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Molecular cloning and characterization of a PR-5 like protein gene from *Brassica campestris* ssp. *chinensis*

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Abstract

Downy mildew caused by *Hyaloperonospora parasitica* is a serious fungal disease in non-heading Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* Makino). Pathogenesis-related 5 (*PR-5*) genes play an important role in plant resistance to disease invasion. In this study, a gene encoding pathogenesis-related 5-like (PR-5L) protein, named *BcPR-5L*, was successfully cloned from non-heading Chinese cabbage. The cDNA sequence of *BcPR-5L* was 747 bp in length. It encoded a protein of molecular mass of 25.78 kDa, an isoelectric point of 4.42, and containing 248 amino acids. Multiple sequence alignment indicated that BcPR-5L protein was highly homologous to other PR-5L proteins identified in 13 different species, with the highest homology to *Brassica rapa*. We analyzed the subcellular localization of BcPR-5L protein by using onion epidermal cells and found that it was localized in the membrane. Real time quantitative PCR analyses revealed that the expression of *BcPR-5L* gene was significantly upregulated after *H. parasitica* infection, and the expression in the resistant cultivar was higher than that in the susceptible cultivar. In summary, our data suggest that *BcPR-5L* gene may play an important role in the resistance of non-heading Chinese cabbage to *H. parasitica* infection.

Additional key words: *Hyaloperonospora parasitica*, non-heading Chinese cabbage, onion, subcellular localization.

Introduction

Downy mildew is one of the most destructive diseases that interfere with the processes of normal growth and development of non-heading Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* Makino) (Liu *et al*. 1997). Downy mildew is caused by the obligate parasite *Peronospora parasitica* (Yerkes and Shaw 1959), but then the Brassica-infecting genus was renamed *Hyaloperonospora parasitica* (Ovidiu and Jamshid 2002). Leaves are the most susceptible parts. At the beginning of the disease, they exhibit pale yellow rounded or polygonal lesions. Then, the leaves with lesions become yellow and brown, and eventually die (Jensen *et al*. 2010). Under appropriate environmental conditions, the rate of repeated infection with *H. parasitica* is high, and the number of damaged plants increases year by year, making the disease a serious limiting factor affecting the stability and yield of many *Brassica* species used as vegetables (Brophy and Laing 1992). To reduce the occurrence of the disease, long-term use of pesticides has been applied, which not only led to pollution of the environment and destruction of ecological balance, but also induced the emergence of pesticide resistance (Vishunavat *et al*. 1998). Therefore, the cultivation of resistant non-heading Chinese cabbage cultivars is the most effective way to prevent *H. parasitica* infections.

Proteins of the PR-5 family, also named thaumatin-like proteins (TLPs), have been detected in a wide variety of plant species, such as *Triticum aestivum* (Fierens *et al*. 2007), *Zea mays* (Perri *et al*. 2011), *Theobroma cacao* (Franco *et al*. 2015), *Manilkara zapota* (Ashok Kumar and Venkatesh 2014), *Nicotiana sanderae* (Pierpoint *et al*. 1992), *Arabidopsis thaliana* (Parker *et al*. 1997), and *Oryza sativa* (Datta *et al*. 1999). PR-5 is a small protein having only 10 conserved cysteine residues (Wang *et al*. 2010). These residues can alter or activate certain proteins that maintain cell integrity, prevent further damage to other organelles, and improve plant resistance to fungal infections (Frendo *et al*. 1992, Shi 2002). So far,

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Abbreviations: AJH - *Brassica campestris* ssp. *chinensis* inbred line Aijiaohuang, GFP - green fluorescent protein; PR-5L - pathogenesis-related 5-like, SZQ - *Brassica campestris* ssp. *chinensis* inbred line Suzhouqing.
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many PR-5 proteins have been identified and differentiated in monocotyledonous and dicotyledonous plants based on their structures, serological relationships, and biological activities (Christensen *et al*. 2010). They have different functions associated with cell signal transduction and antimicrobial activities. Several members of this family display significant *in vitro* inhibitory activity on hyphal growth or spore germination of various fungi probably by changes in membrane permeability (Selitrennikoff 2001).

