Full Length Research Paper

Molecular cloning and characterization of pathogenesis-related protein 5 in *Zea mays* and its antifungal activity against *Rhizoctonia solani*

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We described the cloning and characterization of pathogenesis-related protein 5 gene in maize, named *ZmPR5* (GenBank Accession Number: HM230665). Molecular and bioinformatic analyses of *ZmPR5* revealed an open reading frame (ORF) of 525 bp, encoding a protein of 175 amino acids (aa) and a deduced molecular mass of 17.5 KDa. Homology analysis of the *Zea mays* L. deduced amino acid sequence, indicated homology between 40 and 74% with *Oryza sativa, Hordeum vulgare,* and *Triticum aestivum*, among others. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the expression of *ZmPR5* is constantly higher in the maize resistant inbred line R15 compared with that in the susceptible inbred line 478. Moreover, the *ZmPR5* gene was up-regulated after it was challenged by *Rhizoctonia solani*. We subsequently purified the recombinant protein and analyzed its antimicrobial activities *in vitro*. The results obtained show that the recombinant protein inhibited hyphal growth of *R. solani*. This study suggests that the expression of *ZmPR5* is closely related to maize sheath blight resistance caused by *R. solani*. Further, the antifungal activity of ZmPR5 showed that ZmPR5 plays an important role in the disease resistance response.

Key words: Maize, ZmPR5, banded leaf and sheath blight, pathogenesis-related protein, *Rhizoctonia solani*.

INTRODUCTION

There are approximately 250,000 fungi, distributed widely throughout nearly every ecosystem (Selitrennikoff, 2001). Some organisms are capable of causing serious damage to grain yield and quality of economically important crops. Throughout the evolutionary processes, plants have gradually adapted to environmental changes. Although plants do not have an immune system, potent defense mechanisms exist, including the synthesis of low-molecular-weight compounds, proteins, and peptides with antifungal activities (Selitrennikoff, 2001). These pathogenesis-related (PR) proteins were first described by Van Loon and Van Kammen (1970), when they observed accumulation of various novel proteins after infection of tobacco with tobacco mosaic virus (TMV). PR proteins are classified into 17 families (PR1 to PR17) based on serological and amino acid sequence analyses (Van Loon et al., 2006).

When the host plant is infected by a pathogen, PRs accumulate at the infection site (Antoniw et al., 1983). The production of PRs is considered to be a biochemical mechanism of plant-induced resistance, with their

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Abbreviations: **PRs,** Pathogenesis-related proteins; **SAR,** systemic acquired resistance; **ORF,** open reading frame; **AG1-IA,** anastomosis group IA; **BLSB,** banded leaf and sheath blight; **EST,** expressed sequence tag; **IPTG,** isopropyl-β-D-thiogalactopyranoside; **PAGE,** polyacryamide gel electrophoresis; **HRP,** horseradish peroxidase; **PBST,** phosphate buffered saline with Tween-20; **DAB,** 3,3'-diaminobenzidine.

expression being a symbol of plant disease resistance. Systemic acquired resistance (SAR) is a mechanism of induced defense that confers long-lasting protection against a broad spectrum of microorganisms. Molecularly, SAR induction is generally accompanied by the activation of a set of defense-related genes involved in the formation of chemical barriers. Of these, the pathogenesis-related genes have became a central issue of plant disease resistance research in recent years, with most studies focusing on *Arabidopsis* (Uknes et al., 1992), tobacco (Uknes et al., 1993), rice (Masuta et al., 1991; Agrawal et al., 2001; Mitsuhara et al., 2008), and wheat (Niu et al., 2007).

PR5 proteins share obvious amino acid homology to thaumatin, and are known as thaumatin-like (TL) proteins. Members of the PR5 group have been characterized from corn, soybeans, rice, and wheat, as well as many other plants (Huynh et al., 1992; Koiwa et al., 1997; Ye et al., 1999). Moreover, several lines of evidence indicate that the *PR5* genes are associated with plant resistance to pathogens. In rice, over-expression of thaumatin-like protein (TLP-D34) resulted in enhanced resistance to *Rhizoctonia solani* compared to control plants (Datta et al., 1999). The tobacco thaumatin-like PR proteins exhibited antifungal activity against the phytopathogen *Phytophthora infestans* (Vigers et al., 1992). However, less is known about the function of PR5 genes in *Zea mays*.

Banded leaf and sheath blight (BLSB), caused by *R. solani* AG1-IA, is an important disease in maize world-wide. Few genes related to resistance of this pathogen have been isolated in maize, due to its large genome size, complex genome structure, and quantitative trait nature. These characteristics have hampered the ability to conduct detailed studies of resistance mechanisms and exploitation of resistance resources in maize.

In this study, we isolated the *PR5* gene from AG1-IA-induced high-resistance inbred maize line R15. Expression of the *ZmPR5* gene was analyzed by real-time everse transcription-polymerase chain reaction (RT-PCR) after infection with *R. solani*. We subsequently purified the recombinant protein and carried out analysis of its antimicrobial activities *in vitro*. Our results provide valuable information for comprehensive understanding of PR5 function.

