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Comparative analysis of pathogenesis-related protein 10 (*PR10*) genes between fungal resistant and susceptible peppers

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Abstract To elucidate the functional roles of *PR10* genes from two pepper species during plant-pathogen interactions, *PR10* genes were isolated from fungal-resistant (*Capsicum baccatum* var. PBC80) and fungal-susceptible (*C. annuum* var. Yeoju) pepper fruits infected with anthracnose fungus (*Colletotrichum acutatum*). Despite strong nucleotide sequence identity, there were significant differences in the patterns of gene expression and protein accumulation between the genes from the two host species. Induced expression of the *PR10* mRNA in PBC80 (*bac*PR10) was highly maintained from 24 h after infection (HAI) rather than that in Yeoju (*ann*PR10). These mRNA expression patterns were correlated with the level of respective protein that was detected as two or three bands in each species. Substantial induction of *bac*PR10 proteins was confirmed by 2D-gel analysis followed by immunoblotting. Immunolocalization study showed that deposition of bacPR10 was exclusively observed in the pericarp of PBC80 fruits after fungal infection, suggesting functional significance in defence. Additionally, *in vitro* analysis of the enzymatic properties of PR10 proteins revealed that recombinant bacPR10 had higher ribonucleolytic activity and exhibited less sensitivity to proteinase treatment than did annPR10. Taken together, these results support the idea that relative abundance and prolonged longevity of bacPR10 in PBC80 fruits may contribute to their increased resistance in response to the anthracnose fungus, as compared with Yeoju fruit.

Keywords *Capsicum baccatum* · *C. annuum* · *Colletotrichum acutatum* · 2D analysis

Abbreviations

HAI	Hours after infection
HR	Hypersensitive reaction
P-loop	Phosphate-binding loop
PR	Pathogenesis-related

Introduction

Plants respond to invasions of phytopathogens with coordinated and/or integrated gene expression (Hammond-Kosack and Jones 1996). Various genes

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are induced both at the site of infection and in distal parts of the plant, leading to development of the hypersensitive reaction (HR) and systemic acquired resistance (SAR), respectively (Jackson and Taylor 1996; Durrant and Dong 2004). The expression levels of several genes are often used as indices of plant resistance to pathogen infection. For example, pathogenesis-related (PR) genes are induced early, at higher levels, during incompatible host-pathogen interactions than during compatible interactions (Bell et al. 1986). These genes might play important roles in the resistance of the host against the pathogen.

PR proteins do not usually accumulate in healthy plants, but are induced by pathogen infection or related stresses, and improve the defensive capacity of plants (Van Loon and Van Strien 1999). The PR proteins have been divided into 17 families based on their sequences and biological activities (Vidhyasekaran 2008; Christensen et al. 2002). Although most of PR proteins are extracellular, the first intracellular PR protein, designated PR10, was isolated from cultured parsley cells after treatment with an elicitor (Somssich et al. 1989).

The PR10 proteins are a group of small acidic proteins that are found in both monocots and dicots and are involved in intracellular defence (McGee et al. 2001; Hashimoto et al. 2004; Liu and Ekramoddoullah 2004; Islam et al. 2009: Chen et al. 2010; Lebel et al. 2010). Although fungal or bacterial elicitors rapidly induce *PR10* expression, several *PR10* genes are expressed in a tissue-specific manner during development (Gamas et al. 1998; Jwa et al. 2001; Liu et al. 2005) and are induced by abiotic stresses, hormones, darkness, and physiological stages such as leaf senescence (Iturriaga et al. 1994; Walter et al. 1996; Ziadi et al. 2001; Liu et al. 2006; Kim et al. 2008; Jellouli et al. 2008).

Based on the sequence homologies between a ginseng ribonuclease and PR10 proteins, some PR10 proteins were tested *in vitro* for ribonuclease activity (Bufe et al. 1996; Bantignies et al. 2000; Wu et al. 2003; Srivastava et al. 2006). However, there is no homology between other plant ribonucleases and PR10 proteins, except for the phosphate-binding loop (P-loop; GXGGXG), which is a strongly conserved feature of nucleotide-binding proteins (Saraste et al. 1990). Nevertheless, it has been suggested that intracellular PR10 proteins are capable of cleaving pathogenic RNAs. Park et al. (2004) reported that a cultivar-specific PR10 (CaPR-10) from *C. annuum* var. Bugang became phosphorylated and exhibited RNase activity after inoculation of TMV-P_0, resulting in the cleavage of viral RNA. They suggested that phosphorylated CaPR-10 is an active component of an inducible defence mechanism against pathogens.

The majority of chili peppers, including hot and sweet pepper varieties cultivated worldwide, have been developed from *Capsicum annuum*, which is highly susceptible to anthracnose fungi. To date, no single variety of pepper resistant to anthracnose fungi has been developed. Recently, resistance to fruit-specific anthracnose was identified in two other *Capsicum* species, *C. baccatum* and *C. chinense* (AVRDC 1999). Lesion formation was found to be limited to the site of infection or entirely suppressed in the fruits of several lines of *C. baccatum* (Mahasuk et al. 2009). The mechanism underlying this resistance was examined using cytological and histological techniques (Kim et al. 2004).

We performed a preliminary comparative analysis of several *PR* genes from two pepper species, *C. baccatum* var. PBC80 and *C. annuum* var. Yeoju, which are resistant and susceptible to fruit-specific anthracnose fungus, respectively. There were no significant differences in the nucleotide sequences of PR genes, such as *PR1* and *PR*3 between PBC80 and Yeoju, except *PR10*. Therefore, in this experiment, *PR10* gDNAs and cDNAs were isolated from both pepper species to investigate their sequence similarities, expression patterns at the mRNA and protein levels, and functional activities in order to better understand their roles during plant-pathogen interactions.

