The Pepper Transcription Factor CaPF1 Confers Pathogen and Freezing Tolerance in Arabidopsis

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An ERF/AP2-type transcription factor (CaPF1) was isolated by differential-display reverse transcription-PCR, following inoculation of the soybean pustule pathogen Xanthomonas axonopodis pv glycinis 8ra, which induces hypersensitive response in pepper (Capsicum annuum) leaves. CaPF1 mRNA was induced under conditions of biotic and abiotic stress. Higher levels of CaPF1 transcripts were observed in disease-resistant tissue compared with susceptible tissue. CaPF1 expression was additionally induced using various treatment regimes, including ethephon, methyl jasmonate, and cold stress. To determine the role of CaPF1 in plants, transgenic Arabidopsis and tobacco (Nicotiana tabacum) plants expressing higher levels of CaPF1 were generated. Gene expression analyses of transgenic Arabidopsis and tobacco revealed that the CaPF1 level in transgenic plants affects expression of genes that contain either a GCC or a CRT/DRE box in their promoter regions. Furthermore, transgenic Arabidopsis plants expressing CaPF1 displayed tolerance against freezing temperatures and enhanced resistance to Pseudomonas syringae pv tomato DC3000. Disease tolerance was additionally observed in CaPF1 transgenic tobacco plants. The results collectively indicate that CaPF1 is an ERF/AP2 transcription factor in hot pepper plants that may play dual roles in response to biotic and abiotic stress in plants.

During their life cycle, plants have to deal with various environmental stress conditions. Biotic and abiotic stress factors cause adverse effects on the growth and productivity of crops. To adjust to changes in the environment, plants trigger rapid defense responses via a number of signal transduction pathways. A major target of signal transduction is the cell nucleus, where terminal signals lead to the transcriptional activation of numerous genes. Alterations in the expression of genes coding for transcription regulators greatly influence plant stress tolerance. In Arabidopsis, a number of transcription factor families, each containing a distinct type of DNA-binding domain, such as ERF/AP2, bZIP/HD-ZIP, Myb, WRKY, and several classes of zinc-finger domains, have been implicated in plant stress responses in view of the finding that their expression is induced or repressed under different stress conditions (Rushton and Somsich, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). For example, Arabidopsis plants expressing the tomato (Lycopersicon esculentum) ethylene-response factor (ERF) Pti4 displayed increased resistance to the fungal pathogen Erysiphe orontii, and increased tolerance to the bacterial pathogen Pseudomonas syringae pv tomato. Pti4 may function as a transcriptional activator to regulate the expression of GCC box-containing genes (Gu et al., 2002; Wu et al., 2002). In another case, overexpression of two Arabidopsis ERF/AP2 genes, CBF1/DREBP1B and DREBP1A, resulted in enhanced tolerance to drought, salt, and freezing (Jaglo-Ottosen et al., 1998). These two transcription factors bind the cold-responsive cis-element CRT/DRE and activate the expression of target genes (Kasuga et al., 1999).

Common regulatory components, including phytohormones, are involved in separate signaling pathways. Salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) possibly act as secondary signals following pathogen attack and enhance the expression of many pathogen-responsive genes (Yang et al., 1997). Drought and high salinity lead to the production of high levels of abscisic acid (ABA). Exogenous application of ABA induces a number of genes that are expressed in response to dehydration and cold stress. These findings suggest that differences in expression patterns of biotic- and abiotic-responsive genes are a result of the alternative regulation of transcription factors and phytohormones by diverse stress signals. Recent studies provide evidence for cross-talk between biotic and abiotic stress signaling pathways. For example, the gene expression profiles observed

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during an incompatible plant-fungal interaction overlap with those derived from wounding (Durrant et al., 2000). Experiments with cDNA microarrays reveal that a substantial number of genes are coordinately regulated by different biotic/abiotic stress signals via infection with a fungal pathogen (Schenk et al., 2000) or in conditions of cold/drought stress (Seki et al., 2001). Another global gene expression approach using microarrays with 402 Arabidopsis transcription factors revealed a clear overlap of genes expressed in response to different stress factors. There was also significant overlap with the genes expressed during senescence (Chen et al., 2002). However, despite accumulating data, the molecular mechanisms underlying this cross-talk are largely unknown. Thus, a thorough knowledge of the molecular level of the mechanism of regulation of cross-talk between biotic and abiotic stress signal pathways is essential to understand how plants activate the correct responses to various environmental stress factors.

