PPI1: A Novel Pathogen-Induced Basic Region-Leucine Zipper (bZIP) Transcription Factor from Pepper

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We have isolated a full-length cDNA, *PPI1* (pepper-PMMV interaction 1), encoding a novel basic region-leucine zipper (bZIP) DNA-binding protein, from expressed sequence tags differentially expressed in *Capsicum chinense* PI257284 infected with *Pepper mild mottle virus* (PMMV). *PPI1* encodes a predicted protein of 170 amino acids and contains a putative DNA-binding domain that shares significant amino acid identity with ACGT-binding domains of members of the bZIP DNA-binding protein family. *PPI1* was localized in the nucleus and had transcriptional activation activity in yeast. Transcripts of the *PPI1* gene were preferentially induced during an incompatible interaction by inoculation with PMMV, *Pseudomonas syringae* pv. *syringae* 61, and *Xanthomonas campestris* pv. *vesicatoria* race 3. However, the *PPI1* gene was not induced by abiotic stressors that activate the plant defense-signaling pathway. Our data provide the first evidence that a bZIP transcription factor is preferentially induced by pathogen attack, suggesting that *PPI1* may play a specific functional role in the regulation of expression of plant defense-related genes.

Additional keywords: suppression subtractive hybridization.

Plants resist pathogen attack by activating a wide variety of defense mechanisms, including the hypersensitive response (HR), the induction of genes encoding pathogenesis-related (PR) and defense-related proteins, the production of antimicrobial compounds termed phytoalexins, the generation of reactive oxygen species (ROS), and the reinforcement of cell walls (Reymond and Farmer 1998).

In plants, transcriptional activation of numerous genes, modulated by DNA-binding proteins, plays a fundamental role in the response to pathogen infection (Rushton and Somssich 1998). However, very few transcription factors that respond specifically and directly to pathogens have been characterized in plants.

Eukaryotic transcription factors have been categorized into several families on the basis of their highly conserved DNA-binding domains. These include basic region-leucine zipper (bZIP) proteins, Myb-like proteins, MADS domain proteins, helix-loop-helix proteins, zinc-finger proteins, and homeo-box proteins (Meshi and Iwabuchi 1995; Schwechheimer et al. 1998). The bZIP DNA-binding proteins have been intensively

studied because they play roles in the regulation of several biological processes such as seed-storage gene expression (Albani et al. 1997; Chern et al. 1996), photomorphogenesis (Oyama et al. 1997), leaf development (Yin et al. 1997), flower development (Chuang et al. 1999), abscisic acid (ABA) response (Nakagawa et al. 1996), and gibberellin biosynthesis (Fukazawa et al. 2000). In *Arabidopsis*, about 100 bZIP DNA-binding protein genes are present in the genome (Riechmann and Ratcliffe 2000). Recent studies show that several bZIP DNA-binding proteins appear to be indirectly involved in the plant defense response. For example, *Arabidopsis* NPR1-interacting protein (NIP) belongs to the TGA/ocs element-binding factor (OBF) family of bZIP factors that has been implicated in the activation of salicylic acid (SA)-responsive genes, including *PR-1* (Zhou et al. 2000). In addition, the tobacco bZIP transcription factor, TGA2.2, is a major component of the activating sequence-1 (*as-1*)-binding factor (ASF-1) protein. This protein specifically binds to *as-1*, which is a functionally important element of SA-inducible defense genes such as *PR-1a* (Niggeweg et al. 2000). However, there have been very few reports on the direct induction of bZIP factors by plant pathogens.

In this study, we report the characteristics of a novel bZIP transcription factor, *PPI1*, from pepper. *PPI1* is distinct from other bZIP family members. It shares a limited but significant amino acid homology to other bZIP factors, and its gene expression is preferentially induced following pathogen infection but not following exposure to abiotics such as SA, methyl jasmonate (MeJA), ethephon, H₂O₂, or ABA. We propose that *PPI1* acts as a nuclear factor in a signaling pathway that activates plant defense responses against pathogen attack.

RESULTS

Isolation and amino acid sequence analysis of *PPI1*.