Materials and methods

Fungal and plant materials

C. baccatum var. PBC80 and *C. annuum* var. Yeoju were used as plants resistant and susceptible to the KSCa-1 isolate of *Colletotrichum acutatum* (Fig. 1). Plants were grown at 25°C under greenhouse conditions. Fully grown unripe green fruits of three-month-old pepper plants were used.

Fungal pathogen and inoculation

Inoculum preparation and artificial inoculation procedures were performed as described previously (Kim et

Fig. 1 Development of anthracnose symptoms on susceptible and resistant pepper fruits at 9 days after inoculation with *C. acutatum* KSCa-1. **a** *Capsicum annuum* var. Yeoju; **b** *Capsicum baccatum* var. PBC80

al. 2004). Briefly, the Korean isolate KSCa-1 of *C. acutatum* was grown on potato dextrose agar (PDA; Sigma, USA) at 25°C under fluorescent light with a 16-h light/8-h dark photoperiod. Seven-day-old colonies were flooded with dH_2O and gently scraped from the plates. The inoculum concentration was adjusted to 5×10^5 ml^{-1} of conidia. Two μl of the conidial suspension were injected at three sites on the surface of detached pepper fruits to a depth of 0.8 mm. The inoculated fruits were placed in an acrylic box that was moistened and sealed tightly to maintain the relative humidity, and the box was incubated at 25°C.

Cloning, sequencing, and gene expression

Genomic DNA (gDNA) was extracted from leaves of each species using the DNeasy Plant Maxi Kit (Qiagen, Germany). Sequences encoding orthologs of *bac*PR10 and *ann*PR10 were amplified from gDNA by PCR using gene-specific primers (F, 5′-CTG ACA AGT CCA CAG CCT CAG-3′, and R, 5′-TTA AAC ATA GAC AGA AGG AT-3′) designed against the nucleotide sequence encoding CaPR-10 (Park et al. 2004). To obtain the upstream regions, the gDNAs were digested with EcoRV or HindIII followed by inverse polymerase chain reaction using primer sets (IF1, 5′-CCC CAT CAA GGC TAT TCA A-3′; IF2, 5′-CCT CGC CAA TCC TTC TGT CT-3′;IR1, 5′-GCA ACT GAG GCT GTG GAC TT-3′;IR2, 5′-GCT GTG GAC TTG TCA GTA AAG GT-3′). Total RNA was extracted from infected unripe fruits using the RNeasy Plant Mini Kit (Qiagen, Germany), and reverse transcriptase PCR (RT-PCR) was performed to obtain the cDNAs. Full-length cDNAs and gDNA were inserted into pGEM-T Easy (Promega, USA) and sequenced. Sequence comparisons were performed using BLAST (NCBI). Expression levels were determined using Northern analysis with 10 μg of total RNA extracted from infected fruits. For gene specific probes, the cDNAs were labeled with [α^{32}P] dCTP using a random primer labelling kit (Boeringer Mannheim, Germany).

SDS-PAGE, Western blot analysis, and immunohistochemistry

Samples were homogenized in extraction buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 2 mM DTT, 0.25 M sucrose, and protease inhibitor cocktail) in cold conditions and subjected to centrifugation at 3000×*g* for 15 min. The supernatant was used as total protein. The proteins were separated by 12% SDS-PAGE and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. A 1:2000 dilution of polyclonal anti-bacPR10 antibody was used as the primary antiserum in immunoblot analysis, followed by incubation with a 1:10,000 dilution of horseradish peroxidase-coupled goat anti-rat antibody. For immunolocalization, pepper fruits were fixed in 0.1% glutaraldehyde and 3% paraformaldehyde in 50 mM sodium phosphate buffer (pH 7.0), dehydrated in ethanol, and embedded in paraffin. Tissues were sliced into 10-μm transverse sections. The deparaffinized sections were incubated with anti-bacPR10 antibody (1:2000) for 12 h at 4°C. The primary antibody was detected with FITC-conjugated goat anti-rat antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and observed by fluorescence microscopy (Olympus, Japan). Control experiments using pre-immune serum were unreactive (data not shown).

Two-dimensional gel electrophoresis

Two hundred μg of total protein in 200 μl were extracted with 200 μl of TE-saturated phenol (Bestel-Corre et al. 2002). After centrifugation at 12,000×*g* for 10 min, the upper aqueous phase was removed and the proteins were precipitated with five volumes of 0.1 M ammonium acetate in methanol. The pellet was dried and dissolved in isoelectric focusing sample buffer (9 M urea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate, 0.5% IPG buffer, and 0.01% bromophenol blue). Immobiline DryStrips (pH 4 to 7, nonlinear; 70 mm (Amersham Biosciences, Sweden)) were rehydrated with proteins and focused using an IPGphor system (Amersham Biosciences, USA). The strips were transferred to equilibration buffer (50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 20 mM tributylphosphine) and incubated for 15 min. After electrophoresis in the second dimension, the 2D gels were stained with silver nitrate (Blum et al. 1987) or electrotransferred onto polyvinylidene fluoride (PVDF) membranes. A polyclonal anti-bacPR10 antibody and a secondary anti-rat antibody coupled to horseradish peroxidase were used for immunoblot analysis.