Here, we report the characterization of cDNA encoding new pepper ERF, CaPF1 (Capsicum annuum pathogen and freezing tolerance-related protein 1), which binds to both GCC and CRT/DRE cis-elements. The GCC box and CRT/DRE element have similar core sequences, which are implicated in the activation of different signal transduction pathway-related genes. The issue of whether CaPF1 activates two distinct sets of genes that contain the GCC and/or CRT/DRE element in their promoter region and participates in two different stress tolerance events was investigated. In this article, we elucidate the function of this novel ERF, which may contribute to understanding the molecular mechanisms of cross-talk between biotic and abiotic stress signaling pathways.

RESULTS

Expression of CaPF1 during the Hypersensitive Response of Hot Pepper

During the selection of non-host resistance hypersensitive response (HR)-induced genes in pepper leaves using mRNA differential-display reverse transcription-PCR, we isolated a CaPF1 cDNA fragment with amino acid similarity to other functionally characterized ERF/AP2 family proteins. We examined whether cDNA expression was induced upon pathogen attack. Young pepper leaves (cv Bugang) were syringe infiltrated with a suspension containing either the soybean pustule pathogen Xanthomonas axonopodis pv glycines 8ra (Xag 8ra) or 1 mM MgCl₂ as a control. Non-host HR was noted 18 h after inoculation with Xag 8ra. As shown in Figure 1A, CaPF1 mRNA was induced in both HR and non-HR tissue. However, the abundance and period of the induction were higher and longer, respectively, in HR-occurring tissues. To determine the specificity of CaPF1 in response to HR, we analyzed expression following host resistance-induced HR. Leaves of pepper cultivars ECW-20R (BS2/BS2) and ECW (bs2/bs2) were syringe infiltrated with the pepper bacterial spot pathogen Xanthomonas campestris pv vesicatoria race 3 (Xcv race3), which expresses the avrBS2 gene. Total RNA was extracted from inoculated leaves at different times after infection, and CaPF1 expression was analyzed by northern blotting. Susceptible pepper (cv ECW) infiltrated with Xcv race3 did not exhibit any visible responses until 36 h after infiltration, whereas resistant pepper (cv ECW-20R) developed HR lesions on infiltrated leaf tissues within 24 h (data not shown). Stronger CaPF1 expression was detected in incompatible interactions, while only mild expression was detected in compatible interactions (Fig. 1B). Pepper pathogenesis-related protein 4b (C.J. Park et al., 2001) gene was monitored as a positive control. As expected, up-regulation of the PR4b transcript was only detected in Xcv race 3 (avrBS2) infections of cv ECW-20R (BS2/BS2).

These findings indicate that the CaPF1 expression observed after infection with the HR-inducible bacterial pathogen is consistent and associated with incompatible plant-pathogen interactions.

Isolation and Sequence Analysis of CaPF1

To isolate full-length cDNA, a partial cDNA fragment with sequence similarity to ERF/AP2 family proteins was used as a probe to screen a cDNA library previously constructed from C. annuum (S.Y. Yi, S.H. Yu, and D. Choi, unpublished data). Twelve positive clones were isolated and further analyzed by restriction enzyme digestion and DNA sequencing, resulting in the identification of seven clones with 1.4-kb cDNA inserts. Among these, 5 clones encoded a predicted full-length protein with an open reading frame of 369 amino acids and molecular mass of 41 kD. Nucleotide and protein database searches reveal that the CaPF1 protein contains a 57-amino acid region that constitutes a DNA-binding ERF/AP2 domain, which is highly conserved in members of the ERF/AP2 family of plant transcription factors. CaPF1 contains short clusters of basic residues similar to known nuclear localization sequences (for review, see Dingwall and Laskey, 1991) and an acidic N-terminal region (Fig. 2B).