To study the molecular mechanism controlling the plant defense response, we have identified by suppression subtractive hybridization (SSH) a broad spectrum of *Pepper mild mottle virus* (PMMV) (pathotype P_{1,2})-inducible genes that are differentially expressed during an incompatible interaction possibly mediated by the *L*³ resistance gene of *Capsicum chinense* PI257284. Differentially expressed cDNA clones (205) were isolated and sequenced, and 98 independent clones were obtained (data not shown). One partial cDNA fragment that showed amino acid homology to bZIP DNA-binding proteins was used as a probe to screen a cDNA library previously constructed from *C. annuum* (Lee et al. 2001). Five positive clones were isolated and the longest cDNA, designated *PPI1*, was se-

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quenced completely. *PPI1* was 1,216 base pairs (bp) long and the open reading frame (ORF) predicted a polypeptide of 170 amino acids with a calculated molecular mass of 19.4 kDa (NCBI accession no. AF430372). Amino acid sequence analysis of *PPI1* revealed that it was almost identical to the putative bZIP DNA-binding protein of *C. chinense* (NCBI accession no. AF127797) (Fig. 1). The only amino acids missing in AF127797 were a triplet of asparagines (amino acids at position 117 to 119 of PPI1). The deduced PPI1 protein was found to be homologous to various plant bZIP DNA-binding proteins, which are shown in Figure 1. The *Arabidopsis* ATB2 (NCBI accession no. AF053940) (Rook et al. 1998) and the tobacco BZI-2 (NCBI accession no. AY045570) (Strathmann et al. 2001) are the best-characterized members among them. PPI1 showed sequence similarity to other bZIP DNA-binding proteins from *Arabidopsis thaliana* (NCBI accession nos. AF053939 and AF053940; 42 and 50%, respectively), *Petroselinum crispum* (NCBI accession nos. AJ292744 and AJ292745; 38 and 49%, respectively), *Zea mays* (NCBI accession no. X62745; 29%), *Lycopersicon esculentum* (NCBI accession no. AF176641; 35%), *Nicotiana tabacum* (NCBI accession nos. AY045570 and AB032478; 84 and 38%, respectively), and *Antirrhinum*

majus (NCBI accession nos. Y13675 and Y13676; 34 and 38%, respectively). The overall amino acid sequence identity between PPI1 and other bZIP proteins, except for AF127797, ranged from 29 to 84%, whereas the amino acid identity within their basic and leucine zipper domains (amino acids at position 33 to 112 of PPI1) ranged from 45 to 92%. This region is also known to contain the ACGT recognition motif of plant bZIP factors. This basic motif generally binds to the ACGT core sequence present in the promoters of bZIP-responsive genes, implying that PPI1 is an ACGT-binding factor. There is also an acidic C-terminal region (amino acids at position 113 to 170) that is probably similar in function to acidic activation domains of other transcription factors, suggesting that PPI1 might act as a transcriptional activator.

PPI1 is exclusively localized to the nucleus.

The nuclear localization signal (NLS) sequence (³³KRMISNRESARRSRMRK⁴⁹) of PPI1 is almost identical to the NLS of other bZIP proteins. To confirm the possibility that PPI1 acts as a nuclear factor, we examined the subcellular localization of the PPI1 protein. The ORF of PPI1 was fused to the upstream region of the soluble modified green fluorescent

