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 *PstT1* (A_{600}: 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 *PstT1* were collected in 1.5 ml eppendorf tube and ground in 500 μl GUS extraction buffer (50 mM $NaHPO_4$ pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na_2EDTA, 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^2 D 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 *CDPK*s present in the pepper EST sequence database. One of those genes, referred to *CaCDPK4* (*Capsicum annuum 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/SUPLpredictor.htm) (Fig. 1B). PSORT II (http://www.psort.org/) predicted a putative bipartite nuclear targeting sequence (KKNPNV-SLGETVKARKLK) 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 probed 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*-

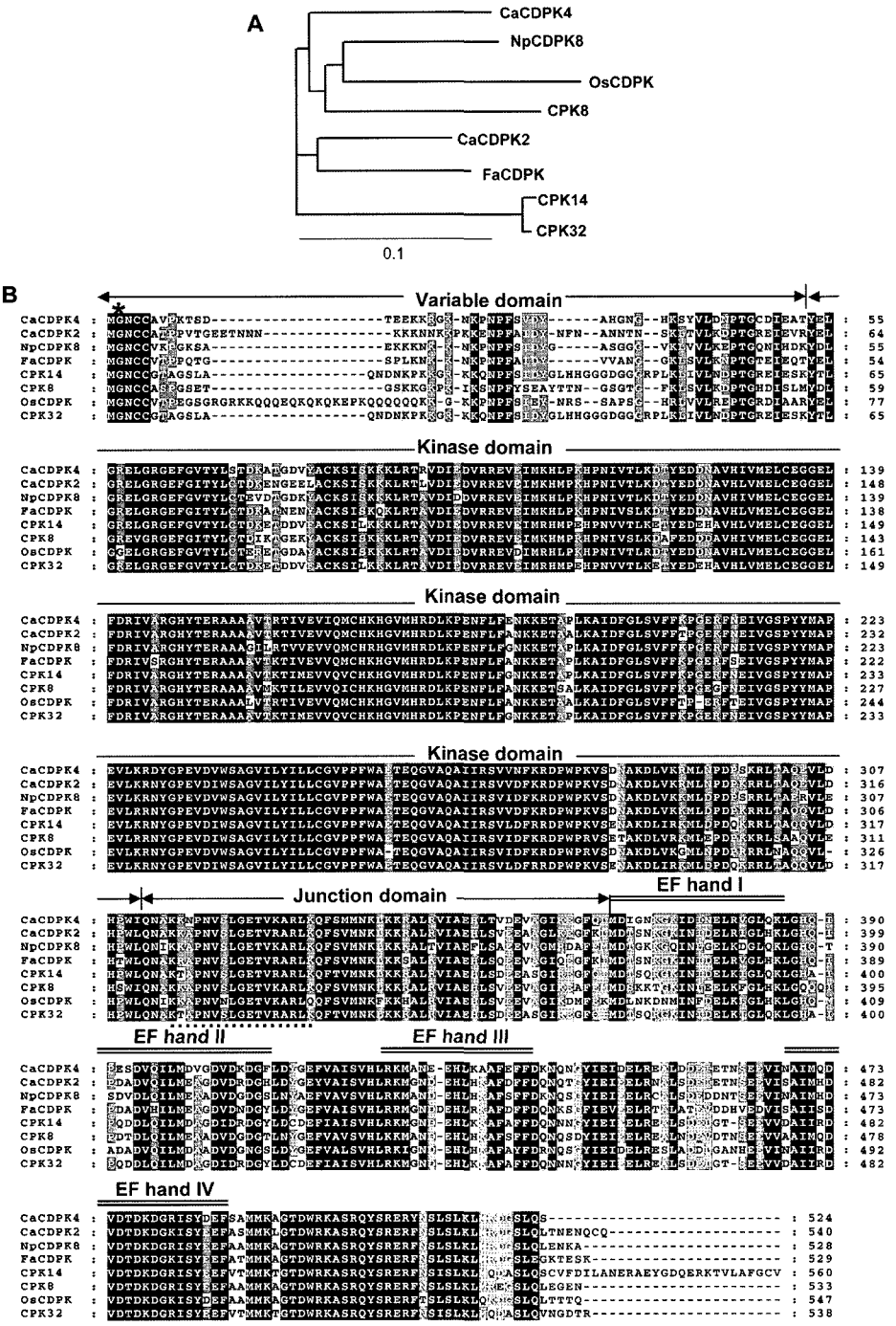

Fig. 1. Phylogenic analysis and structural features of CaCDKP4 and other CDPKs. (A) Evolutionary relationship of CaCDPK4 and other related CDPKs (GenBank accession numbers are given in parentheses). The tree was constructed using TreeViewX version 0.5.0 program (http://jade.cs.pusan.ac.kr/phylodraw/). 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).

