



Expression and functional characterization of two pathogenesis-related protein 10 genes from *Zea mays*

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ARTICLE INFO

Article history:

Received 16 April 2009

Received in revised form

17 July 2009

Accepted 17 July 2009

Keywords:

Antimicrobial

Corn

Expression profiles

PR10

Ribonuclease

ABSTRACT

A novel PR10 gene (*ZmPR10.1*) was isolated from maize and its expression and function were compared with the previous *ZmPR10*. *ZmPR10.1* shares 89.8% and 85.7% identity to *ZmPR10* at the nucleotide and amino acid sequence level, respectively. *ZmPR10* and *ZmPR10.1* were mainly expressed in root tissue with low expression in other tissues. *ZmPR10.1* had significantly lower expression than *ZmPR10* in all tissues examined. The expression of both *ZmPR10* and *ZmPR10.1* was induced by most abiotic stresses including SA, CuCl₂, H₂O₂, coldness, darkness and wounding during the 16-h treatments, and biotic stresses such as *Erwinia stewartii* and *Aspergillus flavus* infection. However, *ZmPR10.1* was induced only 2 HAT and down-regulated thereafter, whereas *ZmPR10* remained induced during the 16-h NAA treatment. Also, inoculation with *Erwinia chrysanthemi* caused about 2-fold induction in *ZmPR10.1* expression 60 HAT but not significant changes for *ZmPR10*. Both *ZmPR10.1* and *ZmPR10* showed RNase activity *in vitro* with an optimal pH and temperature of 6.5 and 55 °C. Their RNase activities were significantly inhibited by low concentrations (1.0 mM) of Cu²⁺, Ag⁺, Co²⁺, SDS, EDTA or DTT. However, *ZmPR10.1* possessed significantly higher (8-fold) specific RNase activity than *ZmPR10*. Also, *ZmPR10.1* showed a stronger inhibition against bacterium *Pseudomonas syringae* pv. *tomato* DC3000 *in vivo* and fungus *A. flavus* *in vitro* than *ZmPR10*, indicating that *ZmPR10.1* may also play an important role in host plant defense.

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Introduction

Plants grown under natural environmental conditions are often attacked by a variety of pathogens including viruses, bacteria, fungi and nematodes. However, plants do not have a circulating adaptive immune system like the one seen in humans. Instead, plants protect themselves with a mechanism consisting of active and passive defenses (Ruiz et al., 2002). In this case, pathogen invasion is recognized by inducible or constitutive proteins in plants that bind specific pathogen-derived proteins (Ođjakova and Hadjiivanova, 2001). Pathogen-related proteins (PR) are a major category of the host proteins induced during pathogen attacks. In the past years, many PR proteins have been identified and categorized into 17 families (PR1 to PR17) based on their different structures and biological activities (Christensen et al., 2002).

The PR10 family is one of the most important families among the 17 PR groups with more than 100 members reported so far from both angiosperms and gymnosperms, including the newly identified *AmPR10* from *Astragalus mongholicus* (Yan et al., 2008). Unlike most other PR proteins with an extracellular destination, PR10 proteins are typically intracellular, small (16–19 kDa) and acidic with similar three-dimensional structures (Ziadi et al., 2001; Liu and Ekramoddoullah, 2006). PR10 proteins have been reported to have various functions ranging from antimicrobial activity, *in vitro* ribonuclease activity, enzymatic activities in plant secondary metabolisms, and roles in abiotic stresses (Liu and Ekramoddoullah, 2006). Therefore, PR10 proteins may play important roles in plant defense against pathogen attack. In addition, some PR10 proteins possess ligand-binding ability like cytokinin-specific binding activity in plant (Fujimoto et al., 1998; Gonneau et al., 2001), and constitutive expression of a PR10 cDNA from *Brassica napus* results in the increase of cytokinin level (Srivastava et al., 2006). These studies demonstrate some PR10 proteins also play roles in plant growth and development through modulating the endogenous cytokinin level. The silencing of *MtPr10-1* from *Medicago truncatula* led to the induction of a new set of PR proteins after infection with *Aphanomyces euteiches* (Colditz et al., 2007), suggesting that there is a relationship between PR10 and other PR proteins.

Abbreviation: ABA, abscisic acid; CHES, 2-(cyclohexylamino) ethanesulfonic acid; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GA, gibberellic acid; GUS, β-glucuronidase; HAT, hours after treatment; IPTG, isopropyl-β-D-1-thiogalactopyranoside; KT, kinetin; MeJA, methyl jasmonate; MES, 2-(N-morpholino) ethanesulfonic acid; NAA, naphthalene acetic acid; Ni-IDA, nickel-iminodiacetic acid; SA, salicylic acid; SDS, sodium dodecyl sulfate

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In addition, many plant species have more than one PR10 protein, such as 18 *PmPR10s* from *Pinus monticola*, 3 *GbPR10s* from *Gossypium barbadense*, 7 *LIPR10s* from *Lupinus luteus*, and 5 *OsPR10s* in *Oryza sativa* (Liu and Ekrasmoddullah, 2006). Recently, a *PR10* gene (*ZmPR10*) was found to be associated with maize kernel resistance to *Aspergillus flavus* infection/aflatoxin contamination, and was cloned from maize based on partial peptide sequences of the protein (Chen et al., 2006). The *ZmPR10* protein over-expressed in *Escherichia coli* possessed ribonuclease activity and inhibited *A. flavus* growth (Chen et al., 2006). The objectives of the present study were to investigate the presence of *ZmPR10* homologues in maize, their differences in expression and in response to abiotic and biotic stresses, and their potential contribution to host resistance. Here, we report the identification of a second *PR10* (*ZmPR10.1*) and its comparisons with *ZmPR10* in expression profiles including tissue-specificity and responses to stresses, RNA degradation ability, and antimicrobial activity.

Materials and methods

Plant materials and growth conditions

Maize (*Zea mays* L.) inbred line MI82, which is resistant to *Aspergillus flavus*, was used in this study. Surface-sterilized seeds were germinated in pots with sterilized potting mix (Piedmont Pacific, Statham, GA) and grown under continuous light ($170 \mu\text{mol m}^{-2}\text{s}^{-1}$) at room temperature (22–25 °C). After 2 weeks of growth, seedlings at the four-leaf stage (V4-stage) were used for various treatments. For tissue-specific expression patterns, the leaves, stalks, sheaths, husks, silks and immature kernels were harvested from plants grown in the greenhouse with natural light. For root tissue, samples were collected from seedlings (V1-stage) grown hydroponically in B5 medium (Gamborg et al., 1968). Wild-type and transgenic *Arabidopsis* (Col-0) plants were grown on the same potting mix as above in a growth chamber under the following light and temperature conditions: 22 °C, 16-h day and 18 °C, 8-h night.

Experimental treatments

For phytohormone and chemical treatments, one of the following solutions of 5.0 mM SA, 100 μM ABA, 100 μM MeJA, 100 μM KT, 100 μM NAA, 100 μM GA₃, 100 μM ethephon, 200 μM H₂O₂, 200 mM NaCl or 1.0 mM CuCl₂ was sprayed on all leaves. Control plants were sprayed with distilled water. The concentration of phytohormone and chemical treatments chosen was based on the studies by Park et al. (2004) and Liu et al. (2006). For cold-treatment, the seedlings were transferred to a 4 °C chamber whereas plants kept at room temperature were used as a control. Potted seedlings kept in the chamber at 23 °C without lights or wounded with a cork borer to produce three 5 mm circles on each leaf were used as the dark and wounding treatments, respectively.

For leaf-pathogen inoculations, *Clavibacter michiganensis* subsp. *nebraskensis*, *Erwinia chrysanthemi* and *Erwinia stewartii*, which cause maize leaf freckles and wilt, stalk and top rot, and Stewart's disease, respectively, were used in this study. Overnight cultures in YDC medium (1% yeast extract, 2% glucose, 2% CaCO₃) were infiltrated into the second and third leaves of the V4-stage seedlings. Leaves infiltrated with YDC medium only were used as a control. Control and treated leaves were harvested at 0, 12, 24, 36, 48 and 60 h after treatment (HAT). For *A. flavus* inoculation, ears of field grown maize plants were inoculated through injection of 5 mL of *A. flavus* conidia suspension ($5 \times 10^6 \text{ mL}^{-1}$) 14 d after pollination (DAP) through the silk channel. Control ears

were wounded with a needle only. Ears were harvested 1, 2, 4, 7, 10, 14, 18 and 22 d after inoculation (DAI), which correspond to various phases of kernel development: mitotic activity (10–14 DAP), DNA polytenization (14–18 DAP), DNA degradation and onset of linear grain-fill (20–22 DAP). All leaves and kernels with or without treatments were frozen in liquid nitrogen after harvest and stored at –80 °C until use.