MATERIALS AND METHODS

Plant materials

High-resistance maize inbred line R15 and high-susceptibility maize inbred line 478 were used in this study. Seeds of both inbred lines were treated with 7% hypochlorite solution for 30 min followed by three washes with sterilized water before being sowed in pots with autoclaved soil. Plants were allowed to grow for 25 to 35 days and were then inoculated with *R. solani* (kindly provided by the Rice Institute of the Sichuan Agricultural University, Sichuan, China) at 28°C. *R. solani* AG1-IA was cultured on potato dextrose agar (PDA) and incubated at 28°C for three to five days before use.

RNA extraction and cloning of *ZmPR5* gene

Total RNA was extracted using Trizol® reagent (Invitrogen, USA) according to the manufacturer's protocol. Five hundred nanograms of DNase-treated total RNA were reverse-transcribed (TaKaRa, Dalian, China). Primers were designed according to the PR5 sequence (GenBank Accession Number: NM_001112232): forward primer 5'-CTCCACAAGAAACAAGGCA-3' and reverse primer 5'–GTACGAAGCACGCACACAA -3'. Amplified products were gel-separated on 1.2% agarose and extracted (Omega, China), then cloned into the pMD18-T vector (TaKaRa, Dalian, China) for sequencing. Three positive clones were sequenced.

Sequence analysis of *ZmPR5* gene

The ExPASy translate tool (http://au.expasy.org/tools/dna.html) was used to deduce the amino acid sequence from the cDNA. Multiple amino acid sequence alignments of different plants were carried out using Genedoc software. Cluster analysis was conducted using DNAstar 7.0 to reveal evolutionary relationships of the proteins from different plants. The putative signal peptide cleavage site was analyzed with the Web-based SignalP program (http://www.cbs.dtu.dk/services/SignalP). The conserved domain between the different plants was predicted at Pfam program (http://pfam.sanger.ac.uk/).

Analysis of *ZmPR5* expression

To analyze the response of the *ZmPR5* gene to *R. solani* challenge, real-time RT-PCR primers (forward primer 5'-GCAGCCAGGAC-TTCTACG-3' and reverse primer 5'-CACAGGCATGGGTCTTCA-3') were designed according to the cloned sequence. The mRNA expression levels of *PR5* were quantified by real-time RT-PCR after inoculating for 0, 6, 24, 36, 48, and 60 h. All samples used in this experiment were sheath from both the resistant and susceptible inbred lines. Actin (GenBank Accession number: 99030435) was used as an internal control.

Real-time RT-PCR was conducted with SYBR Green Mix (TaKaRa, China) using iQ5 (Bio-Rad, China). PCR amplification was performed under the following cycling conditions: 94°C for 3 min, followed by 40 cycles of 94°C for 20 s, 59°C for 20 s, and 72°C for 20 s. Melting curve analysis of amplification products was performed after each reaction to confirm the detection of a single PCR product. All samples to be compared in the same experiments were run on the same plate. The relative expression of target gene in two inbred lines was calculated using the ↵Ct method.

Construction of expression plasmid and optimization of expression conditions

According to the sequences, we designed primers for amplification of the open reading frame (ORF) of *PR5*: forward primer 5'-TCGGAATTCATGGCCGCCGCCTCCTCG-3' (*Eco*RI site is underlined) and reverse primer 5'-ATTTGCGGCCGCTCA-CGGGCAGAAGGTGAGTT-3' (*Not*I site is underlined). The pMD18-*PR5* vector and pET32a (+) were digested with *Eco*RI and *Not*I, respectively. The expected fragments were released from pMD 18-PR5 and subcloned into pET32a(+) vector using T₄ DNA ligase (TaKaRa, Dalian, China). The recovered plasmid was transformed into *Escherichia coli* DH5α and positive clones were selected, which were named pET-32a(+)-*PR5*. The resulting