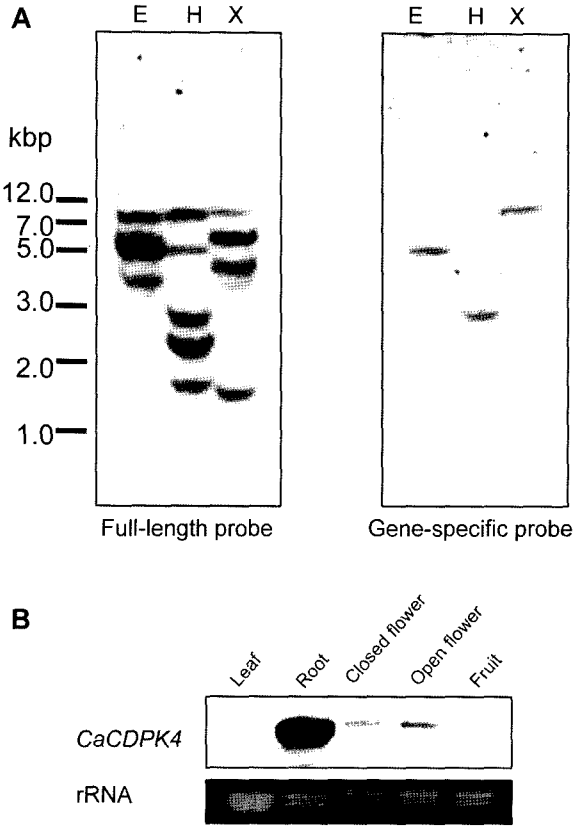

Fig. 2. Genomic DNA gel blot analyses and tissue-specific RNA expression of the *CaCDPK4* gene. (A) Southern blot analyses of *CaCDPK4*. Genomic DNA (20 μg) isolate from chili pepper leaves was digested with *Eco*R I (E), *Hin*d III (H) and *Xba* I (X), separated on 0.8% (w/v) agarose gel, and hybridized with the ^{32}P-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; Reymond 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-