Recombinant bacPR10 and annPR10 proteins and antibody production

The *ann*PR10 or *bac*PR10 open reading frames were amplified from cDNA by PCR and ligated into pGEX-6P-1 (Amersham Biosciences, USA) between the *Eco*RI and *Xho*I sites, creating an in-frame fusion with the sequence encoding glutathione-S-transferase (GST). These GST fusion proteins were expressed in *E. coli* BL21 and purified according to the manufacturer's instructions. The protein concentration was determined using the Bradford method (Bradford 1976). Following digestion with PreScission protease (Amersham Biosciences, USA), the ribonuclease, proteinase resistance, and antifungal activities of the pure proteins (annPR10 or bacPR10) were examined. In addition, purified bacPR10 protein was injected into a rat to raise a polyclonal antibody.

Ribonuclease activity of bacPR10 and annPR10 proteins

RNA degradation assays were performed as described previously (Bantignies et al. 2000). Five μg of total RNA from pepper fruits were incubated with 2 μg of recombinant PR10 protein for 0, 1, 5, 10, 30, 60, or 180 min, and then separated on 1.0% agarose gels.

Proteinase resistance of bacPR10 and annPR10 proteins

Two μg of recombinant PR10 proteins were digested with 1 μg ml^{-1} Proteinase K or PBS buffer at 37°C for 0, 0.5, 1, 1.5, 3, 5, or 10 min, respectively. The proteins were analyzed by SDS-PAGE.

Antifungal activity of bacPR10 and annPR10 proteins

Ten μl of spore suspension (5×10^5 ml^{-1}) and 10 μl of recombinant protein or PBS buffer were applied to cover slips and then incubated in a humidified chamber for 24 h in darkness at 25°C. Fungal viability was estimated by staining germinated spores using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, USA). Fluorescence was observed using a fluorescence microscope (Olympus, Japan) equipped with a FITC filter (excitation 460–490, barrier 520).

Results

Cloning and sequence analysis of *PR10* genes

To isolate *PR10* orthologs, gene fragments were amplified by PCR from gDNA of two peppers using gene-specific primers that were designed based on the nucleotide sequence encoding CaPR-10 (Park et al. 2004). To obtain the upstream regions, two primer sets for inverse polymerase chain reactions (iPCR) were designed in opposite orientation to that of normal PCR using the sequencing data of *PR10* gene fragments. After inverse PCR cloning, 265 bp iPCR products were recovered from genomic DNAs of both species. Finally, based on the sequencing data, the full size of expected fragments were cloned, sequenced, and then designated *bac*PR10 (*C. baccatum* pathogenesis-related protein 10) and *ann*PR10 (*C. annuum* pathogenesis-related protein 10). The 1334- and 1342-bp PCR products from PBC80 and Yeoju, respectively, comprised two exons with an intervening intron and a 3′ UTR (Fig. 2). The sequences were entered into the GenBank nucleotide sequence data-