RNA and DNA isolation

Total RNA from various tissues was extracted using the RNeasy Plant Mini Kit (Qiagen, Stanford, CA) according to the manufacturer's instructions. Genomic DNA was isolated from leaves using the GenElute Plant Genomic DNA Miniprep Kit (Sigma, St. Louis, MO). The quality and concentration of RNA and genomic DNA samples were examined by agarose gel electrophoresis and analyzed with a ND-1000 Spectrophotometer (Thermo, Wilmington, DE), respectively.

Cloning of a new pathogenesis-related protein 10 gene

During a database search with a previously cloned *ZmPR10* (Chen et al., 2006), a sequence (GenBank AC203883) highly homologous to *ZmPR10* was identified. A pair of specific primers (G101: 5'-CTAGGCAACGAGCCGAGCCGTCAG-3' and G102: 5'-GCCGTAGAAAACACAAAAGGCAA-3') was synthesized based on the sequence information of AC203883 to clone this new putative *PR10* gene (termed *ZmPR10.1*) from both the genomic DNA and cDNA of maize leaves. The first strand of cDNA was synthesized with an oligo(dT)₁₆ primer and M-MLV reverse transcriptase (Promega, Madison, WI) under the following conditions: 75 °C for 5 min, 37 °C incubation for 1 h, followed by 75 °C for 15 min. The PCR product, amplified with two gene-specific primers, was gel purified and ligated into a pGEM T-Easy vector (Promega) and confirmed through DNA sequencing. Sequence data from this article have been deposited at GenBank under accession number FJ897503.

Construction of bacterial and plant expression vectors and transformation

The primer pair G103 (5'-ACTCATATGATGGCCTCCACCAACAGCTG-3', *Nde* I site underlined) and G104 (5'-ATAGCGGCCGCTGTAGGCTTCCGGGTG-3', *Not* I site underlined) was used to amplify the coding region of *ZmPR10.1* from cDNA. For *ZmPR10*, the primer pair G105 (5'-CTGCATATGATGGCCTCCGCCAACAGCTG-3', *Nde* I site underlined) and G106 (5'-AATCGCGCCGCTGTAGGCGTCGGGGTTGGC-3', *Not* I site underlined) was used. The resulting PCR products digested with *Nde* I and *Not* I were cloned into the corresponding sites of the pET20b plus vector (Novagen, Madison, WI) to form pET20b-*ZmPR10* and pET20b-*ZmPR10.1*, respectively. After sequencing, the two vectors were transformed into *Escherichia coli* BL21 (DE3) for protein over-expression.

The coding region of *ZmPR10.1* was amplified from cDNA using primer pair G107 (5'-GAGAGATCTGATGCCTCCACCAACAGC-3', *Bgl* II site underlined) and G108 (5'-CGTACTAGTGTGTAGGCTTCGGGTG-3', *Spe* I site underlined). The PCR product digested with *Bgl* II and *Spe* I was cloned into the corresponding sites of pCambia1302 vector (<http://www.cambia.org>) to generate the 35S-*ZmPR10.1::GFP* plasmid, which was verified through DNA sequencing. The plasmid 35S-*ZmPR10::GFP* was constructed in the same manner with primer pair G109 (5'-GAGAGATCTCATGGCTCCGCCAACAGCTG-3', *Bgl* II site underlined) and G110 (5'-CGTACTAGTGTGTAGGCGTCGGGGTTGGC-3', *Spe* I site underlined). Wild-type *Arabidopsis* (Col-0) was transformed with the

Agrobacterium tumefaciens GV3101 harboring the plasmid 35S-ZmPR10::GFP or 35S-ZmPR10.1::GFP using the floral dip method (Clough and Bent, 1998). Also, *Arabidopsis* plants transformed with pCAMBIA1302 only were used as a control. Transgenic plants of homozygous T₃ seeds were used for evaluating their antibacterial abilities.

For assessing the ZmPR10.1 and ZmPR10 expression at protein level indirectly, GUS expression vectors under the control of corresponding ZmPR10.1 and ZmPR10 promoters were constructed. Putative promoter of ZmPR10.1 (pZmPR10.1) and promoter of ZmPR10 (pZmPR10) were amplified based on their sequences from GenBank (AC203883 and AC215303 for ZmPR10.1 and ZmPR10, respectively) using primer pairs, G111 (5'-ACAAAGC-TTGCTGCCGACTCCAAAG-3', *Hind* III site underlined) and G112 (5'-AGTGGATCCGGCTCGTGGTCTGCGATGAGCTTGAC-3', *Bam*HI site underlined), G113 (5'-GAGAAGCTTTGGGTCAGATGTGGCCGATA-3', *Hind* III site underlined) and G114 (5'-CGCGGATCCTACTACTCAGTGATCGATCG-3', *Bam*HI site underlined), respectively. The PCR products digested with *Hind* III and *Bam*HI were cloned into the corresponding sites of pBI121 vector (Clontech, Palo Alto, CA) to produce constructs pZmPR10.1-GUS and pZmPR10-GUS, respectively. The resulting constructs were introduced into *A. tumefaciens* GV3101 and then into wild-type *Arabidopsis* using the floral dip method.

Real-time PCR

For quantitative real-time PCR, cDNA was synthesized by using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster, CA) according to the manufacturer's instructions. The primer pairs G115 (5'-CAAGCTCATCGCAGACCAC-3') and G116 (5'-CGATCTCAACAGTCCAGCTGT-3'), G117 (5'-AGCCTTCAGCTAGCCC-CAAGTT-3') and G118 (5'-GCGGAGGCCATTACTACTTCAG-3'), were used for ZmPR10.1 and ZmPR10, respectively. Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions in a 25 μ L reaction volume containing 25 ng cDNA. The level of 18S rRNA transcript amplified with primer pair G119 (5'-GAGAAACGGCTACCACATCCA-3') and G120 (5'-ACGCG-CCCGGTATTGTAT-3') was used as an internal control. The relative expression of target gene in different tissues of maize or in different transgenic lines of *Arabidopsis* was calculated using Δ Ct method, in which: Δ Ct = Ct_(18S rRNA) - Ct_(target). The data of target gene expression after treatments are the ratio of expression in treated samples compared to controls using $\Delta\Delta$ Ct method ($[Ct_{(18S rRNA)} - Ct_{(target)}]_{treatment} - [Ct_{(18S rRNA)} - Ct_{(target)}]_{control}$).

Fluorometric assay of GUS protein

Tissues from 2-week-old transgenic *Arabidopsis* plants, which were grown on solid medium containing 4.4 g L⁻¹ Murashige and Skoog salt (Sigma), 1% sucrose, pH 5.8, and 0.8% agar, were ground into powder using liquid nitrogen and then suspended in 400 μ L of GUS extraction buffer (100 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT, 1% Triton X-100 and 10% glycerol). After centrifuging samples at 14,000g for 15 min at 4 °C, the supernatant was assayed for protein concentration (Bradford, 1976) and GUS activity (Jefferson et al., 1987). Ten microliters of protein sample was mixed with 90 μ L of GUS extraction buffer containing 2 mM 4-methylumbelliferyl glucuronide (Sigma) and incubated at 37 °C. After 30-min reaction, 10 μ L of mixture was transferred to 190 μ L of 0.2 M Na₂CO₃ and the fluorescence was measured at the excitation/emission wavelengths of 365 nm/455 nm with a Wallac 1420 VICTOR3TM spectrofluorimeter (PerkinElmer, Waltham, MA). Freshly prepared 4-methylumbelliferone (4-MU) from Sigma was

used as a standard. The GUS activity was expressed as nmoles of 4-MU produced per mg protein per min.