To explore further the evolutionary distance among the ERF/AP2 proteins, AP2 domains from different plant species that have relatively high amino acid sequence similarity with CaPF1 were subjected to construct phylogenetic tree using the PhyloDraw program (version 0.8; Fig. 2A). Phylogenetic analysis with ERF/AP2 domain indicated that the CaPF1 is most similar to previously described ERF class B-2 subgroup (RAP2.3, RAP2.2, and RAP2.12; Sakuma et al., 2002). The deduced amino acid sequence of the CaPF1 and those of its homologs are well conserved in different plant species. CaPF1 has 79% identity with JERF1 from tomato (AAK95687), 65% with NtDRF1 from tobacco (Nicotiana tabacum; AAP40022), and 50%
with RAP 2.3 from Arabidopsis (P42736). Conserved domains include the ERF/AP2 domain, putative nuclear localization signals, and a conserved N-terminal motif of unknown function (MCGGAIISD; Fig. 2B). Tournier et al. (2003) recently identified tomato LeERF2, a novel class IV ERF, characterized by an N-terminal signature sequence, MCGGAII/L. This motif was also found in CaPF1; therefore, it could belong to a class IV ERF (Fig. 2B).

Genomic DNA-Blot Analysis and Tissue-Specific Expression of the CaPF1 Transcript

Genomic DNA isolated from pepper was digested with DraI, EcoRI, HindIII, or XhoI. The blot was hybridized to radioactively labeled CaPF1 cDNA (full length) or the 3′ end fragment. Four to five fragments were detected with the CaPF1 full-length cDNA probe, while only a single band hybridized to the 3′ end-specific probe (data not shown). This restriction pattern strongly suggests that the pepper genome contains a single copy of the CaPF1 gene, which belongs to a gene family.

Tissue-specific expression of CaPF1 mRNA was analyzed by northern blotting in eight different tissues. The CaPF1 transcript was less abundant in dormant seeds than germinating seeds (data not shown). Lower levels of CaPF1 transcripts were detected in leaves and seedlings, whereas higher levels of transcripts were detected in floral organs and stem.

Expression of CaPF1 mRNA in Response to Various Treatment Regimes

Ethylene plays important roles in a number of plant stress responses (including response to pathogens) and the expression of ERF genes, including ERF1 and AtERF1 (Solano et al., 1998; Fujimoto et al., 2000). To determine whether CaPF1 expression is regulated by ethylene, we treated 2-month-old pepper plants with ethephon. Expression of ACC oxidase (Wang et al., 2002) was monitored as a positive marker for ethephon treatment. CaPF1 mRNA levels were up-regulated within 30 min of treatment with ethephon (Fig. 3A). Similar to ET, SA, and methyl jasmonate (MJ) are important phytohormones involved in signaling in response to pathogen infections. To determine the possible involvement of the CaPF1 gene in SA and MJ signaling pathways, we examined mRNA expression after treatment with these hormones. As shown in

Figure 1. Expression of CaPF1 mRNA in response to bacterial pathogens. A, Pepper leaves were infiltrated with either a mock solution (1 mM MgCl2) or with solution containing X. axonopodis pv glycines 8ra (non-host pathogen). B, Pepper near-isogenic lines (ECW-20R, resistance; ECW, susceptible) were infiltrated with a pepper leaf spot pathogen, as described in “Materials and Methods.” One blot was hybridized to the CaPF1 probe, while an identical blot was hybridized to the PR4b probe as a positive marker for pathogen infection.
Figure 2. (Legend appears on following page.)
Figure 3A, expression of CaPF1 mRNA was detected following treatment with MJ but not SA. The levels of MJ-regulated hot pepper proteinase inhibitor II (PinII) and SA-inducible pathogenesis-related protein I (PR1) genes (Lee et al., 2002) were monitored as positive markers of each treatment. As expected, both transcripts were induced by MJ and SA, respectively.

We additionally examined the expression of CaPF1 mRNA, following challenge with abiiotic stress. The pepper dehydrin gene (Chung et al., 2003) was used as a positive marker for abiiotic stress treatment. Dehydrin is expressed in spruce seedlings in response to cold, drought, ethylene, as well as treatment with ABA, JA, or wounding (Richard et al., 2000). Pepper plants were grown in soil at 25°C and transferred to low temperatures (4°C) for various periods of time. Northern-blot analysis using a gene-specific probe for CaPF1 revealed an increase in transcript levels within 1 h of cold treatment. Increased CaPF1 transcript levels were observed at all the sampling time points and peaked at 24 h (Fig. 3B). In addition to low temperature, osmotic stress-responsive expression of CaPF1 was monitored following treatment with 0.4 M mannitol (drought mimic conditions) and 0.4 M NaCl. CaPF1 transcript levels were slightly increased within 0.5 and 1.5 h of treatment, which was maintained for 24 h (Fig. 3B). However, CaPF1 transcript level is not responsive to ABA (Fig. 3A). These results collectively indicate that various plant signal, cold, and osmotic stress conditions induce CaPF1 expression.