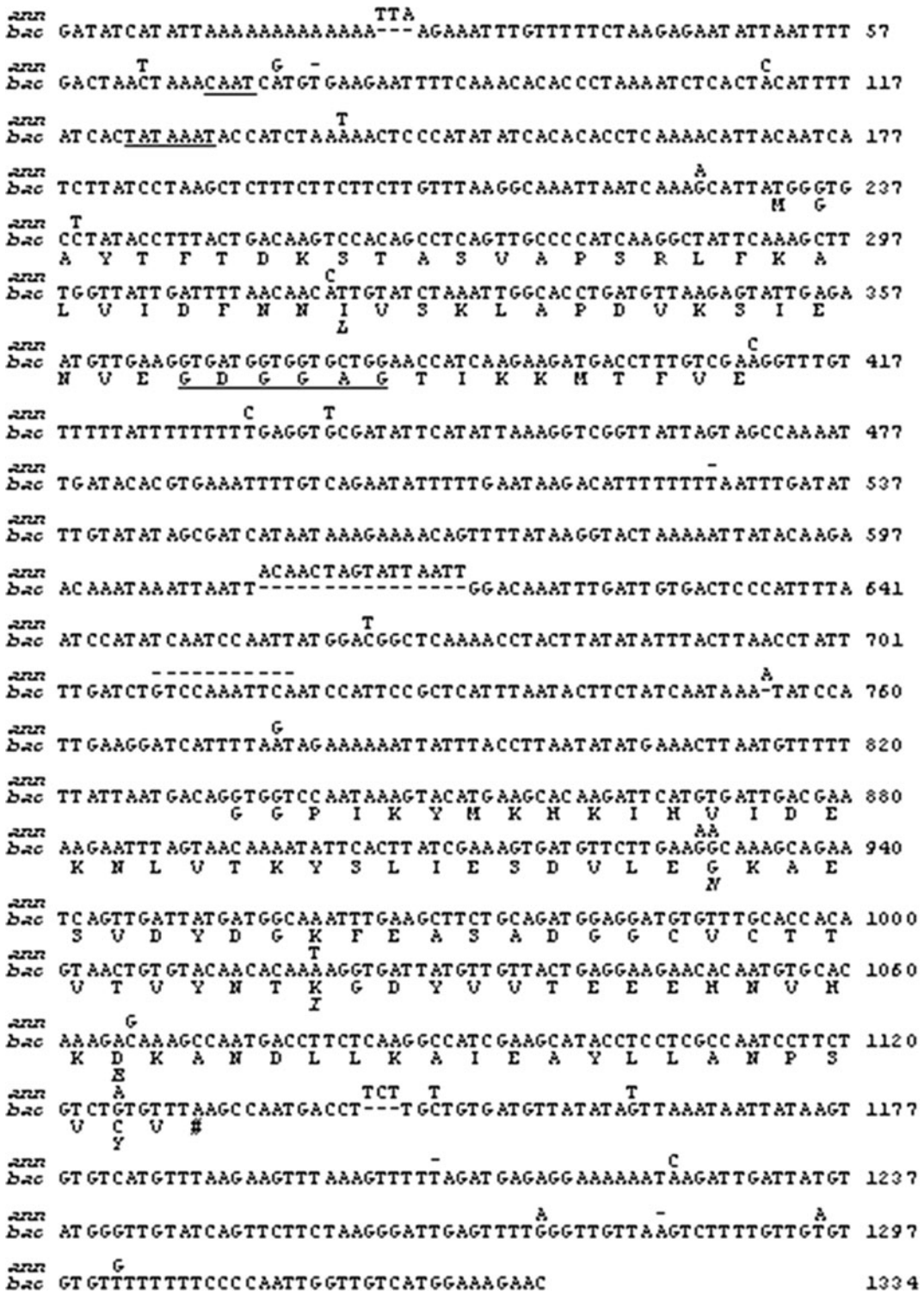

Fig. 2 Alignments of *bac*PR10 and *ann*PR10 gDNA nucleotide sequences and deduced amino acid sequences. Nucleotide and amino acid differences in *ann*PR10 are shown. Putative CAAT, TATA box and P-loop motif are underlined and gaps are represented by -

base under the accession numbers DQ351934 (*bac*PR10) and DQ351935 (*ann*PR10). The 480-bp cDNAs were also generated by RT-PCR from RNA isolated from infected unripe fruits of PBC80 and Yeoju that were developing respective resistant and susceptible symptoms. The resulting cDNAs encode predicted proteins of 159 amino acids with estimated molecular masses of 18 kD. The deduced amino acid sequences of *bac*PR10 and *ann*PR10 share 96% identity. In addition, they contain both a predicted P-loop, which is conserved among PR10 proteins, and several putative phosphorylation sites.

Fungal-induced expression of *PR10* in pepper fruits

The transcript levels of *ann*PR10 and *bac*PR10 were examined in infected fruits of the two pepper species (Fig. 3a). In PBC80, the *bac*PR10 mRNA was induced by the first day following inoculation and maintained thereafter. The expression of the *ann*PR10 mRNA in Yeoju was also induced at the first day but the expression level was lower and slightly declined at 48 h. The result showed that the *PR10* expression levels were significantly stronger in infected PBC80 fruits than in infected Yeoju fruits.

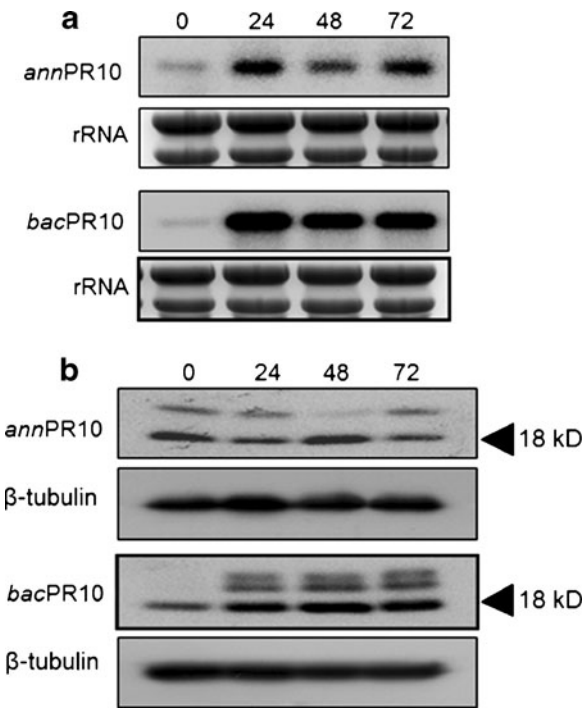

Fig. 3 Differential gene expression and protein accumulation of *ann*PR10 and *bac*PR10 in infected fruits of the two species. **a** Northern blot analysis of total RNA and **b** Western blot analysis on soluble proteins extracted from the fruits of Yeoju or PBC80 at 0, 24, 48, or 72 h after infection (HAI) with *C. acutatum* KSCa-1. The RNAs were electrophoresed and probed with respective cDNA. The immunoblot was incubated with antiserum against *bac*PR10. β-tubulin was used as loading control. Numbers indicate hours after inoculation

Accumulation of PR10 proteins following fungal infection

To characterize the PR10 proteins, a polyclonal antibody was generated against bacPR10 recombinant protein purified from *E. coli*. In a gel blot analysis of the soluble protein fraction of pepper fruits infected with the anthracnose fungus, the anti-bacPR10 antibody specifically recognized three protein bands including the predicted molecular mass of PR10, as well as two higher-molecular-weight proteins (Fig. 3b). In PBC80 fruits that were exhibiting a resistant reaction, the levels of the 18-kD protein induced on the first day after the infection and then maintained. However, in the susceptible fruits of Yeoju, the basal level of the 18-kD protein appeared to have decreased significantly on the first day following infection, and then increased slightly during the following day. The second band was detected in infected PBC80 fruits, but was not observed in Yeoju fruits. However, both PBC80 and Yeoju exhibited the weak third band that cross-reacted with anti-bacPR10 antibody.