Y13675	MAEQ---QRS---TSPGIDD---DERKRRKLSNRESARRSRMRKQRLDEIA 45
Y13676	MAPE---QQS---PNSGNSN---IDERKRRKQSNRESARRSRMRKQRLDEIMA 47
AF176641	MAST---QQP---ASSGSDGQR---YATNDRKRKRMESENRESARRSRMRKQHLLEELMS 51
AB032478	MALT---QQP---ASSGSDGQR---YATNDRKRKRMESENRESARRSRMRKQHLLEELMS 51
AJ292744	MASY---QNQN---QVSSGSDGLRYATFDEKKRKRISNRESARRSRMRKQHVVDLIA 54
X62745	MSSS---SLSPTAGRTSGSDGDS---AADTHRRKRRLSNRESARRSRMRKQHLDELIVQ 54
PPI1	MSSSGTSSGSS---FLQNSGSEEDL---QLLMDQRKRKRISNRESARRSRMRKQKHLDDLMA 58
AF127797	MSSSGTSSGSS---FLQNSGSEEDL---QLLMDQRKRKRISNRESARRSRMRKQKHLDDLMA 58
AY045570	MSSSGTSSGSSFLQNSGSEEDL---QQLVDQRKRKRISNRESARRSRMRKQKHLDDLMA 59
AF053940	MSSSGGTTST---IQTSSGSEE---SLMEQRKRKRISNRESARRSRMRKQKLLDDTLTA 55
AF053939	MSSSSTYRSSSSSDGGINNPSDS---VVTYDERKRKRISNRESARRSRMRKQHVVDLIA 59
AJ292745	MSSSGNSSGSTQKLQNSGSEGD---LMDQRKRKRISNRESARRSRMRKQKHLDELMA 56
Y13675	QESQMDEQNKLRDITNGATQLYNFASDNNVRAQLAELTDRLHSLNSVLQIAS---- 100
Y13676	QESQIQEENKKLQKIIDDSKQLYLNFASENNVRAQLGELTDRLSLNSVLEIAS---- 102
AF176641	QLTQLQNGSTIWREKIESVGRNFHTLDAENNVRAQMAELTERLDSNLSLTRFWA---- 106
AB032478	QMTQLQNGNVLWREKIDAVGRNYLTDAENNVRAQMAELTERLDSNLSLTRFWA---- 106
AJ292744	EMSQSQSNKVVTQKINEATDMFFGVYSENNVRAQLSELTDRLYSLNSVLHIVE---- 109
X62745	EVARLQADNARVAARARDIASQYTRVEQENTYLRARAAELGDRLSYNEVLRRLVE---- 109
PPI1	QVSTLRKENDQILTSMNVTTHQYLNVEAENSILRAQLSELSHRLESNEIIAYMD---AN 115
AF127797	QVSTLRKENDQILTSMNVTTHQYLNVEAENSILRAQLSELSHRLESNEIIAYMD---AN 115
AY045570	QVATLRKENNQILTSMNVTTHQYLNVEAENSILRAQLAELNHRLESNEIIAFLD---AN 116
AF053940	QVNHKKENTEIVTSVITTHQYLTVEAENSILRAQLDELNHRLOSNDIIEFLD---SS 112
AF053939	QINQLSNDNRGILNSLTVTSQLYMKIQAEISVLTADMEELSTRLOSNEIVDLVQ---SN 116
AJ292745	QAQLRKENNQITTTNLTQQFVKVEAENSILRAQMDLTDRLLOSNDILHYINTTTTA 116
Y13675	-----EVSGLVLDIPDIP---DALLEPWQLPCPIQ-----ADIFQ 132
Y13676	-----EVSGMAFDIPAIP---DALLEPWLLPCPIQIPIAA---SADMF 139
AF176641	-----DANGLAVDIPDIP---DTLLEPWQLPCPIQIPITA---SADMF 143
AB032478	-----DANGLAVDIPDIP---DTLLEPWQLPCPIQIPITA---SADMF 143
AJ292744	-----EVSGLAMDIPQYP---DTLMEPWQLPCPAQPIITT---SANMF 146
X62745	-----EFSGVAMDIDQEMPADDPLLRWQLPYPAAMPMPGAPHMLH 150
PPI1	NNNNSCSNGLAMDHN---EPYSFNFAQSDT---VVDGFNMTNSWN---YFCSNQPIMT-AHV 169
AF127797	N---SCSNGLAMDHN---EPYSFNFAQSDT---VVDGFNMTNSWN---YFCSNQPIMT-AHV 166
AY045570	N---NCNGLANMDHNOEPEYSFNFAQNEP---MVDGFNMTNSWN---YLCANQPIMT-ADV 169
AF053940	NNN---NNNNNMGMCSN---PLVGLGECDDF---FVNQMNS-----YIMNQPLMASSDALM 159
AF053939	GAGFGVDQIDGCGFD---DRTVGIDGYD---DMNMSNVNHVGGSVYTNQPIMA-NDINM 170
AJ292745	AAAAATAATTGIDGVFEMDNLFGLDHSSYMNNNNNNSWN---MMYPNQPIMA---DMFM 173
Y13675	C 133
Y13676	H 140
AF176641	F 144
AB032478	F 144
AJ292744	F 147
X62745	Y 151
PPI1	Y 170
AF127797	Y 167
AY045570	Y 170
AF053940	Y 160
AF053939	Y 171

Fig. 1. Comparison of the amino acid sequences of PPI1 (NCBI accession no. AF430372) with other basic region-leucine zipper (bZIP) transcription factors. The dark gray boxes indicate identical residues in amino acid sequences. NCBI accession numbers are as follows: Y13675, bZIP DNA-binding protein from *Antirrhinum majus*; Y13676, bZIP DNA-binding protein from *Antirrhinum majus*; AF176641, *LebZIP1* from *Lycopersicon esculentum*; AB032478, *tbzF* from *Nicotiana tabacum*; AJ292744, *CPRF6* from *Petroselinum crispum*; X62745, *OCSBF-1* from *Zea mays*; PPI1, this work; AF127797, putative bZIP DNA-binding protein from *Capsicum chinense*; AY045570, *BZI-2* from *Nicotiana tabacum*; AF053940, *ATB2* from *Arabidopsis thaliana*; AF053939, *GBF5* from *Arabidopsis thaliana*; and AJ292745, *CPRF7* from *Petroselinum crispum*.