CaPF1 Protein Binds GCC and CRT/DRE Boxes

The observed CaPF1 expression in diverse stress conditions signifies that the protein may function in the activation of numerous stress-responsive genes through binding to one or two cis-acting elements. To test this hypothesis, binding specificity of the CaPF1 protein to known ERF/AP2 factor-binding sequences, GCC box and CRT/DRE cis-element, was evaluated. The entire coding region of CaPF1 was expressed in Escherichia coli by translational fusion with a maltose-binding protein (MBP), and an electrophoretic mobility shift assay was performed. The MBP-CaPF1 fusion protein bound both the GCC-box sequence, and the CRT/DRE cis-element (Fig. 4). To determine the binding specificities, we performed a competition assay by adding unlabeled GCC box and CRT/DRE cis-element to the mobility shift assay. This led to decreased binding of MBP-CaPF1 to the labeled GCC box and CRT/DRE cis-element. Moreover, 50-fold excess of unlabeled GCC box and CRT/DRE DNA resulted in complete loss of binding of the labeled sequences to the MBP-CaPF1 protein. The addition of unlabeled CRT/DRE cis-element DNA (50 ×) to the binding assay decreased MBP-CaPF1 binding to labeled GCC-box DNA. However, addition of 5-fold excess of unlabeled GCC-box DNA resulted in complete loss of binding of the labeled CRT/DRE cis-element DNA to MBP-CaPF1 (Fig. 4). From these results, we conclude that the CaPF1 binds competitively to both the GCC box and CRT/DRE cis-element. Furthermore, binding specificity is higher with a combination of MBP-CaPF1 and GCC.

Overexpression of CaPF1 in Arabidopsis Affects Expression of PR and COR Genes

ERF/AP2s are unique to the plant kingdom and have been characterized in different plants, including Arabidopsis, tomato, soybean, and tobacco. They all possess a number of features in common, such as induction by biotic and abiotic stresses and mediation of the expression of GCC box or CRT/DRE box-containing genes such as PDF1.2 in Arabidopsis. Because pepper is a very recalcitrant species in terms of genetic transformation (Li et al., 2000), we constructed transgenic Arabidopsis plants constitutively expressing CaPF1 under control of the cauliflower mosaic virus 35S promoter to study the function of CaPF1 in biotic and abiotic stress responses of plants. From 22 independent Arabidopsis transgenic lines confirmed by northern- and genomic Southern-blot analyses with the transgene probe, three lines (lines 3, 8, and 22; T3 generation) with a single insertion of the transgene were selected for further analyses. None of these transgenic lines displayed any phenotypic abnormality throughout their life cycle.
To evaluate the role of ectopically expressed CaPF1 in stress-responsive gene expression of transgenic Arabidopsis, northern-blot analysis was performed using genes containing the GCC box (PDF 1.2) and CRT/DRE element (COR47, COR6.6, and COR78/ RD29) in their promoter regions as probes. All the genes tested were constitutively expressed in selected transgenic Arabidopsis lines. Earlier studies show that the expression of PDF1.2 and GST genes in Arabidopsis is dependent on the functions of the JA and ET signaling pathways (Zhou and Goldsbrough, 1993; Penninckx et al., 1998). CaPF1 expression induced an increase in transcript levels of PDF1.2 and GST (Fig. 5). CRT/DRE elements, which contain a conserved 5-bp core sequence (CCGAC), are present in the promoter regions of a number of cold- and dehydration-responsive genes of Arabidopsis, including those designated COR (cold regulated; Thomashow, 1999). CaPF1 transgenic Arabidopsis display COR gene expression in the absence of a low temperature stimulus (Fig. 5). Thus, it seems most likely that CaPF1 is functional in Arabidopsis plants, thereby CaPF1 affects transactivating PR and COR genes.

