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Molecular Characterization of a PR4 Gene in Chinese Cabbage

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Abstract: A cDNA clone for a wound- and pathogen-induced gene in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) was isolated and characterized. The cabbage gene, designated BrPR4, encodes a pathogenesis-related protein 4 (PR4) of 140 amino acids. The BrPR4 protein shows high similarity with wound-inducible antifungal proteins of tobacco, potato, barley, and wheat. The BrPR4 gene is locally induced by a nonhost pathogen, *Pseudomonas syringae* pv. *tomato*, that elicits a hypersensitive response in Chinese cabbage. Treatment of the cabbage leaves with benzothiadiazole (BTH), methyl jasmonate or ethephon showed that the BrPR4 gene expression is strongly induced by ethylene, but not by methyl jasmonate or BTH. The BrPR4 gene is also activated by wounding. Interestingly, however, the wound-inducible BrPR4 gene expression is repressed by salicylic acid or BTH, suggesting that there is cross-talk between salicylate-dependent and -independent signaling pathways.

Key words: *Brassica rapa*, Chinese cabbage, PR4, defense-related

INTRODUCTION

Like animals, plants also have defense mechanisms against various pathogens and pests. One of the best-studied defense mechanisms is systemic acquired resistance (SAR), which is induced locally by pathogen or pest attack and spreads systemically. SAR is also broad-spectrum and long-lasting in effect (Ryals et al., 1996). Defense responses are triggered upon perception of invading pathogens by specific receptors that are encoded by disease resistance genes (Bent, 1996; Dangl and Jones, 2001). This recognition in turn activates signaling components, such as EDS1, NDR1 and NPR1 (Delaney, 1997; Feys and Parker, 2000). Biochemical studies have shown that reactive oxygen species and nitric oxide are important signaling molecules in the activation of disease resistance. These studies have also shown that MAP kinase cascades play a critical role in plant defense responses, as in animals (McDowell and Dangl, 2000; Nuernberger and Scheel, 2001).

The genus *Brassica* includes many important vegetable crops, such as broccoli, cabbage, Chinese cabbage, cauliflower, mustard, rape, kale, and turnip. Although these *Brassica* species have served as good model plants to study self-incompatibility (Takasaki et al., 2000), studies on defense mechanisms in *Brassica* have not been very active. Progress on identifying defense mechanisms in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), an important vegetable crop in Asia, has also been very slow. Previously, we isolated a partial cDNA clone that contained a Chinese cabbage PR4 gene exhibiting strong induction by *Pseudomonas syringae* pv. *tomato* (*Pst*) (Ryang et al., 2002). *Pst* causes bacterial speck disease in tomato and *Arabidopsis* (Bashan et al., 1981; Whalen et al., 1991). In Chinese cabbage, *Pst* induces hypersensitive response (HR), often a prerequisite for induction of disease resistance in plants, although *Pst* does not cause disease (Ryang et al., 2002). Here, we report the cDNA sequence of the full coding region and more detailed expression profiles of the *Pst*-induced Chinese cabbage PR4 gene.

MATERIALS AND METHODS

Plant materials and chemical treatments

Brassica rapa subsp. *pekinensis* (cultivar Norang) seedlings were grown on potting compost after germination. Unless stated otherwise, experiments were performed with cabbage

The nucleotide sequence data that is reported in this paper has been submitted to the GenBank nucleotide sequence database, and assigned the accession number AF528181.