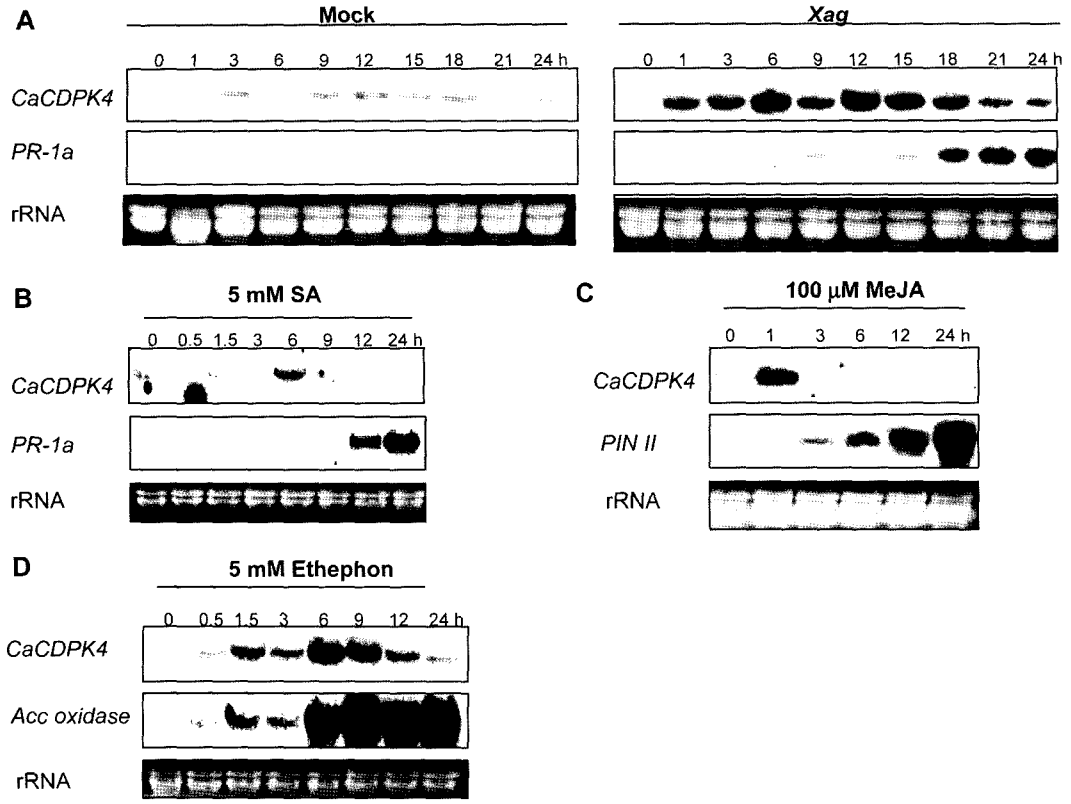

Fig. 3. Expression pattern analyses of *CaCDPK4* in chili pepper leaves treated with incompatible pathogen *Xag*, or various signal molecules. (A) Time-course RNA samples were prepared from the leaves syringe-infiltrated with 10 mM $MgCl_2$ (Mock) or *Xag* suspension cells. (B) RNA blots were carried 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 ^{32}P-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 ^{32}P-labeled *CaCDPK4* and *ACC oxidase* probe as a marker gene. RNA blot analyses were performed as described in experimental procedures.

cation 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 *Cadhn* 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.

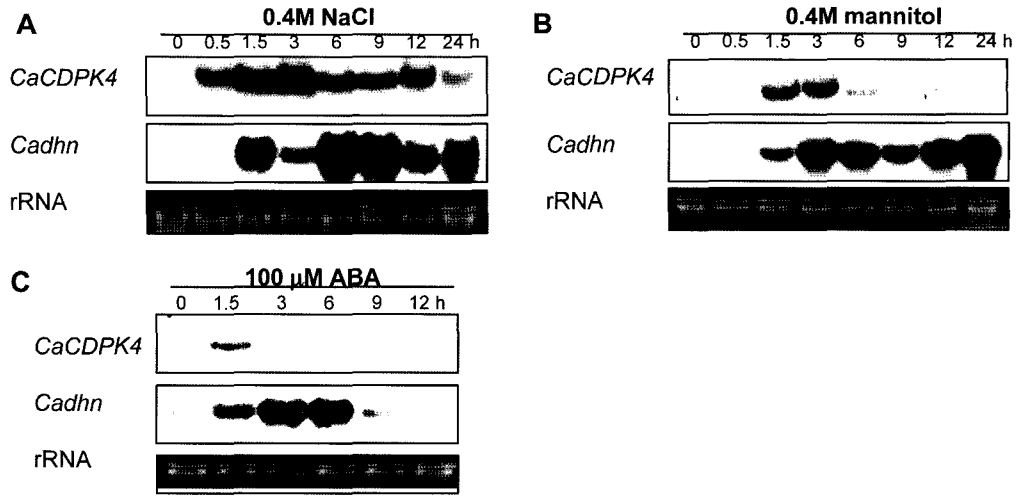

Fig. 4. Time courses of *CaCDPK4* expression in detached chili pepper leaves exposed to osmotic stresses or exogenous ABA feeding. (A) RNA accumulation of *CaCDPK4* and *Cadhn* in detached chili pepper leaves treated with high-salt stress of 0.4 M NaCl. (B) RNA levels of *CaCDPK4* and *Cadhn* in detached 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.

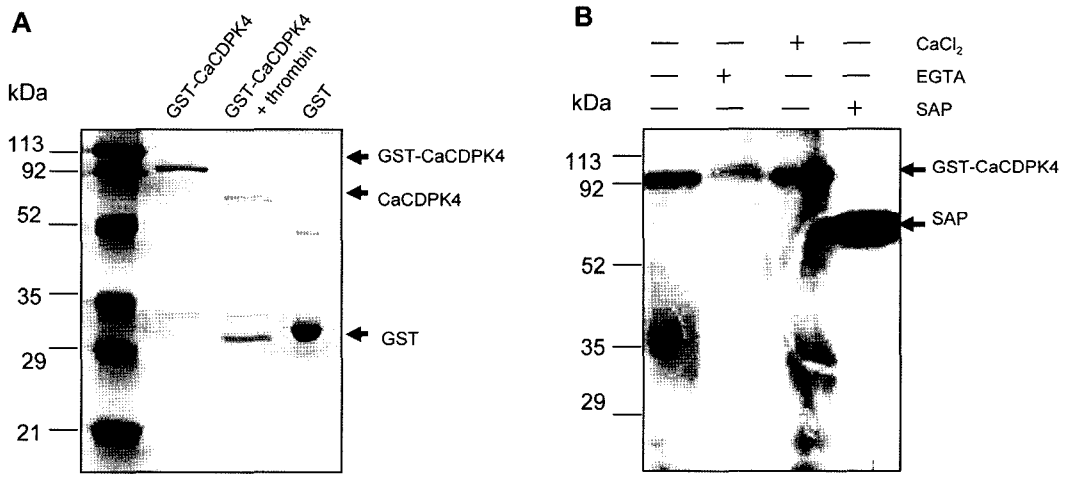

Fig. 5. Purification and *in vitro* autophosphorylation assay of the GST-CaCDPK4 fusion protein. (A) Purification and digestion of GST-CaCDPK4 fusion protein. GST-CaCDPK4 fusion protein was expressed in *E. coli*, purified and concentrated as described in experimental procedures. Samples of a portion 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 $MgCl_2$, 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).