CaPF1 Overexpression Confers Tolerance to Pathogens and Freezing in Transgenic Plants

As shown in Figure 5, the expression of CaPF1 in Arabidopsis led to constitutive expression of the stress-related genes. This raised the possibility that stress tolerance is activated in these plants. The CaPF1 plants were first tested in disease tolerance. Three

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Figure 3. Expression of CaPF1 mRNA in response to chemicals and abiotic stress. A, Expression of CaPF1 mRNA following treatment with ethephon, MJ, SA, or ABA. ACC oxidase, PinII, PR1, and dehydrin genes were used as positive markers for ethephon, MJ, SA, and ABA treatment, respectively. B, CaPF1 expression in response to low temperature, manitol (drought mimic), and high salt. Total RNA was prepared from 2-month-old pepper plants transferred to a 4°C chamber (cold), 0.4 M manitol solution, and 0.4 M NaCl solution, as described in "Materials and Methods." The pepper Dehydrin gene was used as a positive marker for abiotic treatment. Total RNA was extracted from leaf tissues at the different time points indicated.
CaPF1 T₃ generation transgenic Arabidopsis lines (lines 3, 8, and 22) were tested for resistance against *P. syringae* pv *tomato* DC3000 that infects wild-type Arabidopsis Col-0. Leaf bacterial numbers were determined at 0, 3, and 5 d after inoculation. All three plants displayed reduced disease lesion and leaf bacterial numbers compared with the control plant. At 3 d postinoculation, the overexpression of CaPF1 reduces bacterial numbers by 5- to 10-fold (data not shown). As depicted in Figure 6B, 10- to 100-fold reduction in bacterial numbers were detected at 5 d after inoculation in CaPF1 transgenic leaves compared with empty vector-transformed control plants.

To confirm the role of CaPF1 in disease tolerance, transgenic tobacco plants were also generated. Tobacco plants were transformed with the same vector construct used in Arabidopsis study. From 16 independent transgenic lines conformed by northern- and Southern-blot analysis with the transgene probe, 7 lines (lines 2, 3, 6, 9, 12, 13, and 19) with a single insertion of the transgene were selected for further analyses. We observed that all selected seven transgenic lines (T₀ progenies of CaPF1 transgenic tobacco plants; lines 2, 3, 6, 9, 12, 13, and 19) constitutively expressed pathogenesis-related genes, such as PR2, 3, 4, and 5, in absence of pathogen attack (Fig. 7A). Next, we tested for resistance of transgenic tobacco plants (lines 2, 3, and 6) against *P. syringae* pv *tabaci* that infects wild-type tobacco. All three transgenic tobacco lines displayed reduced lesions and leaf bacterial numbers compared with control plants transformed with empty vector (Fig. 7B and C).

For the freezing tolerance test, transgenic (lines 3 and 8) and control Arabidopsis plants were grown in soil at 25°C for 3 weeks. Plants were transferred to −6°C for 24 h and returned to a 25°C growth chamber for 1 week. As a result of two independent experiments, 65% of CaPF1 transgenic Arabidopsis survived. In the same condition, only 17% of nontransgenic Arabidopsis survived (Fig. 8A). To quantify the increase in freezing tolerance, electrolyte leakage was measured following freezing treatment. Electrolyte leakage from frozen and thawed tissues is a sensitive indicator of loss of integrity of the plasmalemma and has been commonly used to assay freezing injury (for review, see Calkins and Swanson, 1990). The electrolyte leakage assay was applied to both nonacclimated and cold-acclimated leaf tissues of transgenic lines 3 and 8, those autoregulated PR and COR genes. The results indicated that the freezing tolerance of nonacclimated CaPF1-expressing Arabidopsis plants was slightly greater than that of nonacclimated control plants; nonacclimated control plants had an EL₅₀ (temperature that caused a 50% leakage of electrolytes) of approximately −6°C, and the two CaPF1-expressing lines had EL₅₀ values of approximately −7°C (Fig. 8B). The freezing tolerance of cold-acclimated CaPF1-expressing Arabidopsis plants was also greater than that of cold-acclimated control plants. CaPF1-expressing plants that had been cold acclimated for 7 d had EL₅₀ values of approximately −9°C. The cold-acclimated control plants had EL₅₀ values of approximately −7°C (Fig. 7C) under these particular conditions.