To examine the PR10 expression patterns in detail, the protein profiles of both species were further investigated by 2D-PAGE combined with immunoblotting using anti-bacPR10 antibody. As shown in Fig. 4, comparison of infected fruits with control Yeoju fruits revealed no changes, except for the expansion of several spots. However, the immunoblot unveiled clear distinctions in the number and position of the PR10 protein spots that were induced by fungal infection in PBC80. Consistent with the results of the 1D blot, the 2D blot showed spots of two different sizes so that the 18 kD spots representing annPR10 protein was distinguishable. The additional spots with slightly higher molecular weight appeared after fungal infection and the intensity of the spots was proportional to that of the lower spots. In contrast to the susceptible fruits, the PBC80 fruits showed massive induction of PR10s in three horizontal rows. It was of interest to determine whether the slowly migrating

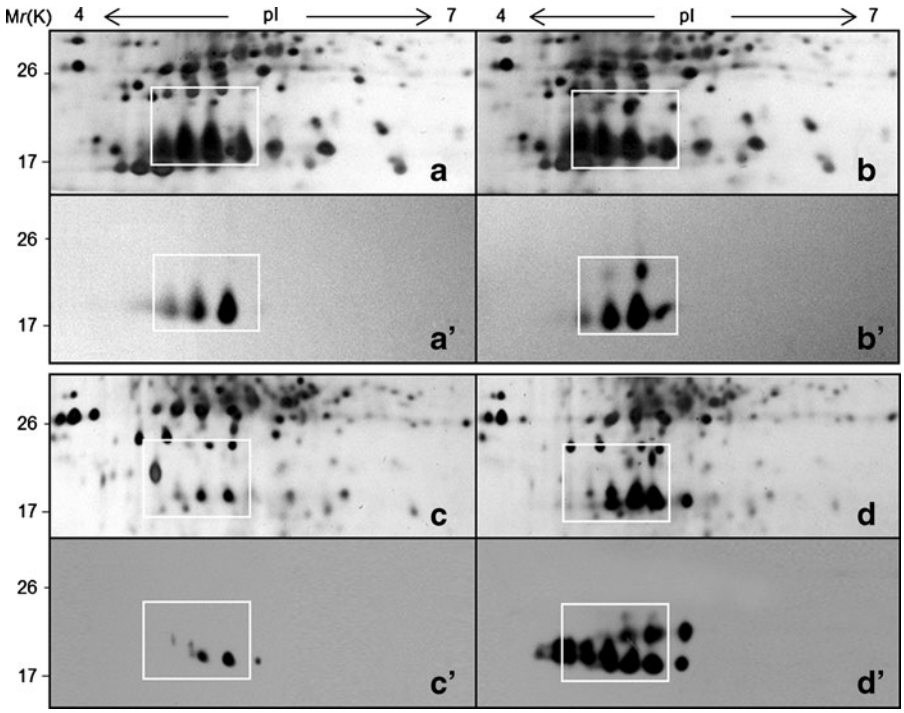

Fig. 4 2D electrophoresis profile and immunoblot of infected fruits of Yeoju and PBC80. Soluble proteins were extracted from infected fruits at 0 and 48 HAI. The first dimension was run on pH 4–7 IEF strips, followed by 12% SDS-PAGE. Next, immunoblotting was performed with a polyclonal anti-bacPR10 antibody. **a** Silver-stained proteins of Yeoju fruits at 0 HAI; **a′** immunoblot of a; **b** silver-stained Yeoju fruits at 48 HAI; **b′** immunoblot of b; **c** silver-stained proteins of PBC80 fruits at 0 HAI; **c′** immunoblot of c; **d** silver-stained PBC80 fruits at 48 HAI; **d′** immunoblot of d. The immunoblots were incubated with antiserum against bacPR10

spots represented a post-translationally modified form of the 18-kD protein. Since PR10 contains several putative phosphorylation sites, we examined the phosphorylation status of each PR10 by treating the crude extract with alkaline phosphatase as described by Park et al. (2004). The patterns of the PR10s did not change after this treatment in 2D blots (Fig. 5). The result supports that most of the protein spots identified in immunoblots represent the isoform of PR10 proteins. It also suggests that significant differences exist between species in the expression of PR10 proteins in infected pepper fruits.

Immunolocalization of PR10 proteins in infected pepper fruits

Immunohistochemical examination was conducted on transverse sections of fruits from both species in order to localize the accumulation of PR10 during fungal infection. Sections were prepared from unripe fruits infected with anthracnose fungus harvested at 0 and 48 HAI (Fig. 6). Under normal conditions, PR10 accumulation was rarely detected in epidermal cells of fruits of both species. Thus the deposition of PR10 was examined in the epidermal cells at the inoculation

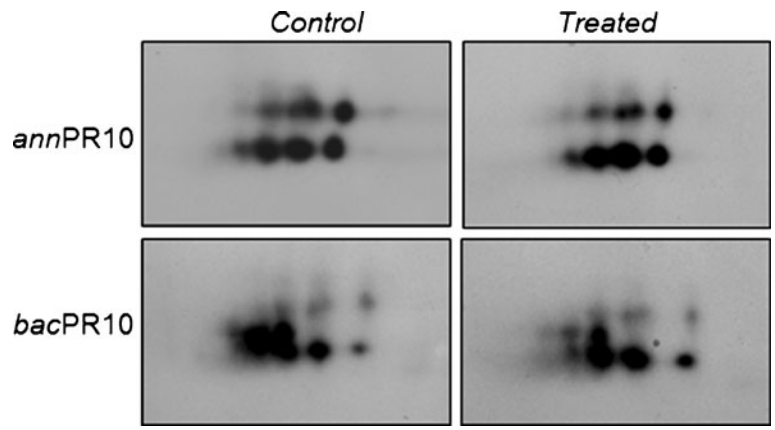

Fig. 5 Analysis of PR10 proteins for phosphorylation. Protein extracts from infected fruits of Yeoju or PBC80 at 24 HAI were incubated in the presence of calf intestinal alkaline phosphatase (50 unit ml^{-1}) and separated by 2D-PAGE. PR10 proteins were visualized by immunoblotting