Over-expression and purification of PR10 proteins in *E. coli*

The pET20b-ZmPR10.1 and pET20b-ZmPR10 constructs contain a His-tag at the downstream of the target gene to allow purification of the over-expressed proteins with a nickel-affinity column. After 3 h of induction by 0.4 mM IPTG at 37 °C according to the manufacturer's instructions, the cells were harvested by centrifugation at 4000g for 10 min at 4 °C. The pellets were resuspended in 20 mM Tris-HCl (pH 7.9) containing 0.5 M NaCl and 5 mM imidazole (Buffer A) and homogenized using a French Pressure Cell Press (SLM Aminco, Lorton, VA). The supernatant of cell lysate was recovered by centrifugation (13,000g, 15 min) and then applied onto a Ni-IDA affinity column (Novagen). The column was washed with 20 volumes of Buffer B (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 60 mM imidazole) and the over-expressed recombinant protein was eluted with Buffer C (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 1.0 M imidazole). After removal of imidazole through fast protein liquid chromatography, the purified proteins were used for biochemical and antifungal assays.

Nucleic acid degradation

For RNA degradation assays, two equal concentrations (10 and 40 μ g mL⁻¹) of the purified recombinant proteins of ZmPR10 and ZmPR10.1 were individually mixed with 10 μ g of total RNA from maize root tissue. For determining potential DNA degradation activities, the two purified proteins were mixed separately with 5 μ g genomic DNA isolated from maize leaves. The mixtures were then incubated for 0.5 h at 50 °C for RNA and at room temperature for DNA (Yan et al., 2008). At the end of incubation, the PR10 protein (both ZmPR10 and ZmPR10.1) was removed by phenol-chloroform (1:1) extraction, and the nucleic acids were separated on 1.5% agarose gels for RNA or 0.8% gels for DNA, and stained with 0.003% ethidium bromide to visualize the extent of nucleic acid degradation. RNase A (New England Biolabs, Ipswich, MA) and RNasin (Applied Biosystems) were included as positive and negative control, respectively. The use of heat-inactivated (boiled for 20 min) PR10 proteins was also included as negative controls.

Assay of ribonuclease activity

The ribonuclease (RNase) activity of these two purified proteins was further quantified using yeast tRNA according to Zhou et al. (2002). Twenty microliters of protein (0.5 mg mL⁻¹) and 10 μ L of yeast tRNA (20 mg mL⁻¹) were mixed with 200 μ L of 50 mM MES (pH 6.0) thoroughly and incubated at 50 °C. After 30 min, the reaction was stopped by adding 1 mL of ice-cold 4 M LiCl to the mixture and left on ice for 15 min. Insoluble tRNA in the reaction mixture was removed by centrifugation (13,000g) at 4 °C for 10 min. The amount of solubilized RNA in the supernatant was determined by UV absorbance at 260 nm (OD₂₆₀) with a Nanodrop ND-1000 spectrophotometer (Thermo). One unit of enzymatic activity is defined as the amount of protein that brings an increase of 1 OD₂₆₀ after 30 min of incubation under the specified condition (Zhou et al., 2002). The positive and negative controls for the activity assay were the RNase A and the Ni-IDA column flow-through fraction, respectively.

Effect of pH, temperature and reagents on the RNase activity

The effect of pH on ZmPR10.1 and ZmPR10 RNase activity was analyzed using 50 mM of the following buffers with pH values

ranging from 3.0 to 11.0: citrate buffer (pH 3.0–4.0), MES buffer (pH 4.5–6.5), Tris–HCl buffer (pH 7.0–8.5) and CHES buffer (pH 9.0–11.0) (Yan et al., 2008). After incubation of PR10 protein and yeast tRNA mixture in these buffers for 30 min at 50 °C the RNA degradation activity was measured as above. The optimal temperatures of RNase activity for ZmPR10.1 and ZmPR10 were determined by assaying RNA degradation at temperatures ranging from 20 to 90 °C in 50 mM MES buffer (pH 6.0) for 30 min. The reaction was terminated and free tRNA was determined as described above. The effect of metal ions (Ca^{2+} , Mg^{2+} , Ag^+ , Zn^{2+} , Mn^{2+} , Fe^{2+} , Cu^{2+} , Co^{2+}), EDTA, protein reducing agent DTT and detergent SDS on RNase activity was also determined. Protein and yeast tRNA were mixed with 200 μL of 50 mM MES buffer (pH 6.0) containing 1.0 mM (final concentration) of each reagent and incubated at 50 °C for 30 min. The RNase activity was then determined as above.

Antimicrobial activity assays

The ability of PR10 proteins to inhibit the growth of *A. flavus*, an opportunistic pathogen that infects maize kernels both pre- and post-harvest and causes aflatoxin contamination in many warm climate regions, was assayed following Chen et al. (2006). Conidia of *A. flavus* (AF13, ATCC 96044) were incubated in potato dextrose broth (PDB) with boiled or native protein at a concentration of 20 or 50 $\mu\text{g mL}^{-1}$ (ZmPR10.1 or ZmPR10) at 30 °C. The germination and growth of conidia were examined at 12 and 20 h after incubation.

The virulent bacterial strain *Pseudomonas syringae* pv. *tomato* DC3000 was used to investigate the antibacterial activity of both ZmPR10.1 and ZmPR10 in *Arabidopsis*. The inoculation of the 6-week-old transgenic plants was performed according to Berger et al. (2007). The overnight culture of *P. syringae* DC3000 in King's B medium (2% peptone, 0.15% K_2HPO_4 , 0.15% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5% glycerol) was diluted 1:50 with fresh medium and incubated for another 2 h at 28 °C. The bacteria were harvested by centrifugation at 5000g for 10 min at 4 °C, washed with 10 mM MgCl_2 for three times, and diluted to a final concentration of optical density (OD_{600}) 0.005 (equivalent to 10^7 cfu/mL). For each inoculation, 10 μL of bacterial suspension was gently infiltrated into the abaxial side of the leaf with a 0.5 mL needleless plastic syringe. Control leaves were inoculated with 10 μL of 10 mM MgCl_2 . Four mature leaves of each plant were inoculated and at least three plants were inoculated for each transgenic line. These infiltrated leaves were detached and photographed 4 d after inoculation. The lesion sizes of infiltrated leaves were measured using APS Assess software (APS Press, Saint Paul, MN).

Data analysis

All real-time PCR and RNase activity experiments were repeated at least three times. The data were analyzed using the Statistical Analysis System (version 9.1, SAS Institute, Cary, NC). Significant changes in gene expression were determined using Fisher's least significant differences ($P = 0.05$).

Results

ZmPR10.1 cDNA cloning and sequence analysis

The cloned cDNA and genomic DNA of ZmPR10.1 were 591 and 668 bp, respectively. Comparison between cDNA and genomic DNA shows that ZmPR10.1 contains one 77-bp intron and two exons of 483 bp in total, capable of encoding a peptide of 160

amino acids (Fig. 1A). There is a typical GxGGxG motif at amino acid residues 48–53 (Fig. 1A, underlined) of ZmPR10.1, which is known as the “P-loop” (phosphate-binding loop) and is frequently found in protein kinases as well as in nucleotide-binding proteins (Saraste et al., 1990). Further, NetPhos, a web-based software (<http://www.cbs.dtu.dk/services/NetPhos/>) predicated that the four serine (Ser13, 102, 120 and 140, in bold italic) and two tyrosine (Tyr122 and 151, in bold italic) residuals are potential phosphorylation sites (Fig. 1A). ZmPR10.1 and ZmPR10 share an 89.8% identity at the DNA level and an 85.7% identity at the protein level (Fig. 1B and C). The predicted molecular mass and isoelectric point for ZmPR10.1 are 16,900.2 Da and 5.36, which are similar to those of ZmPR10 (16,942.3 Da and 5.38) (Chen et al., 2006).