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1         CTCCACAAGAAACAAGGCAGAGCACCAACA ATG GCC GCC GCC TCC TCG TCC TCG GTC CTC
                                         M   A   A   A   S   S   S   S   V   L  10
61  CTG CTG CTC CTG GCC GCC GCC TTG GCT GGC ATG AGC GCC AAC GCC GCC ACC TTC ACC ATC
    L   L   L   L   A   A   A   L   A   G   M   S   A   N   A   A   T   F   T   I  30
121 ACC AAC AAC TGC GGG TTC ACC GTG TGG CCC GCG GCC ACC CCG GTG GGC GGG GGC ACG CAG
    T   N   N   C   G   F   T   V   W   P   A   A   T   P   V   G   G   G   T   Q  50
181 CTG AAC CCG GGC GGG ACG TGG ACC GTC AAC GTG CCG GCC GGC ACC AGC TCC GGC CGC GTG
    L   N   P   G   G   T   W   T   V   N   V   P   A   G   T   S   S   G   R   V  70
241 TGG GGC CGC ACC GGC TGC TCC TTC AAC GGC AAC AGC GGG AGC TGC CAG ACG GGC GAC TGC
    W   G   R   T   G   C   S   F   N   G   N   S   G   S   C   Q   T   G   D   C  90
301 GGC GGC GCG CTG GCC TGC ACG CTG TCT GGG CAG CCG CCG CTG ACG CTG GCC GAG TTC ACC
    G   G   A   L   A   C   T   L   S   G   Q   P   P   L   T   L   A   E   F   T 110
361 ATC GGC GGC AGC CAG GAC TTC TAC GAC ATC TCG GTC ATC GAT GGC TAC AAC CTC GCC ATG
    I   G   G   S   Q   D   F   Y   D   I   S   V   I   D   G   Y   N   L   A   M 130
421 GCC TTC TCC TGC AGC ACC GGC GTG CGG CTG GTG TGC ACG GAC TCT GGA TGC CCC GAC GCG
    A   F   S   C   S   T   G   V   R   L   V   C   T   D   S   G   C   P   D   A 150
481 TAC CAC CAC CCC AAC GAC GTG AAG ACC CAT GCC TGT GGC GGC AAC AGC AAC TAC CAA GTC
    Y   H   H   P   N   D   V   K   T   H   A   C   G   G   N   S   N   Y   Q   V 170
541 ACC TTC TGC CCG TGA TCGGAATAGGCTCTGCATGCATGGGCGAGTGATTTGGATTTGCACAATAATA
    T   F   C   P   *
                                                                              174
607 TTTAGGGAGCGCGTGCATGACACGTGCACGCATTTGTATGTACGCGCGTATACGTACCTACGTAGCTGG
676 ATTTGTGTGCGTGCTTCGTAC
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Figure 1. cDNA sequence of the *ZmPR5* gene and its deduced amino acid sequence. The ORF of *ZmPR5* gene is 525 bp, which lies in 31-555 bp. The upper line is the cDNA sequence, the position is shown at the left. The lower is the deduced amino acid sequence, the position is shown at the right. The putative signal of *ZmPR5* is underlined.

plasmid was used to transform *E. coli* BL21 and was verified by sequencing. Bacteria were grown with two temperature levels at 37 and 28°C respectively to an appropriate density (OD_{600}= 0.6), and induced with 0.1, 0.2, 0.5, 1.0, or 2.0 mM IPTG for 0, 2, 4, 6, 8, and 10 h to identify optimum expression conditions.

SDS-PAGE and western blot

BL21 cells (TIANGEN) expressing the recombinant protein ZmPR5-His were boiled at 95°C for 5min and were then centrifuged for 10 min at 12000 rpm. 10 µl of solubilized protein from the supernatant fraction was mixed with 4* loading buffer subjected to sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred on to a PVDF membrane (Millipore). After blocking for 1 h with 5% milk powder in 1*PBST (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM K_2HPO_4 and 0.1% Tween 20), the membrane was incubated with mouse anti-His-Tag antibody (APPLYGEN) at a dilution of 1:1000 at 4°C overnight, and washed three times for 15 min each in PBST. Subsequently, the membrane was incubated with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) (Boster, China) at a dilution of 1:500 for 3 h at 37°C. After washing twice in PBST, the membrane was displayed (TIANGEN, China) according to the manufacturer's protocol.

Purification and antimicrobial activity assays of ZmPR5 *in vitro*

The pET-32a-ZmPR5 constructs contained a His-tag downstream of the target gene to allow purification of the over-expressed proteins with the His-Bind kit, following the manufacturer's protocol (NovaGen, USA). The induced bacterial cell pellet was collected, lysed, and purified. The recombinant protein in 1×elution buffer was dialysed against 1×phosphate-buffered saline (PBS) with three changes at 4°C over 24 h. The protein concentration was measured according to Bradford (1976) and antimicrobial activity evaluated *in vitro*.

R. solani was cultured on PDA medium at 28°C. Holes 1 cm in diameter were placed on PDA plates (9.0 cm). Differing concentrations of ZmPR5 protein in 1×PBS buffer were added into the three holes (20 µg, 40 µg, and 60 µg), with 1×PBS buffer as control. The plates were incubated at 28°C for 48 h. In addition, *R. solani* were incubated on PDA with PBS buffer (CK) or the purified protein at a concentration of 20 40 and 60 µg (ZmPR5) at 28°C. Germination and growth of *R. solani* were examined at 48 h of incubation under a microscope. The experiment was carried out three times independently, using the same conditions.