A

```
-1965  AGATCTGCTCCTGAGTTATGTAGCTTTCTAATCGCAAATCAAATGTCTCTTCAATTTTTACATGAATAACT
-1894  TGTATTTAAATCAGCTATCAAATACGATATAACTTAAGCACAATTCGATATATACTTTGCATAATTCACCT
-1823  CCAAACCAACTAACAAGCGATCCTTGAATCACCAACTAAACAAAAGGCAGCTCAGTGCAATAAACCTCCCG
-1752  CTATGCGCGGTCCGAGGAAGGGTCGAACTATAATAATTCATTGTATGCAATCTTATCCCACATTTTTATAA
-1681  GATGTTGTTTTCATGACTTGAACTCGTGACCTCTAGATCATACATCAATAACTTTTACCAGTTACGCTAAG
-1610  ACTTCCCTTGGGAATCACCAACCAAACAAAAGGCAGCTCGATGCAATAAATCTTCCGCTATGCGCAGTTTG
-1539  AGTAAGGGCCGAACCACATTAATTTATTGTACGCAATTTTACCCCACATTTTTACAAGATGTTGTTTTCAC
-1468  GACTTAAACTCGTGACCTCCAAGTCATATGTCAACAACTTTTACCATTTACTCTAAGACCAACCTTCGGAA
-1397  TCACCAACCAAACAAATGACAACTCGATACAATAAATCTTTCGCCATGCGCGATCCGAGAAAAGGCCGAAC
                G-box II                  D3 (-1377)
-1326  CACAACAACGTAACTTACCTTATATCATATTTTTACAAGATATTGTTTTTACGACTTGAACTCGTGACCTC
-1255  CAGGTTAGATGTCAACAACTTCTGCCAGTTACTCTACGACTTCCCTTCGAAATCACCAACCAAACAATCCC
-1184  TAAGAAATTAAACATTATTGGAGGGAGAGGGTTGGGGTGGGGGTGGGGGTGGTGGGATAGGACTCGGTGGT
-1113  GATGACAAAAGAAAACAAGATGGAGTACTTTTTGTAAACAAATTATTACTAGCCAATACTTTTTCACCAAC
-1042  ATGCATTTGTTACAAATAATGACATTTTTTTAAAAATAAACAAAACATATACTTAGTACTTTTTTTATTTT
 -971  TTTATTTTTTTATTTTTCAAAACCAAACCATTTTGTTTATTCCAATTGGTTCTTGATTTGGACAATTTGGG
                                                              D4 (-913)
 -900  AAAAACAAGAAGTGGTCAAAAAATACACACACATTCACATTAGAAGTGTTTGAAGCAACAACGTGCATAGT
                                                        G-box I
 -829  TAGGGAAGTTTCCATGAAACTAATTTTGACCTTTGCTCTCTCTCTCTCTCTTCTCTTGTTTCTATAATTTA
       D5 (-833)              W-box                CAAT-box
 -758  GCCTTGTTCACATATTAATTTTCTGTTCAAGATCCTCTTTTTAATCTACAAATACTTTGATTTTTCAATTG
 -687  TTGTACAAAAAACCTCAGGGTTTGTTGTTCCTTTTATTGGGTTTTTGTGAGATCTATGATTTTTCTTAGGTA
       TATA-box
 -616  GTATTTTGGTGTTTTTGAGGATTTAAAGGGACCCCACTTGCTTGTTTTCTTGATTTTTTGTGTAAAAATTG
 -545  GATCTTGGTTGAAGTTTTTGAGGATTTAAGGGAACCCAACTTGCTTGGTTTTCTTGATTTATGTGTTCGTT
 -474  TTTGGAGAAGTTGGATCTTGATTGAAGTTTGACTTAGGGTAAAGTTGCAATCTTGAGATACCCTTTTGGCG
 -403  TTGGAGGTTAATTGAGTGAAAAATTTGGTGTTTTAGCTGTGAAAGTTGAGGTTTTGTTGTGTGGATTTGTT
 -332  GGGGAAAGGTAAAATTTTGAGACCCTTTTTGTTTTAGCTGAAAAATTGGATCTTGGTTGAAGTTTGAGTAG
 -261  TGTAAAGTTGCAATCTTGAAAGAGACTTTTGAGGTTAATAGAGTGGAAATTGGTGATTTAGCTGTGAAAGT
 -190  TGAGATTTTGTTGTGTTGATTTAGTGGGGAAAGGTAAGATTTTTTTGACCCTTTTAGTTTGAGGTGAAAAT
 -119  TGGATTTTTACCTGTGAGAATTGAAGCTTTTTGTTGTGTTAGTTTGGTGGGAAAGTTAAGATCTTGAGACC
  -48  CTTTTAGCTGAAAGTTTTGAGGTACTTGGGTTTTGAAGATCTGGTTCAATG
                -48                                    +1
```