Tissue-specific expression of ZmPR10.1 and ZmPR10 and β -glucuronidase (GUS) activities driven by their promoters

The specific expression of ZmPR10.1 and ZmPR10 in various tissues (root, stem, leaf, sheath, husk, silk and immature kernel) was also determined using quantitative real-time PCR. The results

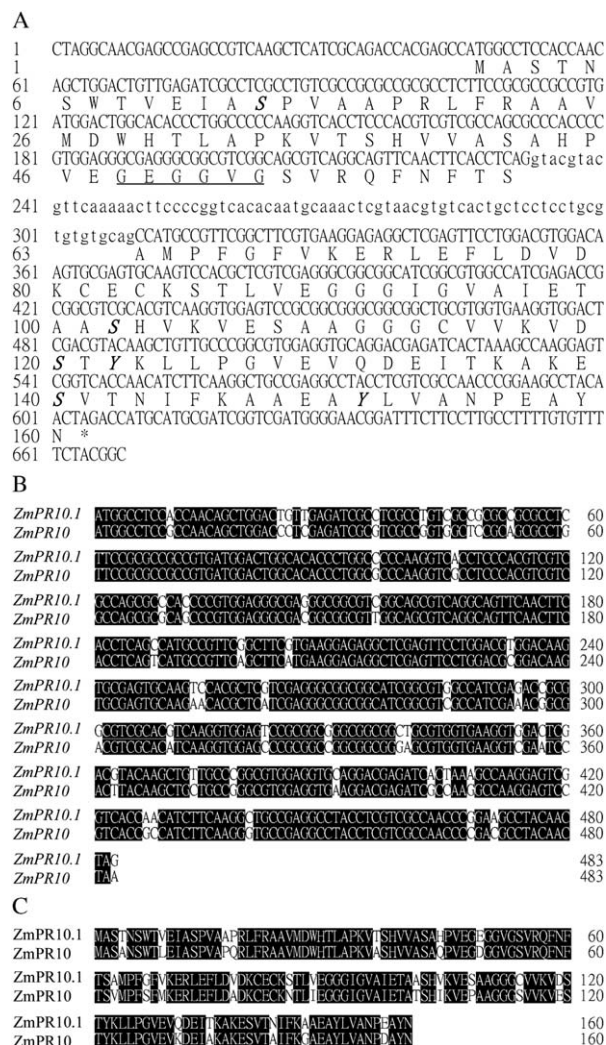


Fig. 1. Nucleotide and protein sequence of ZmPR10.1 and its comparison to ZmPR10. (A) Nucleotide and deduced amino acid sequence of ZmPR10.1 cDNA. The P-loop motif is underlined and the putative phosphorylation sites are marked in bold italics. (B) Alignment of the nucleotide sequences between ZmPR10.1 and ZmPR10. (C) Alignment of the predicted amino acid sequences between ZmPR10.1 and ZmPR10.

showed that both *ZmPR10.1* and *ZmPR10* are expressed constitutively in all tissues examined (Fig. 2). A much higher expression was detected in root tissue than other tissues (6.6–164.5-fold and 11.1–74.6-fold higher than other tissues for *ZmPR10.1* and *ZmPR10*, respectively). The level of expression between the two *PR10* genes was also different. *ZmPR10* expression was significantly higher than that of *ZmPR10.1* in all tissues examined (14.0, 138.1, 5.0, 18.4, 8.3, 8.6 and 10.0-fold for root, stalk, leaf, sheath, husk, silk and immature kernel, respectively) (Fig. 2). The level of *ZmPR10.1* expression was barely detectable in stalk and silk tissues.

GUS activities driven under the promoters of *ZmPR10.1* and *ZmPR10* in transgenic *Arabidopsis* were also compared to estimate their expression differences at protein level. GUS activities were detectable in both leaf and root tissues from transgenic seedlings of either pZmPR10.1 or pZmPR10. The GUS activities in the root and leaf tissues containing the pZmPR10.1 were 0.14 and 0.03 nmoles MU min⁻¹ mg⁻¹ protein, respectively, compared to 8.72 and 0.45 nmoles MU min⁻¹ mg⁻¹ protein in their corresponding tissues expressing the pZmPR10 (Fig. 3).

ZmPR10.1 and *ZmPR10* expression in leaf tissue in responses to phytohormone treatments

The *ZmPR10.1* and *ZmPR10* expression did not change significantly during the 48 h examined in control leaves (sprayed with water) (data not shown). When leaves were treated with abscisic acid (ABA), both *ZmPR10.1* and *ZmPR10* expression showed a significant initial decline to about half of the levels in control leaves for *ZmPR10.1* at 8 h after treatment (HAT) and for *ZmPR10* at 4 HAT, and recovered to the levels in control leaves at 16 HAT (Fig. 4A). Both kinetin (KT) and gibberellic acid (GA) treatments led to a steady increase in expression of *ZmPR10* until 8 HAT, and their expression decreased thereafter (Fig. 4B and C). However, only KT treatment caused a significant initial increase (2.6-fold) in *ZmPR10.1* expression at 2 HAT (Fig. 4B). Its expression did not show significant changes upon GA treatment compared to the control (Fig. 4C). In response to methyl jasmonate (MeJA) treatment, only *ZmPR10.1* showed a significant increase in expression between 2 and 4 HAT (2.3-fold at 4 HAT) (Fig. 4D). When leaves were treated with naphthalene acetic acid (NAA), both genes showed significant induction in expression (about 2-fold) at 2 HAT. *ZmPR10* levels remained high up to 16 HAT, but the level of *ZmPR10.1* declined rapidly to about half of the level in

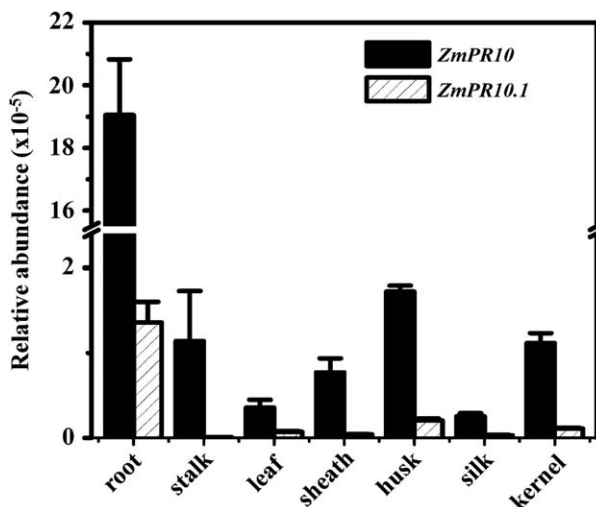


Fig. 2. Tissue-specific expression of *ZmPR10* and *ZmPR10.1* in maize relative to internal control 18S rRNA. Values are means ± SE (standard error, *n* = 4). SE is indicated with a bar.

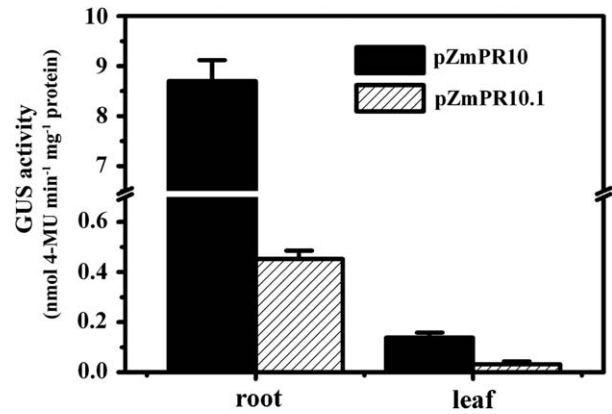


Fig. 3. Activity assay of GUS protein driven by the promoter of *ZmPR10* (pZmPR10) or *ZmPR10.1* (pZmPR10.1). Means are shown with SE calculated from four replications.

the control (Fig. 4E). For salicylic acid (SA) treatment, *ZmPR10.1* and *ZmPR10* expressions increased and peaked at 18 HAT for *ZmPR10.1* (6-fold) and 12 HAT for *ZmPR10* (2-fold) compared to control leaves (Fig. 4F).