RESULTS

Isolation of *ZmPR5*

A specific 697 bp fragment, named *ZmPR5* (GenBank Accession Number: HM230665), was amplified with gene specific primers. The ExPASy translation tool (http://au.expasy.org/tools/dna.html) was used to deduce the amino acid sequence of the cDNA, resulting in a sequence of 174 amino acids (Figure 1). Furthermore, the results indicate an expected acidic protein of 17.5 kD with an isoelectric point of 4.88.
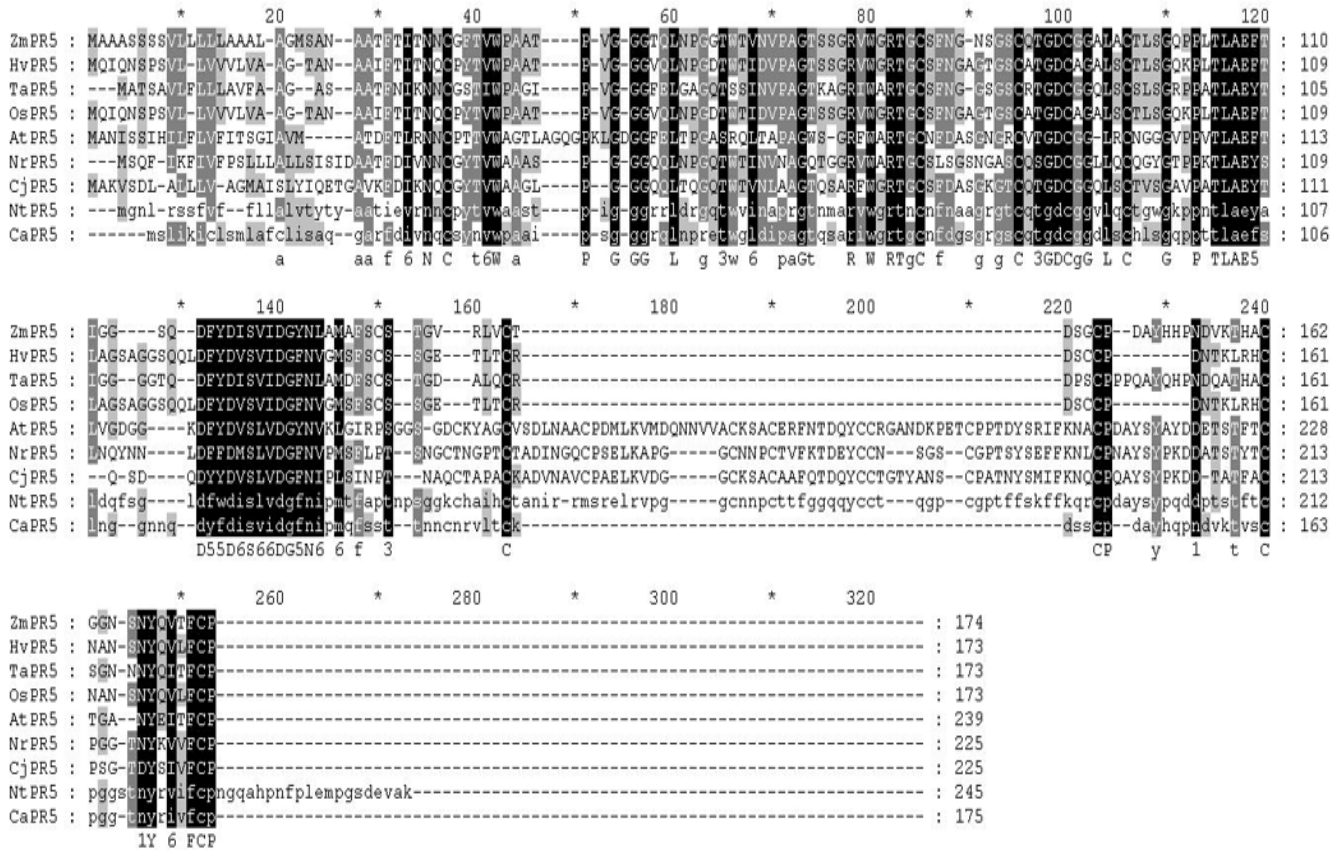
Figure 2. Molecular and bioinformatic analyses of *ZmPR5*. **A,** Alignment of deduced amino acid sequences of PR5 from different plants; **B,** comparison of structural organization of ZmPR5 with members in the thaumatin family; **C,** evolutionary tree of PR5 from different plants. Sequences were downloaded from GenBank and have the following accession numbers: *Zea mays* (ZmPR5), NM_0011122321; *Oryza sativa* (OsPR5), NM_001073864; *Hordeum vulgare* (HvPR5), AM403331; *Triticum aestivum* (TaPR5), AF384146; *Arabidopsis thaliana* (AtPR5), NM_106161; *Nepenthes rafflesiana* (NrPR5), GQ338251; *Cryptomeria japonica* (CjPR5), AB254807; *Nicotiana tabacum* (NtPR5), CAA43854; *Cicer arietinum* (CaPR5), CAA09229. Identical amino acids are shaded in black, and conserved substitution residues are shaded in gray.