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seedlings at the seven- or eight-leaf stage. For the salicylic acid (SA) treatment, fully developed and healthy leaves from the plants were cut into 1 × 1 cm pieces, and floated onto 20 mM MOPS buffer (pH 7.5) containing either 5 mM or no salicylic acid (Sigma Chemical Co., St. Louis, USA) in a 10 cm or 15 cm petri dish. The leaf samples were then transferred to a growth chamber and incubated at 25°C under continuous fluorescent light. After one to three days of treatment, the leaf samples were harvested and frozen immediately in liquid nitrogen. A mixture of 0.3 mM benzothiadiazole (BTH), 1 mM methyl jasmonate (Me-JA) in 0.1% [v/v] ethanol, 1 mM ethephon or 0.1% ethanol were sprayed on leaves of intact plants. BTH (5% active ingredient in wettable powder) was a kind gift from Novartis, Korea. Me-JA and ethephon were purchased from Aldrich Chemical Co. (Milwaukee, USA) or Sigma Chemical Co. (St. Louis, USA).

Pathogen treatment

Pseudomonas syringae pv. *tomato* (*Pst*) 259 was a kind gift from Dr. Cha at the Phytopathogenic Bacteriology Laboratory at Chungbuk National University, Korea. The *Pseudomonas* bacteria for inoculation were freshly grown on NA agar medium (5 g of peptone, 3 g of beef extract, 2 g of yeast extract, and 15 g of Bacto-agar per liter) for one or two days at 30°C. The bacteria were collected by gently scraping the colonies from the agar medium with a sterile glass rod after adding 2 ml of sterile water per 85-mm plate. After centrifugation at 5,000 *g*, the bacterial pellet was resuspended in sterile water and adjusted to an $OD_{600} = 0.1$. Cabbage leaves were inoculated with the bacterial suspension by syringe infiltration. *Pst*-treated cabbage plants were then transferred to a growth chamber and incubated at 25°C under continuous light. Visible HR usually developed 24 to 36 h after inoculation. Control plants were similarly treated with sterile water.

cDNA library screening

The PR4 gene was isolated from a Chinese cabbage cDNA library constructed with the Lambda ZapII vector using mRNA from cabbage leaves inoculated with *Pst*. Aliquots of the library were grown on ten LB agar plates at the density of 2.5×10^4 pfu per plate and the phages were eluted with SM buffer. The phage suspension from each plate was then examined by PCR for the presence of the PR4 clone. One of the PCR primers was a PR4 gene-specific primer, and the other primer was of vector sequence near the cloning site (5' AACAGCTATGACCATGATTAC GCC 3'). The phages in the sub-library fraction yielding positive DNA bands were then diluted and grown on 24 plates at the density of 2,000 pfu per plate. Eluted phages from the plates were again examined by PCR.

To confirm whether the PCR-amplified DNA contained the PR4 gene sequence, DNA blot hybridization was performed using the partial cDNA as a probe. The preparation of alkaline phosphatase-conjugated DNA probe and chemiluminescent detection were carried out as described by Min et al. (2001) using the AlkPhos Direct labeling and detection kit from Amersham Pharmacia Biotech (Buckinghamshire, UK). Finally, the phage sample containing the largest insert was then screened by *in situ* plaque hybridization. Subcloning of the cDNA insert was done by *in vivo* excision of the pBluescript SK phagemid DNA with ExAssist helper phage, as described in Stratagene's protocol. The plasmid clone obtained was designated pBrPR4.

DNA sequence analysis and database search

The nucleotide sequence of the cDNA insert was determined with an Applied Biosystems automatic sequencer at the Macrogen Co., Seoul. The database search and computation were performed at the NCBI (National Center for Biotechnology Information) using the BLAST network service. Motif searches were made using the PROSITE database at http://expasy.hcuge.ch/sprot/prosite.html. Signal peptide prediction was performed using TargetP (Emanuelsson et al., 2000) and http://www.cbs.dtu.dk/services/TargetP/.