The responses of *ZmPR10.1* and *ZmPR10* expression to abiotic stresses

The expression patterns of *ZmPR10.1* and *ZmPR10* in response to abiotic stresses depend on the treatments and a greater induction was usually observed for *ZmPR10.1* than for *ZmPR10* (Fig. 5). H₂O₂ treatment caused a strong induction in the expression of *ZmPR10.1*, which was about 3-fold the level of that in the control samples at 16 HAT, whereas the same treatment led to a modest induction of 68% for *ZmPR10* expression at 16 HAT (Fig. 5A). Both *ZmPR10.1* and *ZmPR10* showed a similar pattern and magnitude of induction in response to CuCl₂ treatment (Fig. 5B). Treatment of leaves with NaCl leads to a pattern of expression different from the above (Fig. 5C). A significant induction (nearly 3-fold) in expression for both *ZmPR10.1* and *ZmPR10* was observed as early as 0.5 HAT. Their levels decreased to normal at 3 HAT. The expression of *ZmPR10.1* and *ZmPR10* under darkness, wounding or coldness was also examined. The results indicated that wounding significantly induced their expressions as early as 2 HAT for *ZmPR10.1* (3.2-fold) and 4 HAT for *ZmPR10* (3.1-fold). Their expressions increased about 5-fold at 16 HAT (Fig. 5D). For coldness treatment, *ZmPR10.1* and *ZmPR10* up-regulated significantly within 2 HAT and peaked at 16 HAT by 3.3 and 2.1-fold, respectively (Fig. 5E). During the 48-h dark treatment, the expression for both *ZmPR10* and *ZmPR10.1* increased significantly 12 HAT, and reached 3-fold higher than that in the control at 24 HAT. Their expressions decreased 48 HAT, but were still higher than that in the control (Fig. 5F).

The responses of *ZmPR10.1* and *ZmPR10* expression to biotic stresses

The expressions of *ZmPR10.1* and *ZmPR10* were investigated after treatment with maize bacterial pathogens. Significant induction of *ZmPR10.1* and *ZmPR10* expression in leaves inoculated with *Erwinia stewartii* was detected 36 HAT. Their expressions continued to increase and reached about 6-fold the level of the control leaves 60 HAT (Fig. 6A). Inoculation with the bacterial pathogen *Erwinia chrysanthemi* caused about 2-fold induction in *ZmPR10.1* expression 60 HAT. However, this treatment did not cause any significant changes in the level of *ZmPR10* (Fig. 6B). A 2-fold induction was observed for *ZmPR10* between 12 and 60 HAT with *Clavibacter michiganensis* subsp. *nebraskensis* (Fig. 6C). The

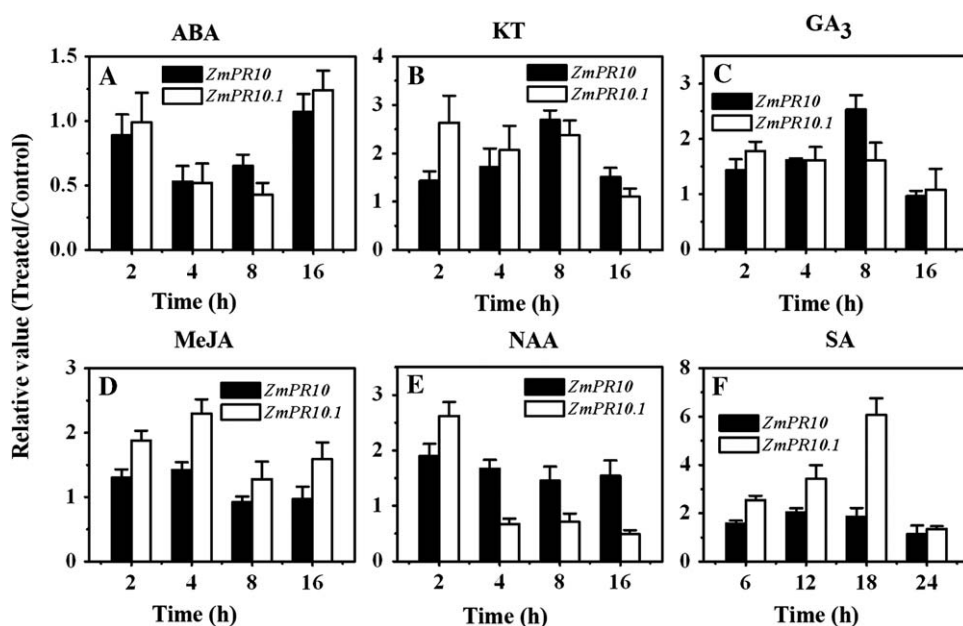


Fig. 4. Expression patterns of *ZmPR10* and *ZmPR10.1* in leaf tissue under various phytohormone treatments: (A) 100 μ M ABA; (B) 100 μ M KT; (C) 100 μ M GA₃; (D) 100 μ M MeJA; (E) 100 μ M NAA; and (F) 5.0 mM SA. Data for each time-point represent the mean of three replications. Plants sprayed with distilled water were used as a control. SE ($n = 3$) is indicated on top of each bar and may not be visible if the value is too small.

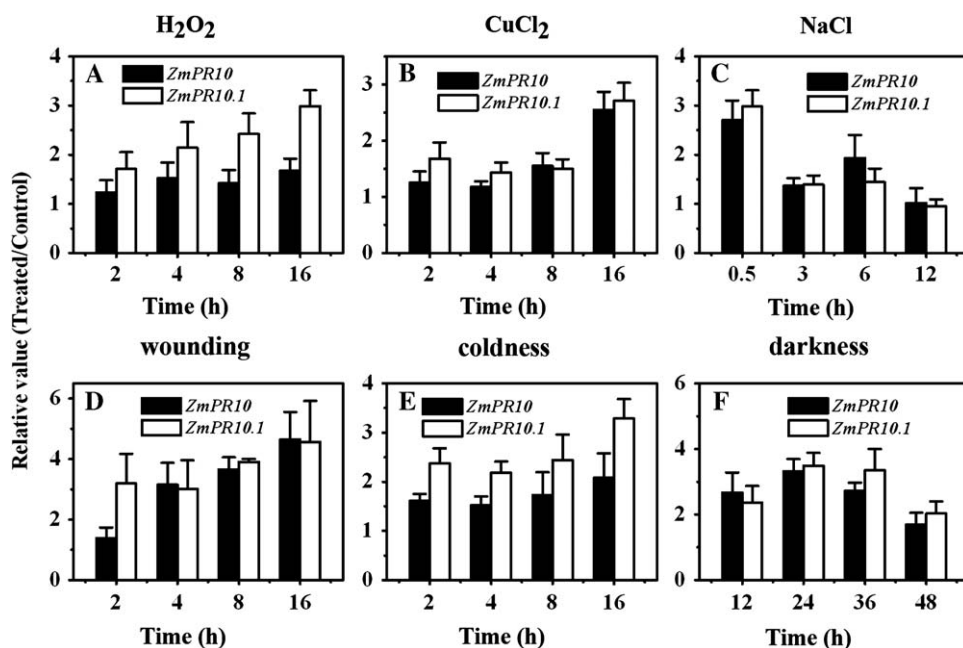


Fig. 5. Expression patterns of *ZmPR10* and *ZmPR10.1* in leaf tissue under abiotic stresses: (A) 200 μ M H₂O₂; (B) 1.0 mM CuCl₂; (C) 200 mM NaCl; (D) wounding; (E) coldness (4 °C); and (F) darkness. Each data point represents the mean value of three replications. Plants sprayed with distilled water and grown at room temperature were used as a control. SE ($n = 3$) is indicated on top of each bar and may not be visible if the value is too small.

same treatment caused a higher induction in *ZmPR10.1* expression, which peaked at 36 HAT (3.6-fold) (Fig. 6C).

Inoculation of maize ears with *A. flavus* caused a significant induction in the expression of both *ZmPR10.1* and *ZmPR10* genes as early as 1 DAI up to 10 DAI compared to non-inoculated control ears. Both of them reached their highest level of induction at 2 DAI (2.7- and 3.1-fold for *ZmPR10.1* and *ZmPR10*, respectively). The expression of *ZmPR10.1* remained high until 10 DAI, whereas *ZmPR10* expression declined rapidly to the control level 14 DAI (Fig. 7). In the control kernels, the expression patterns between *ZmPR10.1* and *ZmPR10* were also different. The expression of

ZmPR10.1 remained steady during the late stages of kernel development; however, the latter continued to increase during kernel development (data not shown).