protein (*smGFP*) gene, which acted as a fluorescent marker. Fusion constructs (*35S::PPII-smGFP*) were introduced into onion epidermal cells by the particle bombardment system to transiently express *smGFP* or *PPII-smGFP* under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, and protein localization was determined with a fluorescence microscope. As shown in Figure 2, the *smGFP* fusion protein was localized only to the nucleus, while the control *smGFP* was uniformly distributed throughout the cell. These results indicate that *PPII* is a nuclear factor.

The acidic C-terminal region of *PPII* mediates transcriptional activation in yeast.

To determine if *PPII* was capable of activating the transcription of reporter genes in yeast, the DNA-binding domain of LexA (Clontech, Palo Alto, CA, U.S.A.) was fused to several constructs of *PPII* (*PPIIF*, residues at position 1 to 170; *PPIIN1*, position 1 to 68; *PPIIN2*, position 1 to 115; *PPIIC1*, position 81 to 170; and *PPIIC2*, position 110 to 170). The transcriptional activation of these constructs was identified by measuring activation of the *LEU2* and *LacZ* dual reporter genes under the control of the LexA operator in yeast (Fig. 3A). Yeast cells carrying the plasmids containing *PPIIN2* ($pI = 8.12$), *PPIIF* ($pI = 5.93$), *PPIIC1* ($pI = 4.31$), or *PPIIC2* ($pI = 4.03$) grew on medium lacking leucine. In contrast, yeast cells carrying the plasmids containing *PPIIN1* ($pI = 10.47$) did not grow. Yeast strains that grew on the medium lacking leucine also induced *LacZ* activity and formed blue colonies in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (Fig. 3B). LexA-*PPIIN2*, LexA-*PPIIF*, LexA-*PPIIC1*, and LexA-*PPIIC2* activated the transcription of the reporter gene by 5.9-, 67.6-, 96.7-, and 82.1-fold compared with the negative control (LexA-Lam), respectively (Fig. 3C). The transcriptional activity of the positive control (LexA-GAL4) was 90.2-fold greater than that of the negative control (LexA-Lam). These observations are consistent with a previous report showing that bZIP DNA-binding proteins have transcriptional transactivation ac-

tivity (Sprenger-Haussels and Weisshaar 2000). Thus, the acidic C-terminal region of *PPII* (residues at position 110 to 170) contains a potential transcriptional activation domain, which also provides strong evidence that *PPII* is a transcriptional activator.

Genomic DNA blot analysis reveals one copy of the *PPII* gene.

Genomic DNA isolated from *C. chinense* PI257284 was digested with *DraI*, *EcoRI*, *HindIII*, or *XbaI*. The blot was then hybridized with radioactively labeled *PPII* cDNA (1,216 bp). Only one band from each digestion was seen on the Southern blot (Fig. 4). This restriction pattern strongly suggested that the pepper genome contained a single copy of the *PPII* gene.

Transcription levels of *PPII* in various tissues.

To investigate the tissue specificity of *PPII* transcription, RNA gel blot analysis was performed. Total RNA was isolated from leaves, stems, flower primordia, open flowers, fruit pericarps, fruit placenta, seeds, and roots of *C. chinense* PI257284, fractionated on a 1.0% agarose gel, and transferred to a membrane. The transferred membrane was hybridized with radioactively labeled *PPII* cDNA (1,216 bp). A 1.2-kilobase transcript was detected in open flowers and seeds at a low level and was very weakly present in stems and flower primordia. In comparison, the transcripts were undetectable in leaves and barely detectable in pericarps, placenta, and roots (Fig. 5).

PPII gene induction patterns by PMMV, *Pseudomonas syringae* pv. *syringae* 61, and *Xanthomonas campestris* pv. *vesicatoria* race 3.

To study *PPII* gene expression in response to pathogen attack, we inoculated *C. chinense* PI257284, which carries the *L³* resistance locus, with PMMV (pathotype P_{1,2}). Inoculated pepper leaves elicited the HR at 3 days postinoculation (data not shown). As shown in Figure 6A, *PPII* was expressed as early as 12 h after PMMV inoculation and its level increased at 24 h after inoculation. At 120 h after inoculation, the level of *PPII* expression decreased, while the level of the *PR-4b* (accession number B1894277) transcript remained high. The *PPII* gene was not expressed when leaves were mock-inoculated with water.