RNA extraction and northern blot analysis

Total RNA was prepared from frozen plant materials using the "hot phenol" method of De Vries et al. (1988). For Northern analysis, 10 μg of total RNA was separated on a 1.0% formaldehyde-agarose gel and blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) using the standard capillary transfer method. After UV-crosslinking at 125 mJ, blots were hybridized using the DNA probe labeled with digoxigenin (DIG). The probe DNA for detecting cabbage PR4 mRNA was prepared by PCR amplification of the cDNA insert of pBrPR4. The amplified DNA was labeled with DIG by random priming reaction. The DNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPD) was obtained using a GAPD cDNA clone (GenBank accession no. AF536826). The DNA probe for a putative cytochrome P450 (CYP83B1) (GenBank accession no. AF528175) was prepared using the cDNA clone (CPE-T9) isolated in our laboratory (Ryang et al., 2002). The DIG-labeling, hybridization, and chemiluminescent immunodetection were performed using kits from Roche Molecular Biochemicals (Mannheim, Germany).

RESULTS AND DISCUSSION

Isolation and characterization of a Chinese cabbage PR4 gene

To obtain a cDNA clone for the *Pst*-induced PR4 gene in

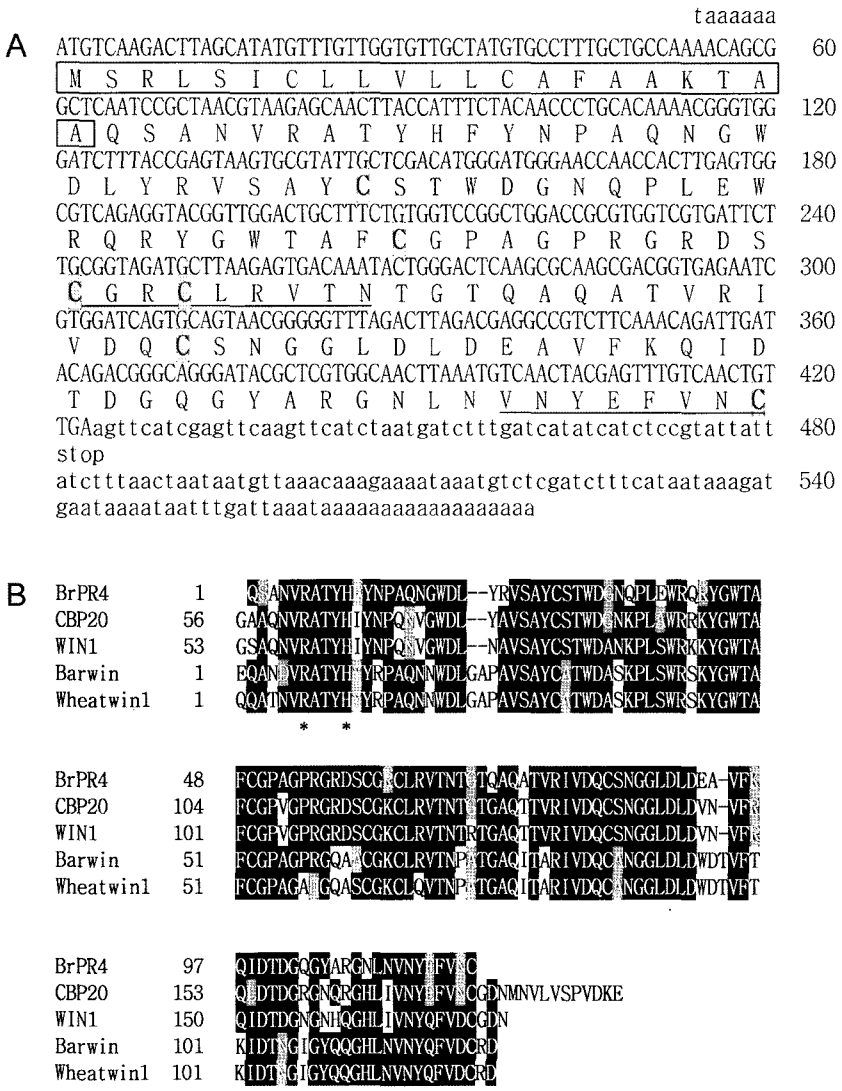

Fig. 1. A PR4 gene in Chinese cabbage. A, Nucleotide and deduced amino acid sequences of BrPR4 gene. 5' and 3' noncoding cDNA sequences are represented by lowercase letters. Putative signal peptide is boxed. Barwin domain signatures are underlined, and the six putative cysteine residues involved in disulfide bonds (C49, C70, C81, C84, C104, and C140) are shaded. B, Multiple sequence alignment of BrPR4 protein and other PR4 proteins from tobacco (CBP20; GenBank accession number AAB29959), potato (WIN1; CAA31851), barley (barwin; P28814), and wheat (wheatwin1; CAA06856). In this alignment, the signal peptides were not included for comparison. In the cases of CBP20 and WIN1, additional N-terminal 55 or 52 amino acid residues of the mature proteins were also excluded. Identical amino acids are boxed, and conserved amino acids or amino acids with similar properties are shaded. Asterisks indicate putative catalytic residues in wheatwin1 for ribonuclease activity.

Chinese cabbage, a cDNA library was screened by PCR and *in situ* plaque hybridization, as described in Materials and Methods. The cDNA clone, designated pBrPR4, contained a 566-bp ORF with 5' and 3' noncoding sequences of 7-bp and 139-bp, respectively (Fig. 1A). Running the TargetP program (Emanuelsson et al., 2000), a prediction program for subcellular localization of proteins, identified a signal