B

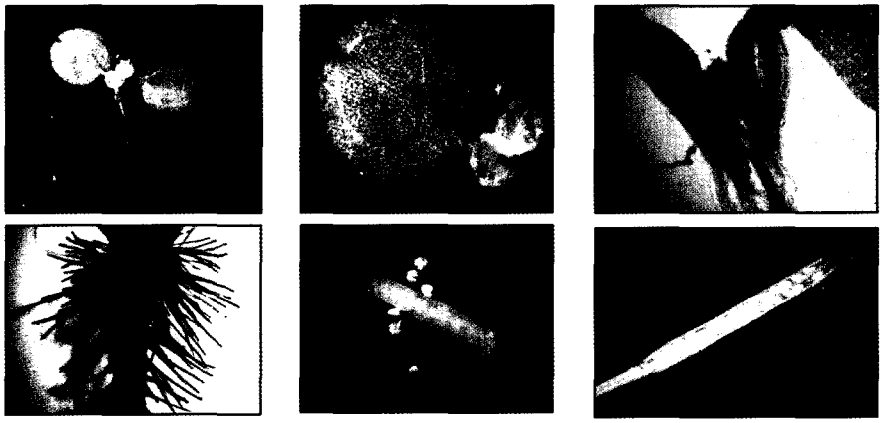

Fig. 6. The upstream DNA sequences of *CaCDPK4* promoter and GUS histochemical staining of the transgenic *Arabidopsis* plants carrying the *CaCDPK4* full-length promoter *GW-GUS* fusion construct. (A) Position of DNA sequences of *CaCDPK4* were counted down from the translational start site of ATG. Positions of D3, D4 and D5 promoter deletions were marked inside. The putative CAAT and TATA boxes are designated and the important *cis*-acting DNA elements are also included: G-box I, G-box II and W-box. The *cis*-acting DNA elements were predicted by the website program (http://intra.psb.ugent.be:8080/PlantCARE/). (B) GUS histochemical staining pictures of *CaCDPK4 GW-GUS* transgenic plants. Top: from left, GUS staining in the germinating seedlings; enlargement of the cotyledon and primary leaf from the germinating seedlings; enlargement of the shoot apex of the seedling at 14 days after germination (DAG), exhibiting the strong staining in the veins. Bottom: from left, GUS staining in the primary root from the seedling at 14 DAG; a flower just after anthesis showing GUS staining in the stigma; GUS expression in the node and the top of the immature silique.

GUS expression was restricted to the stigma of the pistil and to the junction part of silique 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