When pepper leaves were infiltrated with *P. syringae* pv. *syringae* 61, a wheat pathogen, the leaves elicited the HR at 12 h postinfiltration (data not shown). *PPII* transcripts were detected as early as 3 h after infiltration (Fig. 6B), and their levels remained constant up until 24 h after infiltration. Expression of the *PR-4b* gene could be detected at 6 h postinfiltration, and its expression continued to increase over the time course of the experiment.

Furthermore, we studied the induction of *PPII* by inoculating the race 3 (*avrBs2*) of *X. campestris* pv. *vesicatoria*. The expression pattern of the *PPII* gene in leaves was compared between the resistant line and the susceptible line, both near-isogenic pepper lines. The HR symptom was characteristic of *avrBs2* infection to pepper plants, and the *Bs2* gene conferred resistance (Tai et al. 1999). Susceptible plants infiltrated with race 3 of *X. campestris* pv. *vesicatoria* did not exhibit any visible response within 36 h postinfiltration, whereas resistant plants developed an HR on infiltrated leaf tissue within 24 h postinfiltration (data not shown). As shown in Figure 6C, the *PPII* transcript was induced within 8 h after infiltration with incompatible *X. campestris* pv. *vesicatoria* and the detectable levels of *PPII* transcripts remained steady thereafter.

Expression patterns of several PMMV-induced expressed sequence tags were similar to *PPII* expression, regardless of the level of host resistance or nonhost response in this study (data

Fig. 2. Nuclear localization of *PPII*. The *PPII* open reading frame was fused in-frame to the upstream region of the *smGFP* gene. Inner onion epidermal cells were transformed with the fusion constructs by bombardment, and the cells were examined under a confocal laser-scanning microscope (MRC-1024; Bio-Rad, Hercules, CA, U.S.A.) 16 h later. Onion cells transformed with the control *smGFP* are shown in the left panel. Onion cells transformed with *PPII-smGFP* are shown in the right panel. The scale bars are 100 μ m long.

not shown). This suggests that a common mechanism may be involved in the induction of gene expression by plant pathogens.

Expression of *PP1I* is independent of abiotic signals.

The accumulation of endogenous SA, ethylene, or MeJA is common to pathogen-infected plants. *PP1I* transcript levels were examined after exogenous application of SA, ethephon, or MeJA to mature pepper leaves. We found that these compounds did not induce macroscopic cell death (data not shown). As shown in Figure 7A, these compounds also did not induce *PP1I* gene expression, whereas the expression of marker genes such as *PR-1* (BI894280), *ACC oxidase* (BI894281), and *proteinase inhibitor 2* (*Pin2*; BI894279) was induced. *PR-1* mRNA could be detected within 6 h after SA treatment. In addition, the *ACC oxidase* gene and *Pin2* were induced as early as 30 min after treatment with ethephon and MeJA. H_2O_2 and ABA did not induce expression of *PP1I* during the 24 h after treatment, while the marker genes, *GST* (BI894278) and *Dehydrin* (a pepper homolog; provided by D. Choi), were highly expressed 1 to 3 h after treatment (Fig. 7B). Using the same hybridization conditions, the *PP1I* gene was strongly induced at 72 h after PMMV inoculation as a positive control (data not shown).

DISCUSSION

We report here the isolation and characterization of the bZIP DNA-binding protein gene, *PP1I*. The *PP1I* gene was preferentially expressed during incompatible interactions with PMMV, *P. syringae* pv. *syringae* 61, and *X. campestris* pv. *vesicatoria* race 3. *PP1I* is a nuclear protein and is capable of activating transcription in yeast. These results suggest that the *PP1I* protein is a transcription factor that could be part of the plant defense response against pathogen infection.