peptide of 21 amino acid residues. Scanning of the PROSITE database revealed the presence of barwin domain signatures in the cabbage PR4 protein. Barwin is a barley wound-induced seed protein of 125 residues that weakly binds a chitin analog (Svensson et al., 1992). The barwin domain signature 1, C-G-[KR]-C-L-V-N, was found at the positions 81-89 of the cabbage PR4 protein. Another barwin domain signature, V-[DN]-Y-[EQD]-F-V-[DN]-C, was present at the C-terminus. The six cysteine residues involved in disulfide bonds in the barwin protein were also conserved in the cabbage PR4 protein (Fig. 1A), suggesting that overall structure of the cabbage protein might be similar to that of the barwin.

The cabbage PR4 protein exhibited 80% similarity and 69% identity with the *Sambucus nigra* (European elder) PR4 type protein (GenBank accession # Z46946). BLAST search also showed that more than 40 proteins have scores

higher than BLASTP E $<10^{-40}$. These proteins included CBP20, PR-4a and PR-4b proteins of tobacco, and WIN proteins of potato, wheat and barley (WIN1, WIN2, wheatwin1, wheatwin2, and barwin). The comparison of the mature protein of BrPR4 with the mature PR4 proteins of tobacco (CBP20), potato (WIN1), barley (barwin), and wheat (wheatwin1) showed striking similarity among these proteins (Fig. 1B). The WIN proteins have been reported to be induced by wounding and to have antifungal activity (Svensson et al., 1992; Caruso et al., 1996; 2001). CBP20 was reported to be induced by wounding and pathogen (Ponstein et al., 1994). In the cases of WIN1 and CBP20, the N-terminal part of these proteins did not show any similarity with the cabbage PR4 protein. WIN1 and CBP20 had an N-terminal chitin-binding domain, which was not found in the cabbage protein, based on the PROSITE search. When the N-terminal 52 and 55 amino acids of the mature WIN1 and CBP20, respectively, were excluded in the comparison, the remaining part of the proteins showed strong similarity with the cabbage PR4 protein (Fig. 1B).

Recently, it was reported that the wheatwin has ribonuclease activity (Caporale et al., 2004). The authors reported that the wheat PR4 protein showed strong similarity with the RNase A and T1 in three-dimensional structure. They also suggested two His residues (His11 and His113), an acidic residue (Asp92) and a basic residue (Arg7) in the active site (Fig. 1B). The Arg7, His11 and Asp92 residues of wheatwin1 are conserved in the cabbage PR4 protein. However, the second His residue corresponding to the His113 of wheatwin was missing in BrPR4 protein, suggesting that the cabbage protein might not have the ribonuclease activity.