The RNase activity of *ZmPR10.1* and *ZmPR10*

The *ZmPR10.1* was over-expressed in *E. coli* to determine whether it possessed RNase activity similar to *ZmPR10* (Chen et al., 2006). The over-expressed protein accounted for 19.7% of total proteins extracted from *E. coli* (Supplemental Fig. 1). The

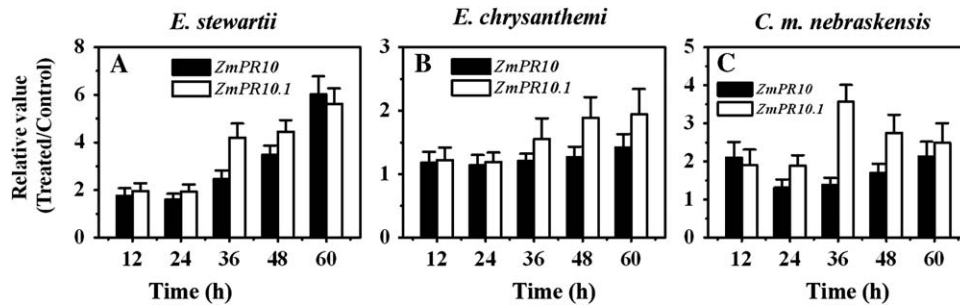


Fig. 6. The effect of bacterial infections on the expression of ZmPR10 and ZmPR10.1 in maize leaves: (A) *Erwinia stewartii*; (B) *Erwinia chrysanthemi*; and (C) *Clavibacter michiganensis* subsp. *nebraskensis*; Leaves infiltrated with YDC medium only were used as a control. Values are means $\pm$ SE ($n = 3$). SE is indicated with a bar.

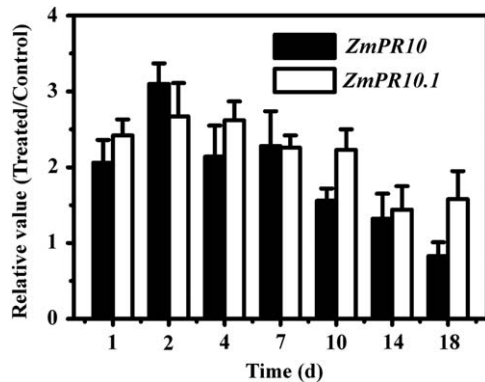


Fig. 7. The effect of *Aspergillus flavus* infection on the expression of ZmPR10 and ZmPR10.1 in immature maize kernels. Ears wounded with a needle only were used as a control. Values are means $\pm$ SE ($n = 3$). SE is indicated with a bar.

recombinant ZmPR10.1 protein was then purified to apparent homogeneity with a nickel-iminodiacetic acid (Ni-IDA) His.Bind affinity column. The recombinant protein contains six histidine residues and five additional amino acids from the vector pET 20b plus at the C-terminus with a total of 171 amino acid residues and a calculated molecular mass of 18.1 kDa, which is similar to the estimated value (Supplemental Fig. 1). Both over-expressed ZmPR10 and ZmPR10.1 appear to be present predominantly in the soluble fraction after French Press based on sodium dodecyl sulfate (SDS)-PAGE.

The possible RNase activity of the purified recombinant ZmPR10.1 was tested with maize root total RNA as a substrate. ZmPR10.1 showed a clear RNase activity against the total RNA (Fig. 8, lanes 4 and 6). No detectable RNA degradation was observed when maize root RNA was treated with proteins eluted from *E. coli* cells containing the vector only (Fig. 8). Further, ZmPR10.1 appeared to have a significantly higher RNase activity than ZmPR10 (Fig. 8). The difference in specific activity between ZmPR10.1 and ZmPR10 was further quantified using yeast tRNA. It was found that ZmPR10 possessed low specific activity (28.3 U mg^{-1} protein), which was only about 12% that of ZmPR10.1 (244.6 U mg^{-1} protein).

Effect of pH, temperature and reagents on the RNase activity

Several buffers with different pH ranges were used in this study to determine the optimum pH for the activities of both PR10 proteins. The highest specific activity for both ZmPR10.1 and ZmPR10 was detected at pH 6.5 (262.2 and 30.0 U mg^{-1} protein, respectively). Their activities declined when the buffer pH either increased or decreased, with ZmPR10.1 showing higher sensitivity to changes in pH than ZmPR10. The lowest activity was observed

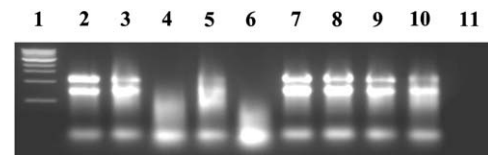


Fig. 8. Ribonuclease activity of the purified recombinant ZmPR10 and ZmPR10.1 using $10 \mu\text{g}$ of total RNA from maize root as a substrate. Lane 1, DNA 1 kb marker; Lanes 2–11 were RNAs after incubating for 0.5 h with the following treatments: protein from *E. coli* harboring plasmid pET20b (lane 2), $10 \mu\text{g mL}^{-1}$ of ZmPR10 (lane 3) and ZmPR10.1 (lane 4), $40 \mu\text{g mL}^{-1}$ of ZmPR10 (lane 5) and ZmPR10.1 (lane 6), $40 \mu\text{g mL}^{-1}$ of heat-inactivated ZmPR10 (lane 7) and ZmPR10.1 (lane 8), 20 U of RNasin along with $40 \mu\text{g mL}^{-1}$ of ZmPR10 (lane 9) and ZmPR10.1 (lane 10), or $10 \mu\text{g mL}^{-1}$ of RNase A (lane 11).

when PR10 proteins were exposed to buffers at pH 3.0 (42.7 and 7.2 U mg^{-1} protein for ZmPR10.1 and ZmPR10, respectively) or pH 11.0 (11.5 and 3.0 U mg^{-1} protein, respectively) (Supplemental Fig. 2A). However, ZmPR10.1 showed a much higher activity (3.9 – 11.1 -fold) than ZmPR10 in these buffers. The optimum temperature for the activity of both proteins was 55°C , with the RNase activity of 255.2 and 29.0 U mg^{-1} protein for ZmPR10.1 and ZmPR10, respectively (Supplemental Fig. 2B). The RNase activity declined to 80.4 and 8.9 U mg^{-1} protein, respectively, for ZmPR10.1 and ZmPR10 when temperature was lowered to 20°C (Supplemental Fig. 2B). When the temperature was raised to 90°C , their activity declined to 46.3% (118.0 U mg^{-1} protein) and 40.4% (11.7 U mg^{-1} protein) for ZmPR10.1 and ZmPR10, respectively. Again, ZmPR10.1 was more sensitive to changes in temperature than ZmPR10 and showed a much higher activity (6.6 – 10.1 -fold) than ZmPR10 upon temperature change.

The effect of metal ions (Ca^{2+} , Mg^{2+} , Zn^{2+} , Fe^{2+} , Mn^{2+} , Cu^{2+} , Ag^+ and Co^{2+}) and other chemicals commonly used during protein isolations (such as ethylenediaminetetraacetic acid (EDTA), DL-dithiothreitol (DTT) and SDS) on enzyme activity was also investigated. Ca^{2+} , Mg^{2+} , Zn^{2+} and Mn^{2+} had little effect on the RNase activity of ZmPR10.1 (96.0% , 99% , 89.8% and 94.9%) or ZmPR10 (83.8% , 83.3% , 95.6% and 93.9%) when they were used at 1 mM final concentration (Supplemental Table 1). However, Ag^+ , Cu^{2+} , Co^{2+} , EDTA, DTT or SDS at 1 mM concentration significantly inhibited the activities of both ZmPR10.1 and ZmPR10, especially for Ag^+ and Cu^{2+} , which reduced ZmPR10.1 activity by 88% and 79% , respectively (Supplemental Table 1).