PP1I is particularly interesting because it has characteristics different from those of other members of the bZIP DNA-binding protein family. First, *PP1I* has a limited but significant amino acid sequence homology with other bZIP transcription factors (29 to 84%) (Fig. 1). The *PP1I* amino acid sequence exhibits the highest identity (84%) with BZI-2 (Strathmann et al. 2001) of tobacco among the functionally characterized bZIP proteins to date. There was even low sequence similarity to the *LebZIP1* protein from tomato (35%) (Stanković et al. 2000), a member of the same solanaceous family. Second, transcripts of *PP1I* are specifically up-regulated during an incompatible interaction between pepper and pathogens (Fig. 6A to C). To our knowledge, this is the first report showing that transcription of a bZIP DNA-binding protein gene is preferentially induced by pathogen infection. It would be interesting to determine if the *PP1I* promoter mediates this pathogen-specific response. Third, treatment of pepper leaves with abiotic compounds such as SA, ethylene, MeJA, H_2O_2 , and ABA did not induce *PP1I* gene expression (Fig. 7). Thus, expression of the *PP1I* gene appears to be induced specifically by biotic stresses. This is surprising because bZIP DNA-binding proteins are induced by phytohormones such as ABA (Nakagawa et al. 1996) and also by other environmental conditions such as low temperature (Aguan et al. 1993), light (Rook et al. 1998), and wounding (Stanković et al. 2000). Fourth, although *PR* genes were induced by most of the biotic and abiotic treatments that were shown previously to be effective for *PR* gene induction (Dong 1998; Reymond and Farmer 1998), induction of *PP1I* gene expression appears to be limited to pathogen infection in this study (Fig. 6A to C). Therefore, *PP1I* gene induction may not be required for expression of the *PR* genes. Our results suggest that *PP1I* may participate in a plant defense-signaling pathway, independent of the relatively well-characterized pathway that is activated in response to SA, ethylene, and MeJA.

The bZIP class of transcription factors has been extensively studied in plants. Plant bZIP proteins share a highly conserved basic domain and bind to target sequences usually containing the G-box ACGT element (Dröge-Laser et al. 1997; Foster et al. 1994; Menkens et al. 1995). Based on sequence analysis, *PP1I* also possesses a highly conserved DNA-binding domain, which may bind the G-box element within promoters.

Nuclear proteins that contain NLS sequences are specifically recognized by receptors and are imported through the nuclear pore complexes into the nucleus (Görlich 1997). NLS sequences are integral parts of nuclear protein structures and usually consist of short sequences with a high proportion of basic amino acids. The *PP1I*-smGFP fusion protein was found exclusively in the nu-

Fig. 3. Transcriptional activation analysis of *PP1I* in yeast. **A**, Diagram of the reporter construct. **B**, LexA-dependent transactivation of *PP1I* in yeast cells. **C**, Transcriptional activation activity of *PP1I* in yeast cells analyzed using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate. 1, LexA; 2, LexA-*PP1I*N1 (amino acids at position 1 to 68); 3, LexA-*PP1I*N2 (position 1 to 115); 4, LexA-*PP1I*F (position 1 to 170); 5, LexA-*PP1I*C1 (position 81 to 170); 6, LexA-*PP1I*C2 (position 110 to 170); 7, LexA-GAL4; and 8, LexA-Lam. One unit of β-galactosidase is defined as the amount that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and D-galactose per min per cell. Data points are means of three replicates with standard error.

cleus (Fig. 2). The putative NLS sequence of PPI1 (³³KRMISNRESARRSRMRK⁴⁹) is a basic domain within the N-terminal region of the protein, and it is a bipartite NLS by two basic amino acids (K and R) at each end. The amino acid sequence alignment of the basic domains of 11 plant bZIP proteins reveals a highly conserved motif of 17 consecutive amino acids, KRxxSNRESARRSRx[R/K]K. It is possible that the dual function of nuclear targeting and DNA binding is conserved in all members of the bZIP family (Varagona and Raikhel 1994).

Recent studies have identified several families of transcription factors that are possibly involved in the plant defense responses. These include W-box-binding WRKY proteins (Wang et al. 1998; Yu et al. 2001), Myb-like proteins (Sugimoto et al. 2000; Yang and Klessig 1996), ethylene-response element-binding proteins (Büttner and Singh 1997; Park et al. 2001; Zhou et al. 1997), bZIP proteins (Büttner and Singh 1997; Després et al. 2000; Niggeweg et al. 2000; Zhang et al. 1999), and NAC proteins (Ren et al. 2000; Xie et al. 1999; S. J. Lee, S. Kim, S. Y. Yi, S. K. Oh, J. S. Lee, M.-C. Suh, K.-H. Paek, D. Choi, and B.-D. Kim, *unpublished data*). However, responses by these factors are not limited to pathogens. Most of the transcription factors may be connected and regulated by a network of signals. Combinatorial interactions among transcription factors may play an important role in mediating plant responses to abiotic/biotic signaling cross-talk (Singh 1998). Since *PPI1* is only induced by pathogens and not by abiotic treatments, it may be involved in a distinct gene regulation pathway that involves factors different from those of other regulatory pathways.