Expression patterns of the cabbage PR4 gene

Figure 2 shows that the BrPR4 gene is strongly induced by *Pst*. Since many defense-related genes are induced by SA, we examined the effect of SA on BrPR4 gene expression. To this end, cabbage leaves were cut into 1 × 1 cm pieces and floated onto MOPS buffer containing 5 mM SA. The result of Northern analysis showed that the BrPR4 gene expression was higher in the leaf squares mock-treated with MOPS buffer (Fig. 2A, M lane) and that the expression of BrPR4 appeared to be repressed by SA (Fig. 2A, S lane). This suggests that expression of this gene is induced by wounding, since the SA and mock treatments involve physical injury to the leaf tissues. The BrPR4 gene was strongly activated 24 h after *Pst* inoculation. Analysis of the leaves distant to the leaf infiltrated with *Pst* showed, however, that the BrPR4 gene was not systemically activated by the pathogen (Fig. 2B).

We examined the effect of other defense response activators, jasmonate and ethylene, since plant defense responses can also be activated *via* SA-independent signaling pathways mediated by jasmonates and ethylene

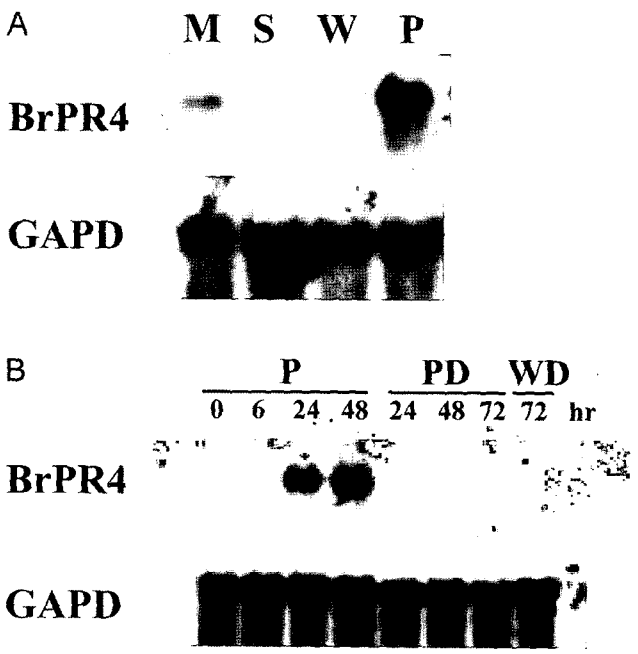

Fig. 2. Northern analysis of the cabbage PR4 gene expression. A, Examination of induction by salicylic acid and pathogen. For salicylic acid treatment, Chinese cabbage leaves were cut into 1 × 1 cm squares, and floated onto either 20 mM MOPS buffer, pH 7.5 (M) or the buffer containing 5 mM salicylic acid (S). For pathogen treatment, Chinese cabbage leaves were infiltrated with either sterile water (W) or *Pst* (P). The leaf samples were collected after 24 h and analyzed by Northern blot hybridization. B, Examination of systemic induction. Chinese cabbage leaves were infiltrated with *Pst*, and the leaf samples were collected after the indicated time. P: *Pst*-infiltrated leaf; PD: leaf distant from the *Pst*-infiltrated leaf; WD; leaf distant from the water-infiltrated leaf. For Northern analysis, 10 μg total RNA from each sample was size-fractionated on a 1% formaldehyde agarose gel. The fractionated RNA was blotted onto a nylon membrane and hybridized with DIG-labeled DNA probe. As a loading control, samples were also hybridized with the cDNA specific for the cabbage glyceraldehyde-3-phosphate dehydrogenase (GAPD). Chemiluminescent detection was done using alkaline phosphatase-conjugated anti-DIG antibody and CSPD.