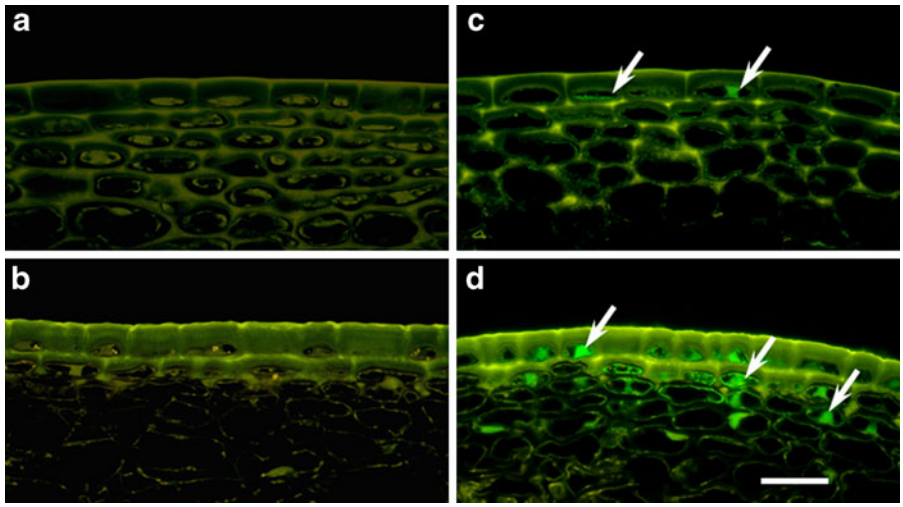

Fig. 6 Immunolocalization of PR10 proteins in infected fruits of susceptible (Yeoju) and resistant (PBC80) species. Sections were prepared from the inoculation sites of pepper fruits at 0 and 48 HAI and incubated with polyclonal anti-bacPR10 antibody. The primary antibody was detected with FITC-conjugated antibody and observed by fluorescence microscopy. Top panel, cross section of infected fruits of Yeoju at 0 (**a**) and 48 (**c**) HAI; bottom panel, cross section of infected fruits of PBC80 at 0 (**b**) and 48 (**d**) HAI. Arrows indicate the PR10 protein in the pericarp of fruits. The bar represents 100 μm

site that was penetrated by the fungi. Following fungal infection, massive accumulation of PR10 proteins was observed in the cytoplasm of cells of the infected PBC80. The result suggests that PR10 proteins may participate in the front line of active defence of resistant fruits. No immunostaining was observed in control sections incubated with preimmune serum (data not shown).

Ribonuclease activity of recombinant PR10 proteins

To elucidate the ribonuclease activities of the two PR10s, recombinant PR10 proteins expressed in *E. coli* were purified and their activities were examined using a previously described method (Bantignies et al. 2000). Five μg of total RNA extracted from unripe fruits were incubated with 2 μg of recombinant annPR10 or bacPR10 protein. Buffer control did not cause degradation of pepper RNA, but both the annPR10 and bacPR10 proteins showed mild RNase activities, as indicated by more rapid migration of the degradation products during agarose gel electrophoresis (Fig. 7). Ribosomal RNA bands began to degrade after 60 and 30 min of incubation with annPR10 and bacPR10, respectively. This result demonstrates that the bacPR10 protein has slightly stronger ribonuclease activity than annPR10.

Proteinase resistance of recombinant PR10 proteins

The proteinase resistance of the PR10s was assessed by incubating the purified recombinant protein (2 μg) with 1 μg ml^{-1} of proteinase K (Fig. 8). The digestion patterns of the PR10 proteins of the two species were compared. Protease treatment of bacPR10 yielded fragments of ~10 and ~7 kD, whereas annPR10 was degraded into a single intermediate band of ~10 kD under the same conditions; bacPR10 was degraded more slowly than annPR10. These results obtained with proteinase K support the idea that the cleavage region of annPR10 is well exposed in the solution, suggestive of some conformational difference between the two PR10s.

Antifungal activity of recombinant bacPR10 and annPR10 proteins

The antifungal activity of the recombinant bacPR10 and annPR10 proteins was investigated to determine their potential biological roles *in planta*. The recombinant proteins were mixed with fungal spores, which were then observed using a microscope. Under normal conditions, the spores began to germinate after 1 h and developed the first appressorium within 3 h. However, the development of the fungus was