Taken together, the data presented in this study strongly indicate that PPI1 is a transcription factor probably involved in the

transcriptional activation that occurs during pathogen attack. It will be important to determine the biological role of PPI1 and its place in the hierarchy of plant defense signaling. Currently, we are generating transgenic tobacco plants with increased or reduced expression of *PPI1* to study its functional role in plant defense.

MATERIALS AND METHODS

Plant materials and pathogen inoculation.

C. chinense PI257284, carrying the *L*³ resistance gene against the *P*_{1,2} pathotype of PMMV, was used for the isolation and expression analyses of genes induced by PMMV. The *L*³ gene confers resistance to the *P*_{1,2} pathotype of PMMV (Berzal-Herranz et al. 1995; Boukema 1982). For the induction of HR, leaves from 7- to 8-week-old *C. chinense* PI257284 were inoculated with sap prepared from *N. benthamiana* leaves infected with PMMV and incubated at 25°C. As a control, leaves were mechanically wounded using Carborundum. To investigate the temporal expression of the *PPI1* gene during a nonhost incompatible interaction, leaves from 7- to 8-week-old *C. annuum* cv. Bukang were inoculated by syringe infiltration with *P. syringae* pv. *syringae* 61 (1×10^8 CFU ml⁻¹). Two near-isogenic pepper cultivars used to investigate the temporal expression of the *PPI1* gene were ECW (*bs2/bs2*, susceptible line) and ECW-20R (*Bs2/Bs2*, resistant line). Bacterial suspensions (approximately 2×10^8 CFU ml⁻¹) of *X. campestris* pv. *vesicatoria* race 3 (*avrBs2*) were used for infiltration. The infiltrated area of resistant plants developed an HR clearly different from that of susceptible plants within 24 h postinfiltration. The infiltrated leaves were harvested at various times and immediately frozen in liquid nitrogen for further analyses.

SSH.

The subtraction was performed with the polymerase chain reaction (PCR)-select cDNA subtraction kit (Clontech) according to the manufacturer's instructions. As the tester, cDNA from pepper leaves sampled 3 days after inoculation with PMMV and undergoing the initial HR was synthesized using *Avian myeloblastosis virus* (AMV) reverse transcriptase while, for the driver, cDNA from noninoculated pepper leaves was synthesized. To generate blunt-ended cDNA fragments that are optimal for subtraction and necessary for adaptor ligation, the tester cDNA was digested with *RsaI* and then ligated to the adaptors as recommended by the manufacturer. After the subtractive hybridization, the subtracted cDNAs were selectively amplified with nested PCR primers to enrich differentially expressed sequences. The amplified fragments were cloned into the pGEM-T easy vector (Promega, Madison, WI, U.S.A.), and the cloned fragments were sequenced with an automatic DNA sequencer (ABI3700; Applied Biosystems, Foster City, CA, U.S.A.). Sequence data were analyzed with BLAST, PSORT, and CLUSTALW programs.

Fig. 4. Southern blot analysis of pepper genomic DNA. Genomic DNA (20 µg per lane) from pepper leaves was digested with *DraI* (D), *EcoRI* (E), *HindIII* (H), or *XbaI* (X) and then fractionated by electrophoresis on an 0.8% agarose gel. The blot was hybridized with the full-length *PPI1* cDNA. The probe was prepared using the random primed DNA labeling method (Feinberg and Vogelstein 1983). Southern blot analysis was performed according to previously reported methods (Church and Gilbert 1984).

Fig. 5. Tissue-specific expression of the *PPI1* gene in pepper. Total RNA was extracted from leaves (LF), stems (ST), flower primordia (FP), open flowers (OF), fruit pericarps (PE), fruit placenta (PL), seeds (SD), and roots (RT). The probe detected transcripts of approximately 1.2 kb in size corresponding to *PPI1* mRNA.

Subcellular localization analysis of the PPI1-smGFP protein.

A chimeric fusion construct, *PPI1-smGFP*, was generated to enable the subcellular localization of the PPI1 protein to be determined. A full-length *PPI1* ORF without the termination codon was prepared by PCR amplification with the *PPI1* cDNA in pBluescript SK-phagemid as a template. Two *PPI1* gene-specific oligonucleotide primers, 5'-GCGCGGATCCATGGCTTCTTCAAGTGGT-3' and 5'-GCGCGGATCCGTACTGCAAGACATGTGC-3', containing *Bam*HI sites, were synthesized and used to amplify the *PPI1* ORF. The PCR-amplified product was digested with *Bam*HI and purified from an agarose gel and then inserted into the *Bam*HI site of the plasmid pSMGFP (David and Vierstra 1996). Subcellular localization of the *PPI1-smGFP* fusion construct was performed as previously described (Lee et al. 2001).