Comparison of antimicrobial activities between recombinant ZmPR10.1 and ZmPR10 protein

The possible antifungal activity of ZmPR10.1 against *A. flavus* was also investigated in this study. ZmPR10.1 significantly inhibited the germination and growth of *A. flavus* at $20 \mu\text{g mL}^{-1}$ (Fig. 9). Further comparison of conidia germination and fungal

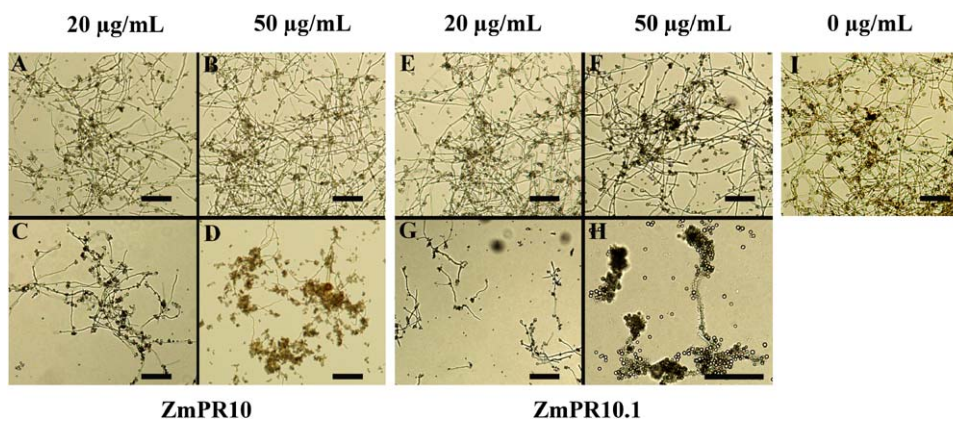


Fig. 9. *In vitro* antifungal activity of ZmPR10 and ZmPR10.1 protein. *Aspergillus flavus* conidia were germinated at 30 °C for 20 h in 10% potato dextrose broth medium containing either purified ZmPR10 (A–D) or ZmPR10.1 (E–H) protein at either 20 (A, C, E and G) or 50 (B, D, F and H) $\mu\text{g mL}^{-1}$. The controls were treated with boiled ZmPR10 (A and B), ZmPR10.1 (E and F) or medium only (I). Bars = 100 μm .

growth in medium containing different concentrations of ZmPR10.1 or ZmPR10 revealed that ZmPR10.1 appeared more potent than ZmPR10 in inhibiting *A. flavus* conidia germination and hyphal growth. When treated with ZmPR10.1, *A. flavus* showed a significantly higher reduction in conidia germination and less hyphal growth compared to that treated with ZmPR10 at either 20 or 50 $\mu\text{g mL}^{-1}$ concentration (Fig. 9).

ZmPR10.1 and ZmPR10 were over-expressed in *Arabidopsis* to evaluate their antibacterial activities. At least 29 and 32 transgenic lines were obtained for ZmPR10.1 and ZmPR10, respectively. The PCR results from genomic DNA with specific primer pairs confirmed the presence of transgene *ZmPR10.1* or *ZmPR10* (Fig. 10A). Real-time PCR of four transgenic lines (1-1, 3-4, 19-1 and 29-2) containing *ZmPR10.1* and four transgenic lines (6-3, 13-3, 28-1 and 32-2) containing *ZmPR10* showed that *ZmPR10.1* expression was significantly lower than *ZmPR10* in transgenic *Arabidopsis* (Fig. 10B). Three transgenic lines (two for *ZmPR10.1* and one for *ZmPR10*) with high level expression of the transgene were further analyzed for their differences in antibacterial activities (Fig. 11). After 4-d incubation with *Pseudomonas syringae* DC3000, severe symptoms (necrosis and chlorosis) around the infiltrated areas were observed in control plants (transformed with vector only) (Fig. 11A). Transgenic plants of *ZmPR10.1* (lines 19-1 and 29-2) showed significant less symptom development (Fig. 11A). However, no significant inhibition on disease development was observed in the transgenic plant of *ZmPR10* (line 28-1) (Fig. 11A) even though its expression level was more than 2-fold higher than that of *ZmPR10.1*. The lesion area of line 28-1 (0.73 cm^2) is not significantly different from the inoculated control plant (0.87 cm^2), which was about twice the lesion area of lines 19-1 and 29-2 (only 0.43 and 0.37 cm^2 , respectively) (Fig. 11B).

Discussion

PR10 is one of the most important families of plant pathogenesis-related proteins that play important roles in defense against microbe attack. In this study, another member of PR10, *ZmPR10.1*, was isolated from maize. Sequence analysis of *ZmPR10.1* showed it lacked a signal peptide like some other members of PR10 family, indicating *ZmPR10.1* is an intracellular protein. The GFP-aided localization confirms that *ZmPR10.1*-GFP fusion protein is expressed in cytosols of transgenic *Arabidopsis* (data not shown), which is different from most other families of PR proteins that are either extracellular or localized in the vacuole (van Kan et al., 1992).

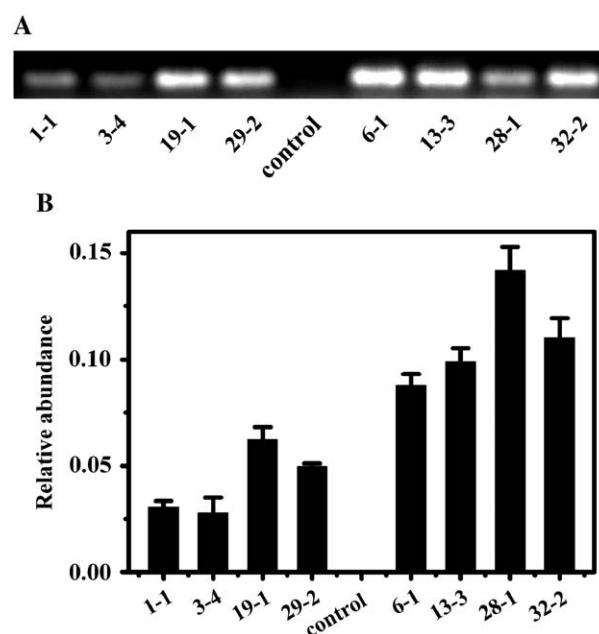


Fig. 10. Expression of *ZmPR10* and *ZmPR10.1* genes in the leaves of transgenic *Arabidopsis* plants. (A) The presence of *ZmPR10* or *ZmPR10.1* confirmed by PCR using gene-specific primer pairs. (B) Real-time PCR determining the relative abundance of *ZmPR10.1* (line 1-1, 3-4, 19-1 and 29-2) and *ZmPR10* (line 6-1, 13-3, 28-1 and 32-2). The plants transformed with pCAMBIA1302 vector only were used as a control. All data represent the mean values of three replications.

For better understanding the differences between the novel *ZmPR10.1* and *ZmPR10*, a series of comparative studies were carried out to assess their potential roles in host disease resistance. Both *ZmPR10.1* and *ZmPR10* showed the strongest expression in root tissue, which is consistent with the expression of other PR10s (Liu et al., 2003; Hashimoto et al., 2004). Further, the higher protein expressions of *ZmPR10.1* and *ZmPR10* in root tissue than in leaf tissue were indirectly demonstrated by the GUS activities of transgenic *Arabidopsis* harboring cloned promoter pZmPR10.1 and pZmPR10.

One possible contributing factor for this high level expression in root tissue is the presence of at least 2–4 root-specific expression elements revealed during the analysis of the cloned promoters of *ZmPR10.1* and *ZmPR10* (Xie et al., unpublished data). Their root-specific expression might also indicate their importance in root defense since there are numerous pathogens in rhizosphere soil.

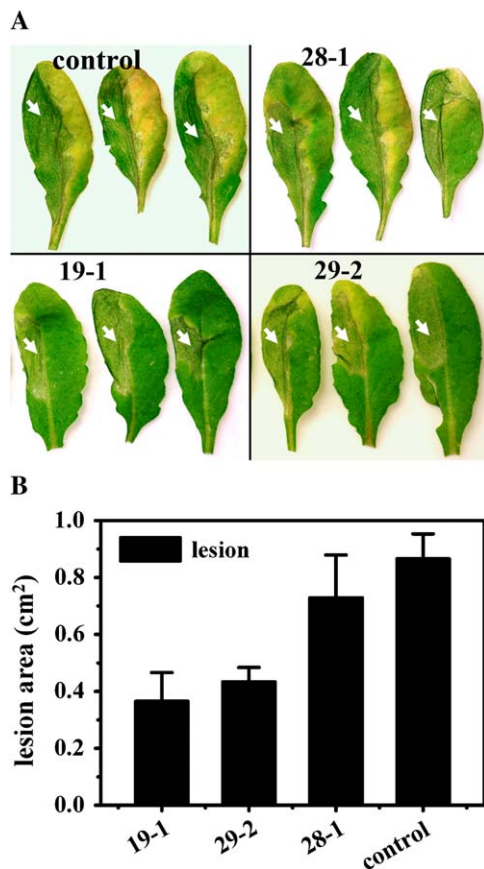


Fig. 11. Antibacterial activity of transgenic *Arabidopsis* plants against *Pseudomonas syringae* pv. *tomato* DC3000. (A) Severity of disease symptoms among control (transformed with vector pCAMBIA302 only), *ZmPR10* transgenic line 28-1 and *ZmPR10.1* transgenic lines (19-1 and 29-2) 4 d after inoculation. The arrows indicate the areas of inoculation. (B) Lesion size of infiltrated transgenic *Arabidopsis* leaves. Means are shown with SE calculated from 12 infiltrated leaves from three plants.