These results indicate that overexpression of CaPF1 confers disease and freezing tolerance in transgenic plants, presumably via activation of the signaling pathway.
DISCUSSION

ERF factors are a subfamily of ERF/AP2 transcription factor that is only present in the plant kingdom. In Arabidopsis, 124 ERF proteins were annotated (Riechmann et al., 2000), and molecular genetic studies for unveiling the roles of this family of genes are actively performed in diverse aspects of biological phenomena. Recent studies revealed the role of some ERF proteins during abiotic stresses of plants (for review, see Kizis et al., 2001; Singh et al., 2002; Shinozaki et al., 2003). In this article, we describe the characterization of cDNA encoding a new pepper ERF, CaPF1, which binds to either GCC or CRT/DRE cis-elements.

CaPF1 Is a Novel Transcriptional Activator

CaPF1 contains a highly conserved ERF domain. However, outside the ERF domain, little sequence similarity exists between CaPF1 and other known ERF proteins (Fig. 2). In vitro sequence-specific DNA-binding activity of ERF domain-containing proteins is well documented. ERF proteins and Pti5 and 6 specifically interact with GCC boxes present in the promoter regions of PR genes (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997). DREB1, DREB2A, and CBF1 proteins bind to the CRT/DRE element containing the core sequence PuCCGAC (Liu et al., 1998). Minor differences in the surrounding common core target sequences of ERF and DREB proteins result in the regulation of distinct target genes (Sakuma et al., 2002). In this article, electrophoretic mobility shift assays with GCC or CRT/DRE cis-elements demonstrate that CaPF1 binds both sequences, and overexpression of CaPF1 in Arabidopsis induces constitutive expression of PR and COR genes (Figs. 4 and 5). One of the tobacco ERFs, Tsi1, could also bind both the GCC box and the CRT/DRE box. However, transgenic tobacco plants overexpressing Tsi1 did not show induced expression of rd29A, which contains a CRT/DRE box in its promoter region, under normal growth conditions (J.M. Park et al., 2001).

JERF1 (AY044235) and NtDRF1 (AY286010) have the most similarity in amino acid sequence of CaPF1 protein. Interestingly, the three ERFs (CaPF1, JERF1, and NtDRF1) contain a novel, highly conserved N-terminal motif of unknown function (MCGGAIISD; Fig. 2B). Tournier et al. (2003) isolated four new members of the ERF family from tomato (LeERF1–4), and phylogenetic analysis indicated that LeERF2 belongs to a new ERF class, which is characterized by a conserved N-terminal MCGGAIISD/L sequence. Based on dual DNA-binding activities of the CaPF1 protein to both the GCC and CRT/DRE boxes and N-terminal MCGGAIISD/L signature sequence, we conclude that CaPF1 is a novel ERF protein.

CaPF1 Is Responsive to Various Stress Factors

ERF family of genes plays various roles in plant growth, development, and response to different environmental stress factors (Okamuro et al., 1997). The pepper ERF CaPF1 gene also responds to pathogen attack and various abiotic stresses (Figs. 1 and 3). CaPF1 transcripts are up-regulated during an incompatible interaction between pepper and bacterial pathogens (Fig. 1). One possible role of CaPF1 in response to pathogens is the orchestration of the pathway that involves the expression of biotic and abiotic stress-responsive genes.
correct temporal response in defense-related gene expression. In plants, pathogen infection generates multiple defense-response signaling pathways. One is mediated by SA, which culminates in the activation of pathogenesis-related protein genes. Signaling through the synergistic action of JA and ET is also involved in stress responses of plants and operates in a SA-independent fashion. The JA/ET pathway involves the induction of PR3, PR4, and PDF1.2 (Moller and Chua, 1999). In our experiment, CaPF1 transcript level is not responsive to SA. However, ET or JA induces CaPF1 expression in pepper within 1 h of treatment (Fig. 3A), and elevated PDF1.2 and GST transcript levels were detected in CaPF1-overexpressing Arabidopsis (Fig. 5). These results indicate that CaPF1 may regulate defense-related genes, in part, through the JA/ET pathway.

The CaPF1 transcript level is not responsive to ABA (Fig. 3A), like other ERF/AP2-type transcription factors (Liu et al., 1998). Induction of several abiotic stress-inducible genes, such as rd29A and COR, is known to mediate by ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki, 1994; Shinozaki et al., 2003). Our study showed that the CaPF1 mRNA is induced by low temperature, mannitol, and high salt (Fig. 3B), and constitutive expression of CaPF1 in transgenic Arabidopsis plants results in enhancing the expression of rd29A and COR genes containing
These results indicate that CaPF1 may play a role in regulating COR class of genes through ABA-independent pathway. Taken together, our data suggest that CaPF1 is a multiple stress-responsive factor responding to both biotic and abiotic stressors, including pathogen, low temperature, salt, and water stress.