Sequence analysis of *ZmPR5*

Multiple amino acid sequence alignments of different crop species were carried out using Genedoc software. Homology analysis indicated that the deduced amino acid sequence from *Z. mays* L. shares homology between 40 and 74% with *Oryza sativa, Hordeum vulgare, Triticum aestivum, Arabidopsis thaliana, Nepenthes rafflesiana, Cryptomeria japonica, Nicotiana tabacum,* and *Cicer arietinum*. Similarities were found with *O. sativa* (73%)*, H. vulgare* (72%), and *T. aestivum* (74%) (Figure 2A). A main domain, thaumatin, which comprises amino acids 32 to 147, was found in ZmPR5 using http://pfam.sanger.ac.uk/. Conserved regions between the different plants showed that all of the PR5 proteins have the same thaumatin structure (Figure 2B). A cluster analysis was assembled from the homologous proteins retrieved from the database using DNAstar 7.0, showing relatedness between PR5 from *O. sativa, H. vulgare*, and *T. aestivum* (Figure 2C). Analysis with the

SignalP tool revealed that the putative signal peptide cleavage site lies in the region between amino acids 1 and 23.

Analysis of *ZmPR5* expression

Expression of *ZmPR5* after infection with *R. solani* was investigated by real-time RT-PCR. *Actin* gene was chosen as the reference gene. Of the two inbred lines, expression of *ZmPR5* was generally up-regulated. In R15, after treatment with *R. solani*, the expression of *ZmPR5* continued to increase and reached a level 16-fold that of the control leaves after 60 h (Figure 3A). In line 478, the same treatment produced a significant change in *ZmPR5* expression, which peaked 60 h after treatment with *R. solani* (10-fold) (Figure 3B). Furthermore, expression in high-resistance line R15 was significantly higher than the high-susceptibility line 478 (Figure 3C). The results demonstrate that *PR5* may be
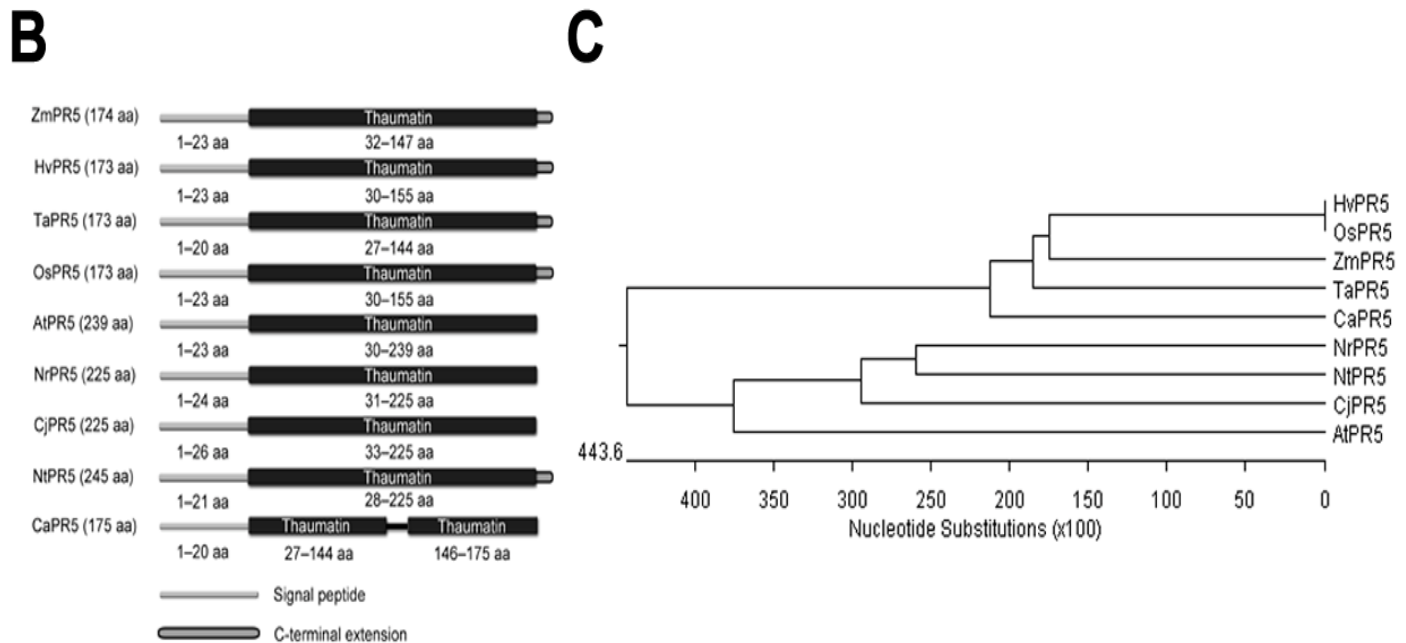
Figure 2. Contd

closely associated to maize sheath blight resistance.