In a previous study (Xiao *et al*. 2016), we used complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) technique to analyze the expressions of different genes after inoculation of non-heading Chinese cabbage with *H. parasitica*. Among them, we selected one gene for resistance to *H. parasitica*, the bioinformatics analysis of which showed that it belongs to the pathogenesis-related 5-like protein gene family. Thus, we cloned it and named it *BcPR-5L*. The aim of this study was to analyze the PR-5L protein sequences in different species. Further, we studied the expression of *BcPR-5L* after inoculation of non-heading Chinese cabbage with spores of *H. parasitica* by real time quantitative PCR and finally the subcellular localization of the BcPR-5L protein in the onion cells.

Materials and methods

Plants and treatments: The *Brassica campestris* ssp. *chinensis* inbred lines Suzhouqing (SZQ, disease-resistant) and Aijiaohuang (AJH, susceptible) were used as experimental materials. The spores of *Hyaloperonospora parasitica* were collected from diseased leaves according to Sun *et al*. (2014), 1/10 volume of dimethylsulphoxide (DMSO) and 1/20 of *Difco Skim Milk* were added, and the mixture was stored at -80 °C before used. At the fourth-leaf stage of development of SZQ and AJH plants, conidia were sprayed on the upper surface of the leaves (about 50 mm^3 per leaf); leaves sprayed with sterile water served as control (Liu *et al.* 1997). In total, 20 true leaves of 20 plants were taken at 24, 48, 72, and 96 h after inoculation and stored at -80 °C before used.

Isolation of RNA and cDNA synthesis: RNA extraction was performed using the *RNase Free DNase I* kit (*TaKaRa*, Dalian, China), and DNA was removed using the RNA clean kit (*Tiangen*, Beijing, China). The concentration, purity, and completeness of RNA were determined by a nucleic acid detector, and by 1.0 % (m/v) agarose gel electrophoresis. The synthesis of cDNA was carried out according to the instructions provided in the RNA PCR kit (*AMV Ver.2.1*; *TaKaRa*).

Molecular cloning of *BcPR-5L* gene: The full-length cDNA sequence of *BcPR-5L* was isolated by homologous cloning, and submitted to *NCBI* GenBank with the accession number of MG686084. The primers are in the Table 1 Suppl. The PCR mixture (20 mm^3) included 1 mm^3 of each primer, 1 mm^3 of the template, 0.2 mm^3 of *ExTaq* DNA polymerase (5 U mm^{-3}), 2 mm^3 of 10× *Ex Taq* buffer (Mg^{2+} free), 2 mm^3 of *dNTP* mixture (each 2.5 mM), 2 mm^3 of $MgCl_2$ (25 mM), and 10.8 mm^3 of distilled H_2O. Pre-denaturation was performed at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, at 54 °C for 30 s, and at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplified product was electrophoresed in 1.2 % (m/v) agarose gel. The DNA fragments were recovered using the gel extraction kit (*CoWin Biosciences*, Beijing, China) and then ligated to the PMD18-T vector (*TaKaRa*). The ligation products were transformed into *Escherichia coli* (strain DH-5α) cultivated at 37 °C on Luria-Bertani (LB) medium containing ampicillin. After 12 h, single colonies were picked and sequenced successfully by *TSINGKE*, Beijing, China.

Sequence analysis and homology comparison of *BcPR-5L*: The *SIGNALP 4.0* server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. Homologous sequences were aligned using *BLAST* (http://www.ncbi.nlm.nib.gov/blast). The *DNAMAN6.0* software was utilized to calculate the theoretical isoelectric point and protein molecular mass, and perform multiple alignment analysis. The *MEGA5* software (neighbor-joining method) was employed to construct a phylogenetic tree, and the correction parameter Bootstrap was repeated 1 000 times. The conserved motif was analyzed online *via* the *MEME* website (http://meme-suite.org/tools/meme), with the following parameter set: the site distribution was zero or one occurrence per sequence; the number of motifs selected was 10. Structural predictions were obtained with the *Phyre2* application (http://www.sbg.bio.ic.ac.uk/phyre2/).