Yeast transactivation assay.

The *Eco*RI-*Xho*I restriction fragments of the truncated forms of *PPI1* were ligated into the *Eco*RI-*Xho*I site of pLexA (Clontech) to make in-frame fusions with the LexA DNA-binding domain. The resulting plasmids were named pLexA-PPI1F (amino acids at position 1 to 170), pLexA-PPI1N1 (position 1 to 68), pLexA-PPI1N2 (position 1 to 115), pLexA-PPI1C1 (position 81 to 170), and pLexA-PPI1C2 (position 110 to 170). These were cotransformed into the yeast strain, EGY48, with p8op-lacZ, which contains a *LacZ* reporter gene under the control of a LexA operator. For controls, pLexA, pLexA-Lam, and pLexA-GAL4 were used. Enzymatic assays for β -galactosidase in the yeast clones containing LexA fusion proteins were performed using *o*-nitrophenyl β -D-galactopyranoside (ONPG) as a substrate, according to the manufacturer's instructions (Clontech).

Fig. 6. Induction of *PPI1* by pathogens in pepper. **A**, Induction of *PPI1* by *Pepper mild mottle virus* (PMMV) (pathotype P_{1,2}) in *Capsicum chinense* PI257284 that carries the *L*³ resistance gene. Leaf samples were harvested at indicated time points and total RNA was extracted. Upper panels and lower panels show hybridizations with *PPI1* and *PR-4b* (a positive marker for pathogen treatment) probes, respectively. Twenty micrograms of total RNA was used per lane. **B**, Induction of *PPI1* by *Pseudomonas syringae* pv. *syringae* 61 in *Capsicum annuum* cv. Bukang. **C**, Expression pattern of *PPI1* in two near-isogenic lines (*C. annuum* ECW-20R, resistant; *C. annuum* ECW, susceptible) inoculated with *Xanthomonas campestris* pv. *vesicatoria* race 3.

Genomic DNA blot analysis.

Genomic DNA was isolated from mature leaves of *C. chinense* PI257284 as described (Lee et al. 2001). DNA was treated with RNase, and 20- μ g samples were digested to completion with *Dra*I, *Eco*RI, *Hind*III, or *Xba*I. The digested genomic DNA was separated by electrophoresis on an 0.8% agarose gel, denatured, and blotted onto a nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Southern blotting was performed as described previously (Church and Gilbert 1984), and the membranes were hybrid-

ized with a full-length *PPII* cDNA probe labeled with 32 P (5×10^6 disintegrations per min [dpm]).

Chemical treatments.

For chemical compound treatments, SA was dissolved in water at a concentration of 5 mM; MeJA and ethephon were dissolved in 10% acetone at concentrations of 100 μ M and 5 mM, respectively; ABA was dissolved in 0.1% ethanol at a final concentration of 100 μ M; and H_2O_2 was diluted in water at a final concentration of 5 mM. These chemicals were sprayed

Fig. 7. *PPII* gene expression in response to abiotic treatments. **A**, Expression patterns of the *PPII* gene after exogenous treatment with salicylic acid (SA), ethephon, or methyl jasmonate (MeJA). Total RNA was extracted at different time points from pepper leaves treated with 5 mM SA, 5 mM ethephon, or 100 μ M MeJA. *PR-1*, *ACC oxidase*, and *Pin2* genes were used as positive markers for the SA, ethephon, and MeJA treatments, respectively. **B**, *PPII* expression in response to 5 mM H_2O_2 or 100 μ M abscisic acid (ABA). *GST* and *Dehydrin* were used as positive markers for H_2O_2 and ABA treatments, respectively.

onto the matured leaves of *C. chinense* PI257284. For all the experiments, leaves were pooled from several plants grown under the same conditions, quickly frozen in liquid nitrogen, and stored at -70°C until used.

Gene expression analysis.

Total RNA was extracted from different tissues of *C. chinense* PI257284 by a previously described method (Choi et al. 1996). Twenty micrograms of total RNA was denatured and electrophoresed with 1.0% agarose gels containing formaldehyde and transferred onto nylon membranes (Amersham Pharmacia Biotech). The membranes were hybridized with ^{32}P -labeled (5×10^6 dpm) probes.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BLAST program: <http://www.ncbi.nlm.nih.gov/BLAST/>
 CLUSTALW program: <http://www2.ebi.ac.uk/clustalw/>
 PSORT program: <http://psort.nibb.ac.jp/>