SDS-PAGE and western blot analysis

E. coli BL21 cells were transformed and expression conditions were optimized at 37°C and 28°C, after induction with 0.1, 0.2, 0.5, 1.0, or 2.0 mM IPTG for 0, 2, 4, 6, 8, and 10 h, respectively. The mRNA encoding ZmPR5 was fused to His-Tag coding sequence at the 3' end, for comparison to cells containing recombinant plasmid without induction. The results obtained show a distinguishable extra band around 36.2 kD and the size of the ZmPR5-thioredoxin fusion protein in *E. coli*. The optimal induction occurred at 37°C with 0.6 mM IPTG at 6 h (data not shown).

The recombinant plasmid was transformed into the host strain BL21 (DE3) and induced at 37°C with 0.6 mM IPTG at 6 h. The expressed proteins were analyzed by SDS-PAGE and western blot analysis. Western blot analysis revealed the presence of one protein in the induced cells with apparent molecular weight of approximately 36.2 kD, which is bound to the anti-His-Tag antibody (Figure 4A). For analysis of antimicrobial activity of the fusion protein *in vitro*, the protein was purified by SDS-PAGE, which revealed highly pure protein (Figure 4B).

Purification and antimicrobial activity assays of ZmPR5 *in vitro*

The recombinant protein concentration was measured

with the Bradford method (Bradford, 1976). The possible antifungal activity of ZmPR5 against *R. solani* was investigated *in vitro* with three replications. The results obtained show that the ZmPR5 protein exhibited an inhibitory effect *in vitro* on hyphal growth of *R. solani.* The *R. solani* treated with ZmPR5 at 60 µg/ml showed significantly slower hyphal growth compared with the PBS control (Figures 5A and B).

DISCUSSION

PR5 is one of the most important members of the families of plant pathogenesis-related protein that play important roles in plant defense against pathogen attacks. In this study, we cloned ZmPR5 which encodes a novel antimicrobial protein from maize. Expressions of PRs are associated with a plant resistance response and the activation of defense mechanisms (Guo, 2008). Alexander et al. (1993) found that constitutive *PR1* expression resulted in increased tolerance to two oomycete pathogens in *PR1-a* transgenic tobacco plants. Further, Sarowar et al. (2005) showed that over-expression of *PR1* enhanced resistance of pepper plants to fungi.

Earlier studies reported up-regulation of *PR1, PR2, PR5,* and *PR10* when rice, wheat, and corn were exposed to biotic or abiotic stresses (Mitsuhara et al., 2008; Niu et al., 2007; Sarowar et al., 2005). In this study, real-time RT-PCR confirmed up-regulation of *PR5* expression in the high-resistance line R15 and the high-susceptibility line 478. Moreover, *PR5* expression was much higher in R15 than in 478. There was an obvious positive relationship between *PR*5 gene
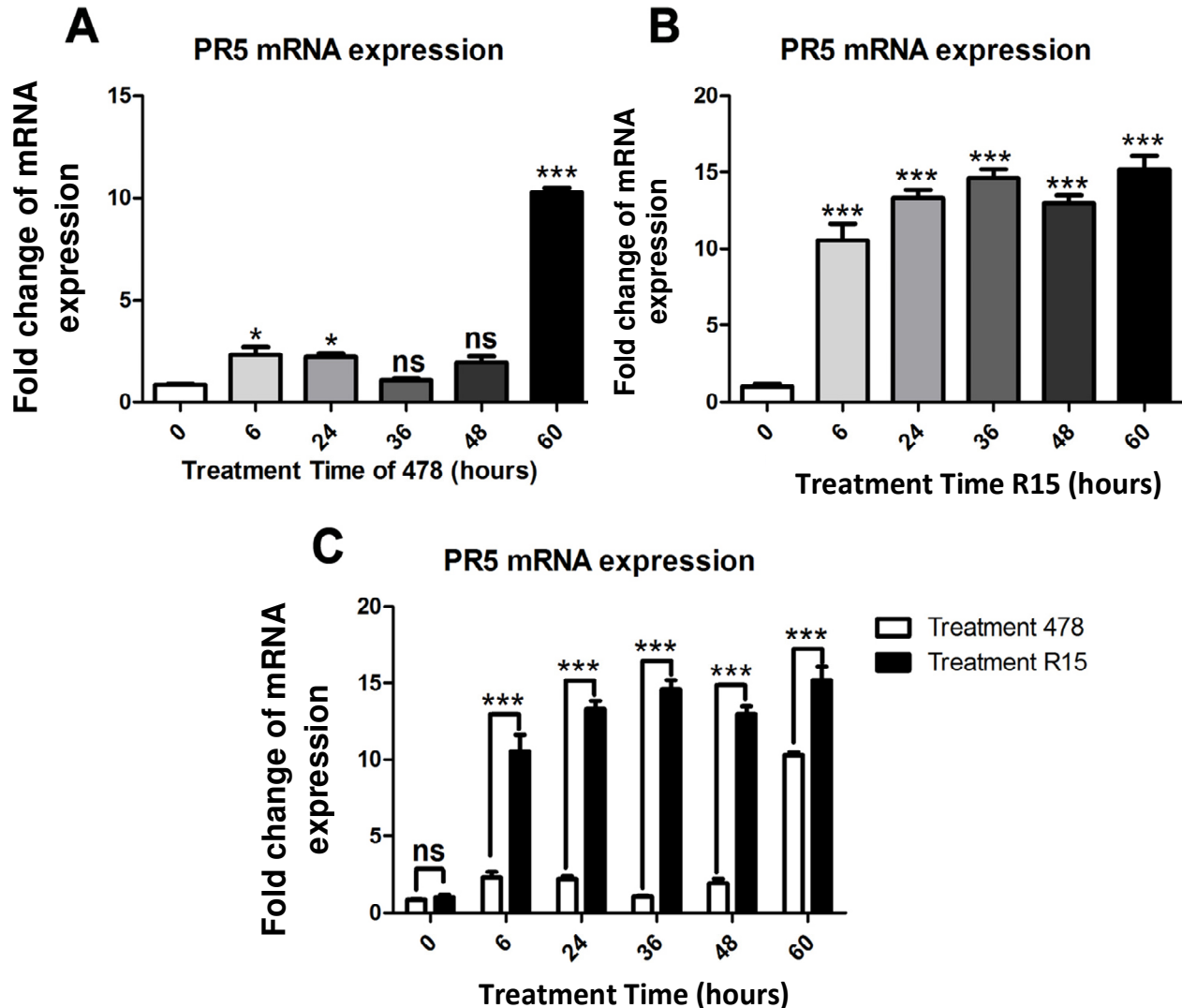
Figure 3. PR5 mRNA expression in two inbred lines. A, PR5 mRNA expression in R15 after treatment; B, PR5 mRNA expression in 478 after treatment; C, comparison of PR5 mRNA expression in the two inbred lines.