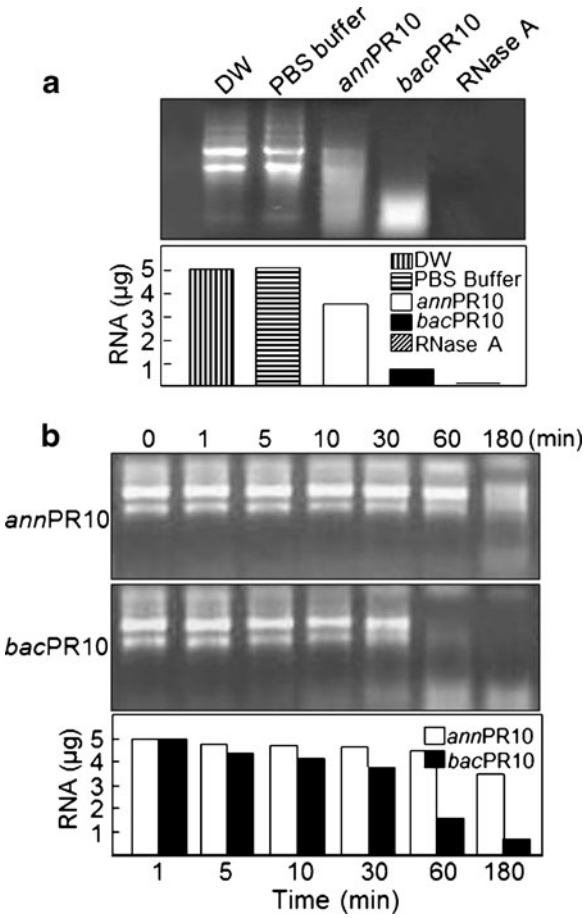

Fig. 7 Ribonucleolytic activities of recombinant PR10 proteins on pepper RNA. Gel electrophoresis was performed to separate hydrolyzed RNAs on a 1.0% agarose gel. Each reaction mixture, containing recombinant PR10 protein (2 μg) and total RNA (5 μg) from pepper fruit, was incubated for 180 min at 37°C (**a**) or for 0, 1, 5, 10, 30, 60, or 180 min (**b**) at 37°C. **a** Lane 1, RNA + DW; lane 2, RNA + PBS buffer; lanes 3–4, RNA + recombinant PR10 protein; lane 5, RNA + RNase A. **b** RNA + recombinant annPR10 or bacPR10 protein. The relative amounts of RNA after incubation with PR10 protein as shown in (**b**) are depicted in the bottom panel. The results shown are representative of three independent experiments

severely impaired by incubation with either of the recombinant proteins. During incubation with low concentrations (~0.05–0.5 μg) of recombinant protein, the hyphal tip tended to elongate and branch without developing appressoria (Fig. 9), and germination was completely blocked by 1 μg ml^{-1} of either protein (data not shown). In addition, fungal viability, as estimated by staining with a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, USA), was strongly affected by protein concentrations as low as 0.05 μg ml^{-1}, without significant difference between the species.

Discussion

To elucidate differences between pepper species that are resistant and susceptible to anthracnose fungus, several *PR* genes, including *PR1*, *PR3*, and *PR10*, were isolated and their expression patterns compared in infected fruits of PBC80 and Yeoju, which are resistant and susceptible to anthracnose fungus, respectively. There were few differences in the nucleotide sequences of the *PR* genes between two species, except for that of *PR10*. Therefore, *PR10* orthologs were isolated from the two host species and compared. The gDNA sequences of *ann*PR10 and *bac*PR10 were almost identical (96%), and the cDNA and deduced amino acid sequences showed 98 and 96% identity, respectively. Although the two *PR10* sequences showed a very high degree of homology, the highest nucleotide sequence identities for *bac*PR10 with genes from other species were 87 and 83% to *PR10* sequences from *Capsicum chinense* (*PR10*; accession number AJ879115) and *Solanum virginianum* (*SsPR10*; accession number AY660753), respectively. Despite the high sequence similarities between the two genes, they were differentially expressed in response to invasion of an anthracnose fungus. Induced expression of the

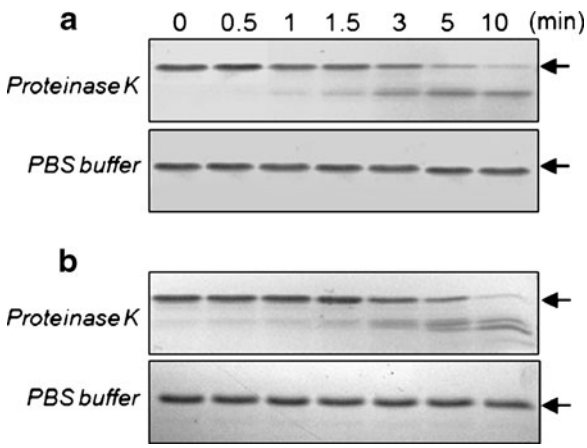

Fig. 8 Protease resistance of annPR10 (**a**) and bacPR10 (**b**) proteins. Recombinant protein (2 μg) was treated with 1 μg ml^{-1} proteinase K or PBS buffer at 37°C for 0, 0.5, 1, 1.5, 3, 5, or 10 min, and then separated using SDS-PAGE. *Arrows* are indicated 18 kD PR10 proteins. The test was conducted four times to confirm the consistency of the results