Overexpression of CaPF1 in Arabidopsis Confers Tolerance against Various Stresses

The hypothesis that CaPF1 may play a role in biotic/abiotic stress resistance in plants is supported by the results of CaPF1-overexpressing transgenic Arabidopsis and tobacco plants. Overexpression of CaPF1 resulted in constitutive overexpression of stress-related genes such as PR and COR and stress tolerance under normal growth condition. It has been reported that overexpression of the Pti4, an ERF/AP2-type factor of tomato, in Arabidopsis activated the expression of GCC box-containing PR genes and exhibited increased resistance against pathogens (Gu et al., 2002). Overexpression of DREB1A, a stress-inducible ERF/AP2-type transcription factor, was also shown to enhance tolerance to multiple abiotic stresses via expression of COR genes containing DRE/CRT in their promoter regions (Kasuga et al., 1999). To date, only
one example of ERF/AP2-type factor was shown to confer tolerance to both pathogen and abiotic stress by overexpression of a single transcription factor. Overexpression of Tsi1, a tobacco ERF/AP2-type factor, in tobacco improved tolerance to salt and pathogens (J.M. Park et al., 2001). Tsi1 was also reported to have dual binding activities to GCC and DRE/CRT cis-acting elements. However, overexpression of the Tsi1 in tobacco resulted in enhanced expression of PR genes but not rd29A. In this study, we show that the pepper ERF/AP2-type factor can confer disease and freezing stress tolerant in transgenic plants with constitutive expression of both PR and COR genes.

Recent studies in molecular and genomic analyses on the complex cascades of gene expression in abiotic stress response identified specificity and cross-talk in stress signaling (Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki et al., 2003). Using full-length cDNA microarray, Seki et al. (2001) found that many genes are induced by both drought and cold stress and suggest the existence of cross-talk between the drought and cold stress. In this study, we described elevated expression of PR and COR genes and enhanced tolerance to pathogen and freezing tolerance in an ERF protein overexpressor plant. This result together with others (J.M. Park et al., 2001) could be a starting point to study the cross-talk between biotic and abiotic stress-responsive gene expression in plants. Global gene expression study using CaPF1-overexpressing transgenic plants and transgenic approach using mutants in hormone signaling may provide new insight into the roles of CaPF1 in biotic and abiotic stress responses in plants.

**MATERIALS AND METHODS**

**Isolation of CaPF1 cDNA and Sequence Analysis**

The hot pepper (*Capsicum annuum*) cDNA library was constructed from mRNA prepared from 8-week-old plants inoculated with *Xanthomonas axonopodis* pv *glycines* and screened with a random prime-labeled CaPF1 differential-display reverse transcription-PCR fragment (421 bp) as a probe. Plaques (5 × 10³) were screened at 42°C using the hybridization and washing conditions described by Choi et al. (1996). The DNA sequences of CaPF1 cDNA clones were determined using standard procedures (Sambrook et al., 1989).

**Plant Materials, Growth Conditions, and Treatment**

Arabidopsis (ecotype Colombia) and tobacco plants (*Nicotiana tabacum* cv *Xanthi nc*) were grown in a chamber (16 h of light and 8 h of darkness at 25°C). For growth under sterile conditions, Arabidopsis and tobacco seeds were macerated in a 1.5-mL microcentrifuge tube containing 200 μL of 10 mM MgCl₂. Samples were diluted in 10 μL MgCl₂ and plated on selective medium (Luria-Bertani containing 100 μg/mL rifampicin).