Earlier studies reported that *PR10* expression changed in response to salt stress in rice (Moons et al., 1997), drought stress in pine (Dubos and Plomion, 2001), UV exposure in lupine (Pinto and Ricardo, 1995), coldness in peach (Wisniewski et al., 2004) and heavy metals in birch (Koistinen et al., 2002). The *PR10* expression in pine was also induced by wounding (Liu et al., 2003) and hormone treatment such as JA, SA, GA₃ and ABA (Jwa et al., 2001; Zhou et al., 2002; Liu et al., 2006; Kim et al., 2008). In this study, effects of various abiotic treatments (phytohormone, chemicals, wounding, coldness and darkness) on *PR10* expression were investigated. Both genes showed similar responses in most cases, which can be grouped into three different patterns. When exposed to treatments such as SA, CuCl₂, H₂O₂, coldness, darkness or wounding, mRNA levels of both *ZmPR10.1* and *ZmPR10* up-regulated gradually in the 16-h of treatment. The expression of *ZmPR10.1* and *ZmPR10* down-regulated initially but recovered later during the ABA treatment. The third pattern is that the expression of both *ZmPR10s* up-regulated initially, then down-regulated when exposed to treatments such as KT, GA, MeJA and NaCl. The similar response of *ZmPR10.1* and *ZmPR10* to the same stress might be the result of high sequence homology (about 60% identity) between their putative promoter sequences and the presence of several stress-responsive *cis*-elements in both promoters, such as ABA-responsive element, W-box element, dark-responsive element (Xie et al., unpublished). However, in response to NAA treatment, *ZmPR10.1* expression exhibited a pattern similar to the third one, whereas *ZmPR10* expression showed a pattern

similar to the first one. While in most cases the magnitude of responses between the two genes was similar, *ZmPR10.1* showed a consistent higher induction than *ZmPR10* in responses to MeJA, SA, H₂O₂ and coldness treatments.

Many *PR10* genes were up-regulated when plants were exposed to biotic stresses, such as viruses (Xu et al., 2003), bacteria (Robert et al., 2001) or fungi (Jwa et al., 2001). Further studies found that some *PR10* proteins showed antimicrobial activities *in vitro* against bacteria, fungi and viruses, such as Ocatin (Flores et al., 2002), SsPR10 (Liu et al., 2006) and CaPR10 (Park et al., 2004). In this study, the expressions of *ZmPR10.1* and *ZmPR10* were significantly induced in response to bacterial *E. stewartii* inoculation on young maize leaves and to fungal *A. flavus* inoculation on maize immature kernels. Also, the antimicrobial activities of *ZmPR10s* were evaluated both *in vivo* through inoculation of transgenic *Arabidopsis* expressing *ZmPR10.1* or *ZmPR10* and *in vitro* using purified *PR10* proteins against *A. flavus*. Transgenic *Arabidopsis* plants expressing *ZmPR10.1* exhibited significant higher resistance toward *P. syringae* DC3000 infection *in vivo* with smaller lesion size than plants expressing *ZmPR10* at a much higher level. This might be the result of a higher RNase specific activity of *ZmPR10.1* compared to *ZmPR10*. When *ZmPR10.1* and *ZmPR10* proteins purified from *E. coli* cells over-expressing these genes were examined *in vitro*, both proteins exhibited a clear *in vitro* antifungal activity against *A. flavus*. These data indicate the potential involvement of both genes in host defense against bacterial and fungal pathogens. However, the mechanism of *ZmPR10s*' antimicrobial activities is still unclear. Lam and Ng (2001) reported that a heterodimeric RNase protein from roots of *Panax notoginseng* possessed both antifungal activity and translation-inhibiting activity. Another study by Chadha and Das (2006) suggested that the ribonuclease function and internalization into sensitive fungal hyphae is essential for AhPR10 antifungal activity.

Therefore, the present study further examined whether *ZmPR10.1* possesses RNase activity to understand the possible mechanism of its strong antimicrobial activity. The present study revealed that *ZmPR10.1* not only possesses RNase activity as *ZmPR10*, but also shares similar biochemical characteristics as *ZmPR10*, such as the same optimum pH (6.5) and temperature (55 °C), and similar inhibitions by chemicals such as Ag⁺, Cu²⁺, Co²⁺, EDTA, DTT and SDS. However, *ZmPR10.1* has over 8-fold higher specific RNase activity than *ZmPR10*, which might be a contributing factor for the observed higher antimicrobial activities by *ZmPR10.1*. Also, *ZmPR10.1* showed higher sensitivity to pH and Tm change than *ZmPR10*. It is unclear to us regarding these differences in biochemical characteristics since both proteins share more than 85% identity (with 23 amino acid differences out of 160 amino acid residues). However, similar results were found in the study of a PR protein (LIPR10.1B) from yellow lupine. While LIPR10.1B displayed RNase activity, its homologue (76% identity) from the same species with the same conserved glycine-rich loop (GxGGxG), LIPR10.1A, showed no RNase activity at all (Biesiadka et al., 2002). Wu et al. (2003) reported that SPE-16 showed no RNase activity after site-directed mutagenesis of only three amino acid residues (E95A, E147A and Y149A). These studies along with our data suggest that amino acid residues other than the three conserved amino acid residues (D76, G111 and G112) reported by Liu et al. (2006) also are critical for the activity since these three amino acid residues are all present at similar positions in LIPR10.1A/LIPR10.1B and *ZmPR10*/*ZmPR10.1*.

In conclusion, a novel *PR10*, *ZmPR10.1*, was isolated from maize that shares 89.8% of homology to the previously reported *ZmPR10*. *ZmPR10.1* had significantly lower expression levels than *ZmPR10* in all tissues investigated. Both *ZmPR10s* showed similar expression profiles when exposed to most of biotic or abiotic stresses except

for NAA and *E. chrysanthemi* treatment. Although *ZmPR10* expresses at a consistently higher level than *ZmPR10.1* in all tissues examined, *ZmPR10.1* may play as important a role in host defense as *ZmPR10* based on its higher specific RNase activity, and a higher resistance it conferred in transgenic *Arabidopsis* to a bacterial pathogen with a much lower expression compared to *ZmPR10*.

Acknowledgements

We thank Drs. M. Catherine Aime, Kenneth E. Damann and James V. Moroney for critical reviewing of the manuscript and Nicole Hazard for technical assistance. This study was supported by USDA cooperative agreement 58-6435-6-055. Published with the approval from the Director of the Louisiana Agricultural Experiment Station as Manuscript no. 2009-240-2574.

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jplph.2009.07.004.

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Supplementary data

Supplemental Table 1. Effect of reagents on the RNase activity of ZmPR10.1 and ZmPR10

Reagents (1 mM)	ZmPR10.1* (%)	ZmPR10* (%)
None**	100 ± 5.2	100 ±10
AgCl	12.1± 3.3	24.6 ± 7.3
CaCl ₂	96 ± 5.0	83.8 ± 12.3
CoCl ₂	29 ± 6.4	47.2 ± 19.4
CuCl ₂	21.3 ± 4.7	34.7 ± 10.9
MgCl ₂	99 ± 10	83.3 ± 10.2
MnCl ₂	94.9 ± 10.6	93.9 ± 7.6
ZnCl ₂	89.8 ± 5.3	95.6 ± 11.2
DTT	26.9 ± 7.6	32.7 ±7.7
EDTA	54.1 ± 15.5	60.2 ± 8.7
SDS	43.8 ± 4.5	54.4 ± 7.3

* Data shown are means of three replicates ± standard error.

**The reaction without reagents serves as a control.