(Pieterse and van Loon, 1999). To see how the BrPR4 gene responds to ethylene or jasmonates, cabbage leaves were treated with Me-JA or with ethephon, an ethylene-releasing compound. We also used BTH, one of the most potent SAR-inducing chemicals (Goerlach et al., 1996). In this experiment, the chemicals were sprayed onto the leaves so that the leaves would not be injured. As shown in Fig. 3A, the BrPR4 gene was not induced either by BTH or Me-JA. To verify that appropriate induction conditions were used, we included as a positive control the expression of the Me-JA-inducible Chinese cabbage gene encoding a putative CYP83B1, which was isolated in our laboratory as *Pst*-induced genes (Ryang et al., 2002). CYP83B1 is an oxime-metabolizing enzyme in the glucosinolate biosynthesis (Hansen et al., 2001). The cabbage cytochrome P450 gene was induced by both BTH and Me-JA, as shown in Fig. 3A. Taken together, these results clearly showed that the BrPR4

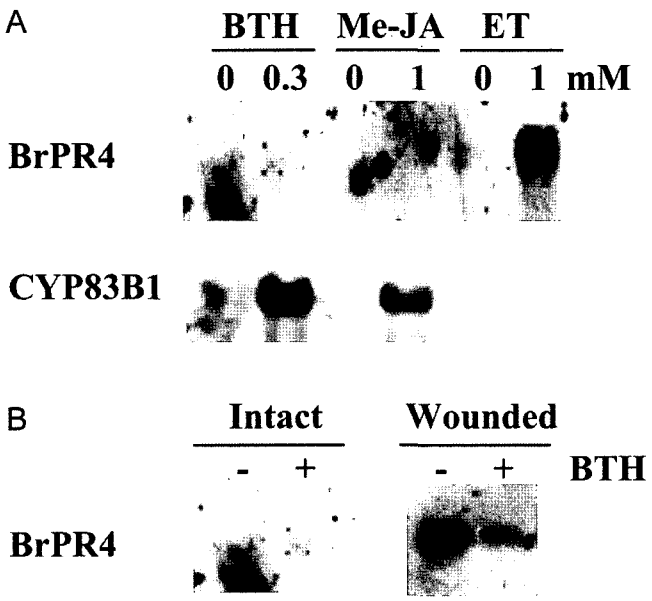

Fig. 3. Effect of BTH, methyl jasmonate and ethylene. A, Expression of the BrPR4 gene in intact leaves. 0.3 mM of BTH, 1 mM methyl jasmonate (Me-JA) in 0.1% ethanol, 1 mM ethephon solution, or 0.1% ethanol and sterile water (control) was sprayed onto cabbage leaves. After 24 h the leaf samples were collected and analyzed by Northern blot hybridization using DIG-labeled probes, as described in Fig. 2. B, Wound-inducible PR4 gene expression and the effect of BTH. In one BTH treatment, intact leaves were sprayed with 0.3 mM BTH solution. In the other experiment, the leaves were cut into 1 × 1 cm pieces and floated onto BTH solution. 24 h after the treatment, the leaf samples were collected and analyzed by Northern blot hybridization using DIG-labeled DNA probe, as described in Fig. 2.

gene is induced by ethylene, but not by BTH or Me-JA.

The result shown in Fig. 3 indicates that the BrPR4 gene is wound-inducible. To address directly whether the cutting itself induced the gene expression, we cut the leaves into 1 x 1 cm squares and treated the squares with BTH as we had treated them with SA. The result was then compared with that of BTH treatment in intact leaves. The result showed that wounding strongly induced expression of the BrPR4 gene (Fig. 3B). Again, expression of the BrPR4 gene was repressed by BTH, suggesting that the induction of BrPR4 gene expression by wounding is antagonized by the presence of SA or BTH. Inhibition of the wound response by SA is a well-known phenomenon (Doares et al., 1995; Penta-Cortes et al., 1993). Since the BrPR4 gene is activated by ethephon, its suppression by SA or BTH seems to be an outcome of cross-talk between the SA-dependent and ethylene-dependent pathways.

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