**DNA and RNA Gel-Blot Analyses**

Genomic DNA was isolated from mature leaves of pepper *cv* Bugan. Genomic DNA samples (20 μg) were digested to completion with *DraI*, *EcoRI*, *HindIII*, or *XbaI*. Digested genomic DNA was separated by electrophoresis on a 0.8% agarose gel, denatured, and blotted onto a nylon membrane (Amersham Pharmacia, Uppsala). Southern blotting was performed as described previously (Church and Gilbert, 1984), and membranes were hybridized with the CaPF1 cDNA probe (full length or gene specific) labeled with [α-32P]dCTP. RNA was extracted from pepper or Arabidopsis plants, using the procedure of Yi et al. (1999). For northern-blot analyses, total RNA was separated on formaldehyde-containing agarose gels and blotted onto nylon membranes following standard procedures (Sambrook et al., 1989). Equal loading of RNA was verified by visualizing of RNA following staining with ethidium bromide. Blots were hybridized with [α-32P]dCTP-labeled probes. Arabidopsis-specific probes were generated via PCR amplification with gene-specific primers: *ATGST1* (1890–1955), 5'-GAGTTCTTCTATGCCTTCTCAC-3', 5'-GGCTAGCTTACGCGCCT-3'; *COR6.6* (XS0893), 5'-AGTATCCGGCATGCCGACTG-3', 5'-CACCGTATACGCTTCAAGGAGAA-3'; *COR7* (X90899), 5'-CGAG-5'; *ATGCTCCACCCTCCACATC-3'. The Arabidopsis RD29A, PDF1.2, and PR2 probes were synthesized as described earlier (Uknes et al., 1992; Penninckx et al., 1996; J.M. Park et al., 2001). All the amplified DNA fragments were cloned into a pGEM-Teasy vector (Promega, Madison, WI), and partial DNA sequences were determined for confirmation of correct gene. Pepper cDNA clones (PR1, PR4, Pit1, ACC oxidase, and dihydroze cDNA) and tobacco cDNA clones (PR2, 3, 4, and 5) used in this study were isolated previously from pepper and tobacco (Lee et al., 2002; Chung et al., 2003).

**Preparation of Recombinant Proteins and Electrophoretic Mobility Shift Assays**

The coding region of CaPF1 was cloned into pMAL (New England Biolabs, Beverly, MA) and expressed in *Escherichia coli* BL21 cells (Amersham Pharmacia). A MBP-CaPF1 fusion protein was purified using amylose resin, according to the manufacturer’s instructions (New England Biolabs). For the electrophoretic mobility shift assay, both strands of the following oligonucleotides were synthesized for the GCC box (ATAAGCACCCCGC-C-TAAAAT; Ohme-Takagi and Shinshi, 1995) and CRT/DRE element (ATT-TCATGCCGACCTTGGTTTATTTC; Stockinger et al., 1997). Double-stranded oligonucleotides were labeled with [32P]-y-ATP (5,000 Ci mmol⁻¹; Amersham) by treatment with T4 polynucleotide kinase (Promega) and purified on Sephadex G-25 columns (Roche, Mannheim, Germany), according to the manufacturer’s instructions. Radiolabeled probes were incubated with 1 μg protein in 10 μL 1 × binding buffer (HEPES KOH, pH 7.5, 25 mM/KCl, 40 mM/EDTA, 0.1 mM/glycerol, 10% DTT, 1 mM/PMSF, poly d(C)/500 μg) for 20 min at room temperature before loading onto a 4% polyacrylamide gel. Electrophoresis was performed at 100 V at room temperature. For competition assays, the protein was incubated with cold probe for 15 min at room temperature, then incubated further 20 min after adding radioactive probe.

**Plant Transformation**

CaPF1 full-length cDNA was constructed into a polylinker site of a binary vector, pMBP-1, a derivative of pBI121, in the sense orientation. Constructs were introduced into *Agrobacterium tumefaciens* strain C58C1. Arabidopsis plants used for transformation were grown in 8-cm pots filled with soil at 25°C for 5 weeks and transformed by vacuum infiltration, as described by Beckold

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Electrolyte Leakage Measurement

Electrolyte leakage tests were performed essentially as described (Warren et al., 1996) with minor modifications. Five-week-old seedlings were incubated in growth chambers at 25°C (for nonacclimated plants) or 4°C (for cold-acclimated plants). After 7 d, young leaves were harvested, washed, and 4 leaves per plant were placed in 5-mL aliquots of 0.4 M sorbitol (Sigma, St. Louis). Tubes were equilibrated to either −2°C or 0°C in a cooled incubator (MR-153; Sanyo, Osaka), and allowed to remain there for 24 h. The cooled incubation was then ramped down to −10°C at a rate of 2°C d−1. The cold-treated tubes were held at 4°C for 2 h and then warmed to room temperature. Electrical conductivity was measured (model 455C, Istek conductivity meter; Seoul, Korea), after which the tubes were autoclaved to release all electrolytes for the second determination of the total content of electrolytes in each sample.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ246274.

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LITERATURE CITED