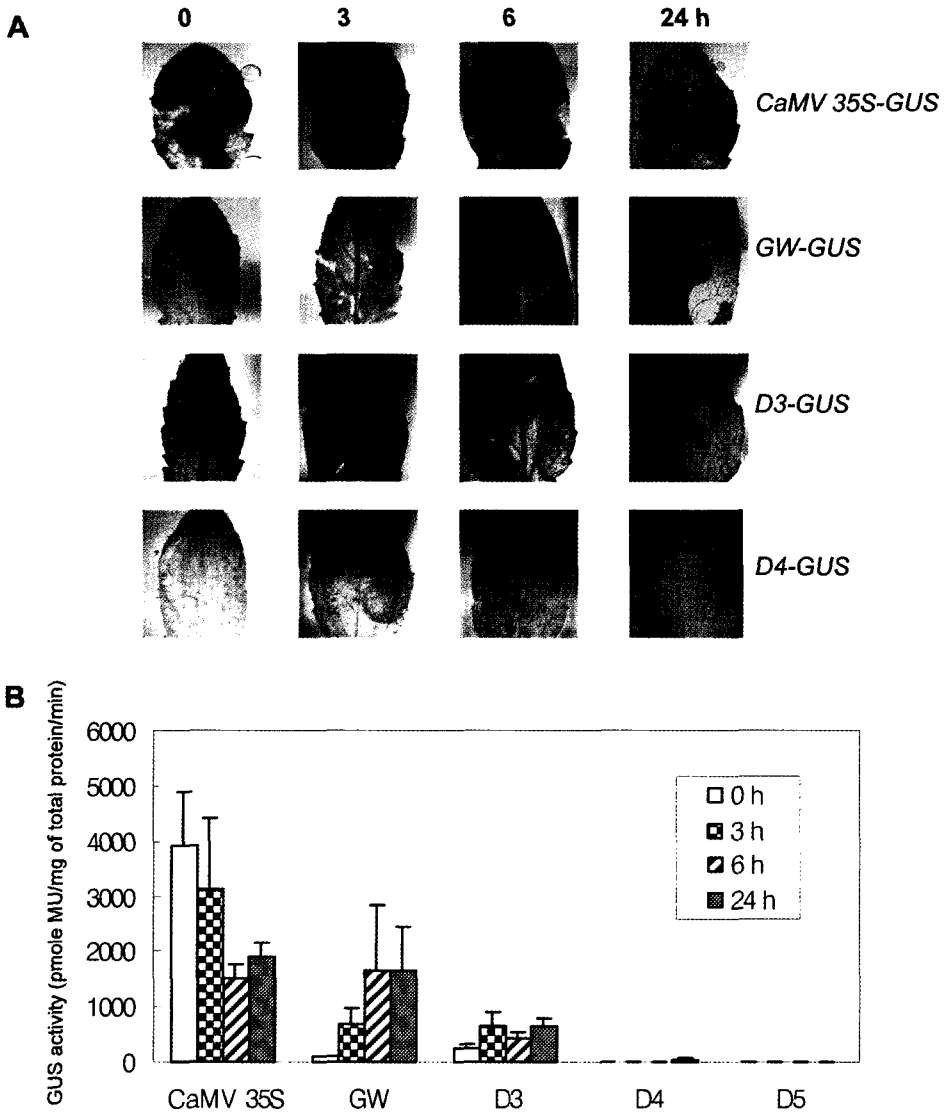

Fig. 7. GUS histochemical and fluorometric assays in response to incompatible pathogen infection in *CaMV 35S-GUS*, *CaCDPK4* promoter *GW-*, *D3-*, *D4-* and *D5-GUS* transgenic *Arabidopsis*. (A) GUS expression pattern in mature leaves from *CaMV 35S-GUS* and *CaCDPK4 GW-*, *D3-* and *D4-GUS* transgenic 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 *PstT1* infection (Fig. 7B). Theses 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 *NtCDPK2* 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 ethepon 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 *Cadhn* than mannitol stress (Fig. 4A and 4B; Chung et al., 2004). The levels of *CaCDPK3* and *Cadhn* 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 *Cadhn* (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 *PstT1* 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.

Acknowledgements

This work was supported in part by grants from the Plant Diversity Research Center (PDRC), the Crop Functional Genomics Center (CFGC) of the 21st Century Frontier Research Program of MOST to D. C. and E. C. is supported by the Promising Women Scientist Program of KOSEF (Korea Science and Engineering Foundation).

References

An, G., Ebert, P. R., Mitra, A. and Ha, S. B. 1988. Binary vector. In: Plant Molecular Biology Manual. A3. ed. by S. B. Gelvin, R. A. Schilperoot and D. P. S. Verma, pp. 1-19. Kluwer Academic Publishers.

Arabidopsis Genome Initiative. 2000. Analysis of the genome of the flowering plant *Arabidopsis thaliana*. *Nature* 408:820-826.

Asano, T., Tanaka, N., Yang, G., Hayashi, N. and Komatsu, S. 2005. Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: comprehensive analysis of the CDPKs gene family in rice. *Plant Cell Physiol.* 46:356-366.

Botella, J. R., Arteca, J. M., Somodevilla, M. and Arteca, R. N. 1996. Calcium-dependent protein kinase gene expression in response to physical and chemical stimuli in mungbean (*Vigna radiata*). *Plant Mol. Biol.* 30:1129-1137.

Bray, E. A. 1993. Molecular responses to water deficit. *Plant Physiol.* 103:1035-1040.

Chehab, E. W., Patharkar, O. R., Hegeman, A. D., Taybi, T. and Cushman, J. C. 2004. Autophosphorylation and subcellular localization dynamics of a salt- and water deficit-induced calcium dependent protein kinase from ice plant. *Plant Physiol.* 135:1430-1446.

Cheng, S. H., Willman, M. R., Chen, H. C. and Sheen, J. 2002. Calcium signaling through protein kinases. The Arabidopsis Calcium-dependent protein kinase gene family. *Plant Physiol.* 129:49-55.

Chico, J. M., Raíces, M., Téllez-Iñón, M. T. and Ulloa, R. M. 2002. A calcium-dependent protein kinase is systemically induced upon wounding in tomato plants. *Plant Physiol.* 128:256-270.