expression and plant resistance, indicating that *PR5* is involved in *Z. mays* resistance and closely associated with maize banded leaf and sheath blight.

Of the characterized PRs currently known, PR-1, PR-2, PR-3, and PR-4 showed antimicrobial activity *in vitro*. PR-1 protein exhibited strong differentiation-inhibiting activity towards *Uromyces fabae* infection structures (Rauscher et al., 1999), and PR-2 and PR-3 exhibit β-1, 3-glucanase and chitinase activities, respectively (Mauch et al., 1988; Ji and Kuć, 1996). *O. sativa* L pathogenesis-related protein 4b (OsPR-4b) expressed in *E. coli* exhibited antifungal activity *in vitro* against the sheath blight fungus (Zhu et al., 2006).

This study shows that ZmPR5 exhibited antimicrobial activity against *Rhizoctonia* fungus *in vitro*, and anti-fungal properties were enhanced as protein concentration increased. PR5 protein may cause cell per-

meability changes in fungal cells through destruction of pathogen cell membranes as reported previously (Vigers et al., 1992). This study describes for the first time a *PR5* gene in maize and the antifungal activity of PR5 protein. Further elucidation of the relationship between ZmPR5 and maize resistance is needed and shall be carried out in the future.
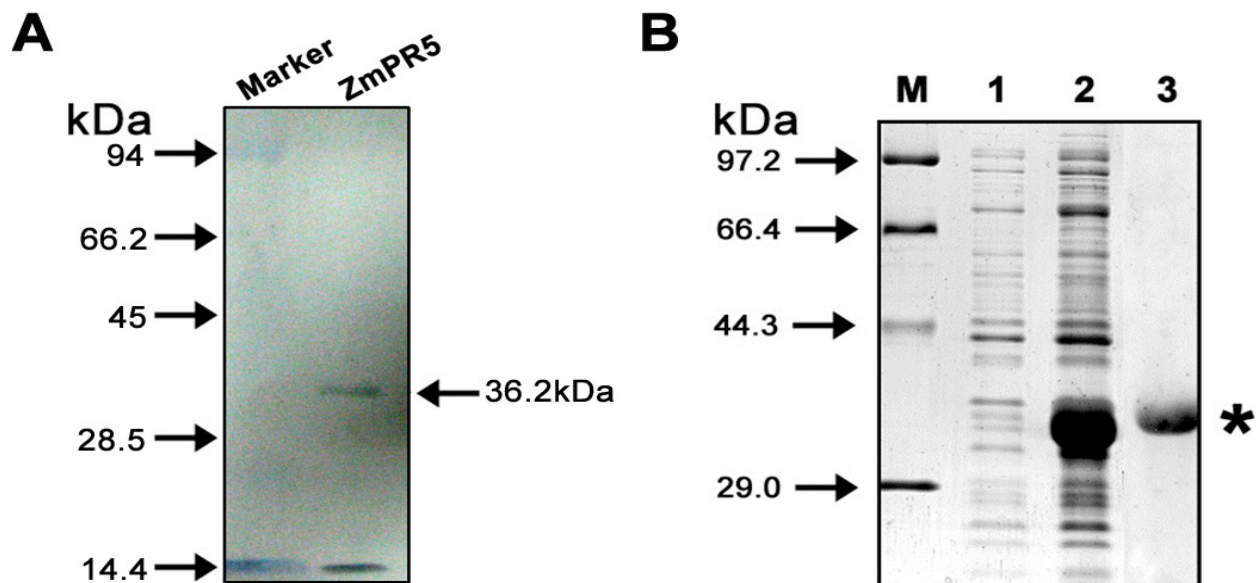
ACKNOWLEDGEMENTS