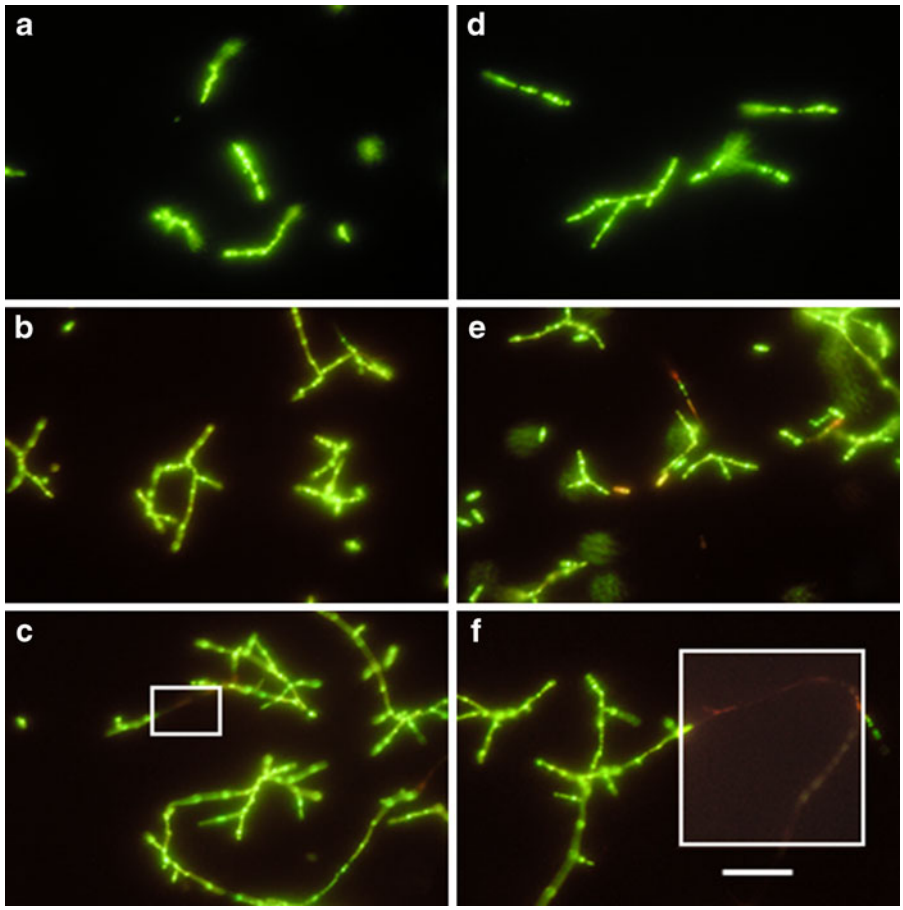

Fig. 9 Loss of viability and growth inhibition of anthracnose fungus treated with recombinant PR10 proteins in vitro. Fungal growth was assessed by fluorescence microscopy at 24 h after protein treatment. *Left panel*, **a** 0, **b** 0.05, or **c** 0.5 μg ml^{-1} of recombinant annPR10; right panel, **d** 0, **e** 0.05, or **f** 0.5 μg ml^{-1} of recombinant bacPR10. The boxed area represents unviable fungus exhibiting red fluorescence. The results shown are representative of at least three independent experiments. The bar represents 40 μm

*bac*PR10 mRNA in PBC80 were maintained during infection period, whereas a transient decrease in the expression of *ann*PR10 mRNA was observed on the second day following the infection of Yeoju fruits (Fig. 3a). Although the induction of *PR10* genes has been observed in several plant species after pathogen attack (Lo et al. 1999), this is the first description of a differential pattern of induction between fungal-resistant and -susceptible species.

Furthermore, we examined whether the pattern of accumulation of PR10 over time was associated with the development of anthracnose symptoms in infected fruits of each species. Immunoblot analysis showed that the level of an 18-kD protein (PR10) differed in infected fruits depending upon the extent of fungal resistance (Fig. 3b). In addition to the 18-kD protein, a higher band was also observed in both species. Interestingly, an intermediate band was clearly detected in infected fruits of PBC80, but not in those of Yeoju. Therefore, to examine the PR10 proteins in each species in more detail, the number and positions of PR10 spots were observed in infected pepper fruits using immunoblots of 2D gels (Fig. 4). Unlike the 18-kD and additional spots, the intermediate spot was not detected in a 2D gel blot of Yeoju fruits, consistent with the result of the 1D blot. It is possible that this intermediate band is a phosphorylated form of bacPR10 resulting from the resistance response, as suggested by Park et al. (2004). Thus, we examined whether the shifted spots represent a version of the 18-kD protein that is post-translationally modified by phosphorylation. The pattern of the spots did not change after alkaline phosphatase treatment, suggesting that the additional spots with higher molecular mass are other members of the PR10 family.

When the infection with an anthracnose fungus resulted in the development of typical symptoms on *C. annuum* fruits, no lesions formed on fruits of the resistant *C. baccatum*. An immunohistochemical investigation showed that the infection caused massive

accumulation of a PR10 protein within the cells located in inoculated surface of the fruit. This is of interest because the selective induction of PR10 bands was also observed in the immunoblot in the present study. These results suggest that the enhanced accumulation of bacPR10 protein in the fruits may be associated with the expression of *PR10*s. Moreover, the differential PR10 accumulation pattern in infected fruits of resistant and susceptible peppers indicates that species-specific control of expression occurs during interaction with a phytopathogen.

Since the recombinant PR10 proteins (bacPR10 and annPR10) exhibited ribonuclease activity, we investigated their antifungal activity in order to examine the biological properties of these proteins. No significant difference was observed in the degree of antifungal activity of the two proteins. However, bacPR10 showed greater resistance to proteinase K than annPR10. This result suggests either that the cleavage site is not identical in the two PR10 proteins, or that the cleavage regions are well exposed in the solution, implying that some conformational difference exists between the two proteins. The protein resistance may represent a method to increase the duration of bacPR10 activity during the resistance reaction of PBC80 fruits.

In this study, a comparison between the two orthologs was conducted based on the assumption that the genes encoding PR10 proteins, as well as their regulation, would be conserved among the plant genomes. However, different expression patterns were elicited by a pathogen, depending on the degree of resistance exhibited by the fruits of the peppers. There was a distinct and differential expression pattern for bacPR10 proteins in infected *C. baccatum* fruits. The substantial induction and extended longevity of bacPR10 contribute to the specific accumulation of the protein in high amounts in infected fruits of *C. baccatum*. Otherwise, the two pepper species do not share a common regulatory mechanism at either the transcriptional or post-translational level for the functional activation of *PR10* genes. This result has highlighted some significant species differences that merit future study. We are currently investigating the differences in the regulatory regions of the *PR10* orthologs in order to better understand the regulation of gene expression in defence responses. In addition, studies on the *PR10* isoforms to show functional evidence in transgenic plants would be helpful as a way of confirming the present results.

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