Choi, D., Kim, H. M., Yun, H. K., Park, J. A., Kim, W. T. and Bok, S. H. 1996. Molecular cloning of a metallothionein-like gene from *Nicotiana glutinosa* L. and its induction by wounding and tobacco moasaic virus infection. *Plant Physiol.* 112:353-359.

Chung, E., Kim, S. Y., Yi, S. and Choi, D. 2003. *Capsicum annuum dehydrin*, an osmotic-stress gene in chili pepper plants. *Mol. Cells* 15:327-332.

Chung, E., Park, J.M., Oh, S.-K., Joung, Y.H., Lee, S. and Choi, D. 2004. Molecular and biochemical characterization of the *Capsicum annuum calcium-dependent protein kinase 3* (*CaCDPK3*) gene induced by abiotic and biotic stresses. *Planta* 220:286-295.

Church, G. M. and Gilbert, W. 1984. Genomic sequencing. *Proc. Natl. Aca. Sci. USA* 81:1991-1995.

Clough, S. J. and Bent, A. F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735-743.

Dammann, C., Ichida, A., Hong, B., Romanowsky, S. M., Hrabak, E. M., Harmon, A. C., Pickard, B. G. and Harper, J. F. 2003. Subcellular targeting of nine calcium-dependent protein kinase isoforms from *Arabidopsis*. *Plant Physiol.* 132:1840-1848.

Dellaporta, S. L., Wood, J. and Hicks, J. B. 1983. A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* 1:19-21.

Ecker, J. R. 1995. The ethylene signal transduction pathway in plants. *Science* 268:667-675.

Evans, N. H., McAinsh, M. R. and Hetherington, A. M. 2001. Calcium oscillations in higher plants. *Curr. Opin. Plant Biol.* 4:415-420.

Harmon, A. C., Putnam-Evans, C. and Cormier, M. J. 1987. A calcium-dependent but calmodulin-independent protein kinase from soybean. *Plant Physiol.* 83:830-837.

Harper, J. F., Huang, J.-F. and Lloyd, S. J. 1994. Genetic identification of an autoinhibitor in CDPK, a protein kinase with a

calmodulin-like domain. *Biochem.* 33:7267-7277.

Harper, J. F., Breton, G. and Harmon, A. 2004. Decoding Ca^{2+} signals through plant protein kinase. *Annu. Rev. Plant Biol.* 55:263-288.

Heath, M. C. 2000. Hypersensitive response-related death. *Plant Mol. Biol.* 44:321-334.

Hrabak, E. M., Chan, C. W., Gribskov, M., Harper, J. F., Choi, J. H., Halford, N., Kudla, J., Luan, S., Nimmo, H. G., Sussman, M. R., Thomas, M., Walker-Simmons, K., Zhu, J. K. and Harmon, A. C. 2003. The Arabidopsis CDPK-SnRK superfamily of protein kinases. *Plant Physiol.* 132:666-680.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO. J.* 6:3901-3907.

Kang, J. Y., Choi, H. I., Im, M. Y. and Kim, S. Y. 2002. Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell* 14:343-357.

King, E. O., Ward, M. K. and Raney, D. E. 1954. Two simple media for the demonstration of phycocyanin and fluorescin. *J. Lab. Clin. Med.* 44:301-307.

Knight, H., Trewavas, A. J. and Knight, M. R. 1997. Calcium signaling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J.* 12:1067-1078.

Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lee, S., Kim, S. Y., Chung, E., Joung, Y. H., Pai, H. S., Hur, C. G. and Choi, D. 2004. EST and microarray analyses of pathogen responsive genes in hot pepper (*Capsicum annuum* L.) non-host resistance against soybean pustule pathogen (*Xanthomonas axonopodis* pv. *glycines*). *Funct. Integrative Genomics* 4:196-205.

Lee, S. S., Cho, H. S., Yoon, G. M., Ahn, J.-W., Kim, H.-H., Pai, H.-S. 2003. Interaction of *NtCDPK1* calcium-dependent protein kinase with NtRpn3 regulatory subunit of the 26S proteasome in *Nicotiana tabacum*. *Plant J.* 33:825-840.

Lu, S. X. and Hrabak, E. M. 2002. An *Arabidopsis* calcium-dependent protein kinase is associated with the endoplasmic reticulum. *Plant Physiol.* 128:1008-1021.

Ludwig, A. A., Romeis, T. and Jones, J. D. G. 2004. CDPK-mediated signalling pathways: specificity and cross-talk. *J. Exp. Botany* 55:181-188.

Marcotte, W. R. Jr., Russell, S. H. and Qatrano, R. S. 1989. Abscisic acid-responsive sequences from the *Em* gene of wheat. *Plant Cell* 1:969-976.