Figure 4. Western blot and SDS-PAGE of recombinant thioredoxin-ZmPR5 fusion protein. A, Western blot analysis; B, SDS-PAGE of the recombinant thioredoxin-ZmPR5 fusion protein purified after expression in *E. coli.* Lane M, Molecular markers; lane 1, non-induced; lane 2, induced with 0.6 mM IPTG; lane 3, recombinant thioredoxin-ZmPR5 fusion protein purified.
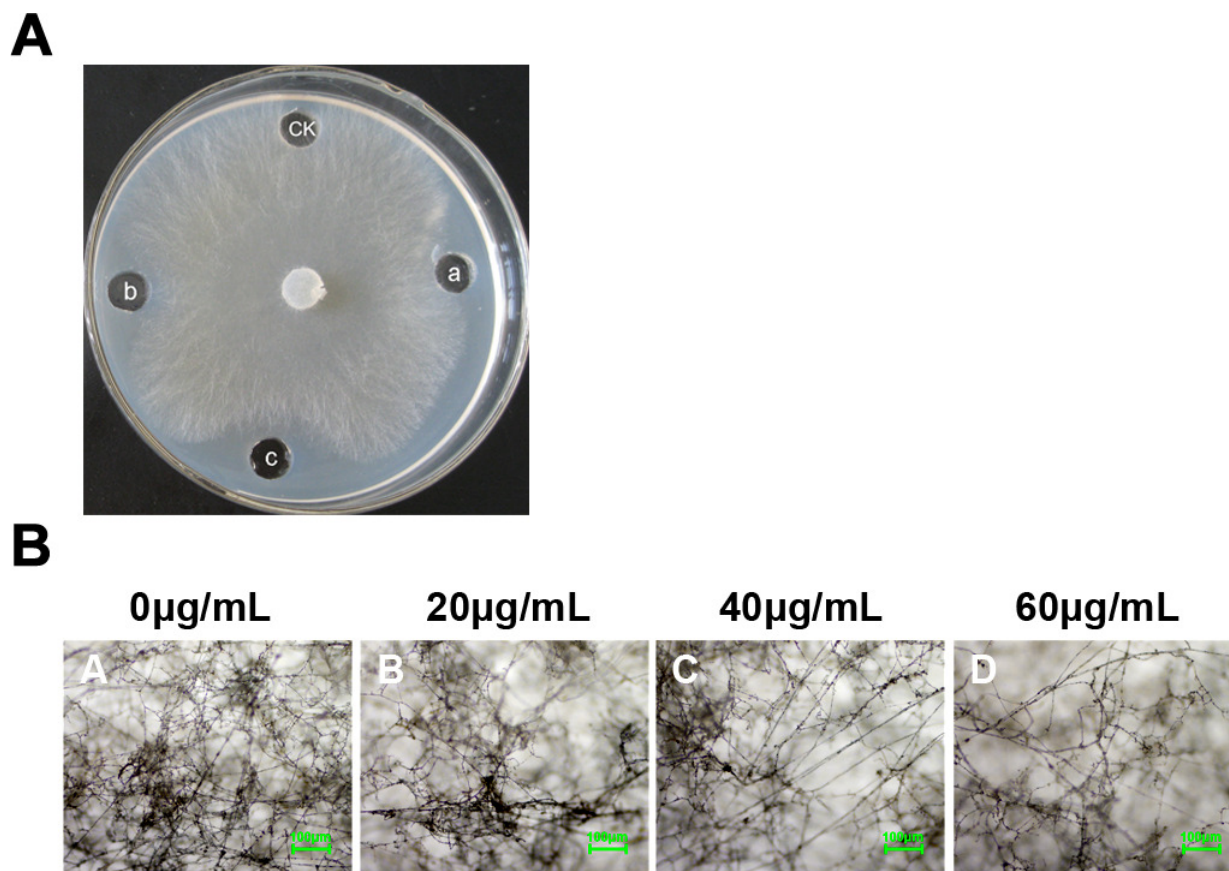

Figure 5. Antifungal bioassay of ZmPR5 to *Rhizoctonia solani in vitro*. A, antifungal activity of ZmPR5 protein in culture dish; CK, 1×PBS buffer. a, 20 µg protein; b, 40 µg protein; c, 60 µg protein. B, antifungal activity of ZmPR5 protein under a microscope. Controls were treated with PBS buffer. Bars = 100 µm.

and helped improve the manuscript.

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