Murillo, I., Cordero, E. J. M. and Segundo, B. S. 2001. Transcriptional activation of a maize calcium-dependent protein kinase gene in response to fungal elicitors and infection. *Plant Mol. Biol.* 45:145-158.

Niggeweg, R., Thurow, C., Weigel, R., Pfitzner, U. and Gatz, C. 2000. Tobacco TGA factors differ with respect to interaction with NPR1, activation potential and DNA-binding properties. *Plant Mol. Biol.* 42:775-788.

Notredame, C., Higgins, D. and Heringa, J. 2000. A novel method for multiple sequence alignments. *J. Mol. Biol.* 302:205-217.

Oh, S.-K., Park, J. M., Joung, Y. H., Lee, S., Chung, E., Kim, S.-Y., Yu, S. H. and Choi, D. 2005. A plant EPF-type zinc-finger protein, *CaPIF1*, involved in defence against pathogens. *Mol. Plant Pathol.* 6:269-285.

Park, B. K. and Hwang, I. 1999. Identification of *hrcC*, *hrpF*, and *miaA* genes of *Xanthomonas campestris* pv. *glycines* 8ra: roles in pathogenicity and inducing hypersensitive response on non-host plants. *Plant Pathol.* 15:21-27.

Patharkar, O. R. and Cushman, J. C. 2000. A stress-induced calcium-dependent protein kinase from *Mesembryanthemum crystallinum* phosphorylates a two-component pseudo-response regulator. *Plant J.* 24:679-691.

Prescott, A. and Martin, C. 1987. A rapid method for the quantitative assessment of levels of specific mRNAs in plants. *Plant Mol. Biol. Rep.* 4:219-224.

Putnam-Evans, C., Harmon, A. C., Palevitz, B. A., Fechheimer, M. and Cormier, M. J. 1989. Calcium-dependent protein kinase is localized with F-actin in plant cells. *Cell Motil. Cytoskeleton* 12:12-22.

Putnam-Evans, C. L., Harmon, A. C. and Cormier, M. J. 1990. Purification and characterization of a novel calcium-dependent protein kinase from soybean. *Biochem.* 29:2488-2495.

Reymond, P. and Farmer, E. E. 1998. Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* 1:404-411.

Roberts, D. M. and Harmon, A. C. 1992. Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:375-414.

Romeis, T., Ludwig, A. A., Martin, R. and Jones, J. D. G. 2001. Calcium-dependent protein kinases play an essential role in a plant defence response. *EMBO. J.* 20:5556-5567.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. 2000. Over-expression of a single Ca^{2+}-dependent kinase confers both cold and salt/drought tolerance. *Plant J.* 23:319-327.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual, Cold Spring Harbor, Cold Spring Harbor Laboratory Press, USA.

Sanders, D., Pelloux, J., Brownlee, C. and Harper, J. F. 2002. Calcium at the crossroads of signaling. *Plant Cell* 14:S401-S417.

Satterlee, J. S. and Sussman, M. R. 1998. Unusual membrane-associated protein kinases in higher plants. *J. Membrane Biol.* 164:205-213.

Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N. and Shinozaki, K. 1994. Two genes that encode Ca^{2+}-dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana*. *Mol. Gen. Genet.* 244:331-340.

Whalen, M. C., Innes, R. W., Bent, A. F. and Staskawicz, B. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3:49-59.

Whitelaw, C. A., Le Huquet, J. A., Thurman, D. A. and Tomsett, A. B. 1997. The isolation and characterization of type II metallothionein-like genes from tomato (*Lycopersicon esculentum* L.). *Plant Mol. Biol.* 33:503-511.

Xing, T., Wang, X.-J., Malik, K. and Miki, B. L. 2001. Ectopic expression of an Arabidopsis calmodulin-like domain protein

kinase–enhanced NADPH oxidase activity and oxidative burst in tomato protoplasts. *Mol. Plant-Microbe Interact.* 14:1261-1264.

Yi, S. Y., Kim, J. H., Joung, Y. H., Lee, S., Kim, W. T., Yu, S. H. and Choi, D. 2004. The pepper (*Capsicum annuum* L.) transcription factor, *CaPF1*, confers pathogen and freezing tolerance in *Arabidopsis*. *Plant Physiol.* 136:2862-2874.

Yoon, G. M., Cho, H. S., Ha, H. J., Liu, J. R. and Pai, H.-S. 1999. Characterization of *NtCDPK1*, a calcium-dependent protein kinase gene in *Nicotiana tabacum*, and the activity of its encoded protein. *Plant Mol. Biol.* 39:991-1001.

Yu, D., Chen, C. and Chen, Z. 2001. Evidence for an important role of WRKY DNA binding proteins in the regulation of *NPR1* gene expression. *Plant Cell* 13:1527-1539.