Expression pattern of *BcPR-5L*: Real time qPCR was conducted using a *7500* real-time PCR system (*Applied Biosystems*). Specific primers of *BcPR-5L* and *β-actin* were designed using the *Primer Premier 5.0* software (Table 1 Suppl.). The *SYBR Premix Ex Taq*™ kit from *TaKaRa* was used for the real time qPCR reactions. The $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) was used to calculate relative gene expression. The differences among samples were analyzed by one-way *ANOVA* using the LSD test at a significance level of $P < 0.05$.

Subcellular localization: The *pEZS-NL-GFP* (*Biovector NTCC*, Beijing, China) was utilized as an expression vector. Specific primers contained 21 bp sequences that are homologous to the ends of *pEZS-NL-GFP* (Table 1

Suppl.). The empty expression vector was cleaved with *EcoRI* and *KpnI*. Fusion cloning reaction was performed using the *Quick-Fusion Cloning* kit (*Selleck*, Shanghai, China). The preparation of the micro-bullets was performed according to the method of Yang *et al*. (2011). Fresh inner skin of the onion (*Allium cepa* L.) was peeled and placed on MS medium. The epidermis was put upwards and closely contacted with the medium. The expression and control plasmids were transformed into onion epidermal cells by gene marking (1 000 psi). After bombardment with gene gun (*PDS-1000/He*, *Bio-Rad*, California, USA), the onion epidermal cells were incubated at 25 °C for 16 h, and the results were observed with confocal laser microscope under white radiation in the bright field or observed in the blue radiation (488 nm) with fluorescence microscope (*LSM780*, *Carl Zeiss*, Jena, Germany). Finally, the onion epidermes were placed in 300 g dm^{-3} sucrose solution for 10 min to observe the fluorescence after plasmolysis.

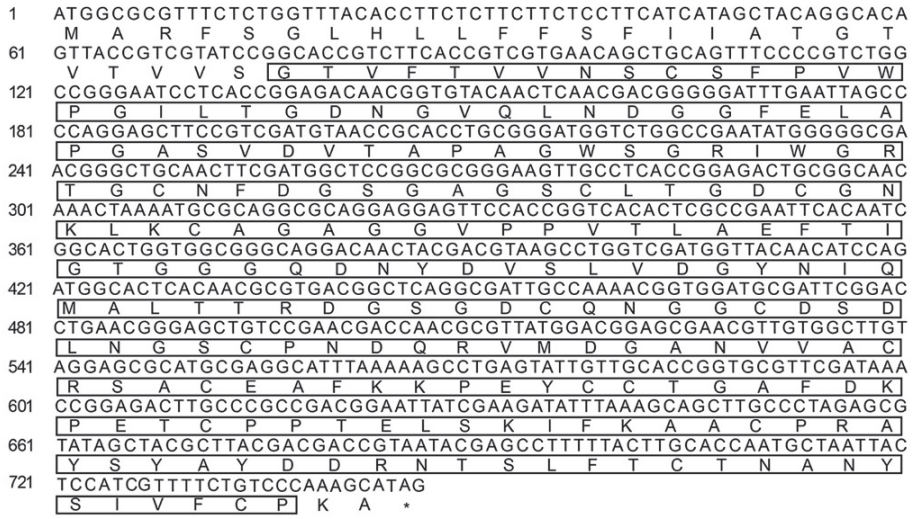

Fig. 1. Nucleotide sequence and the deduced amino acid sequences of BcPR-5L. The thaumatin family domain is indicated with a *box*.

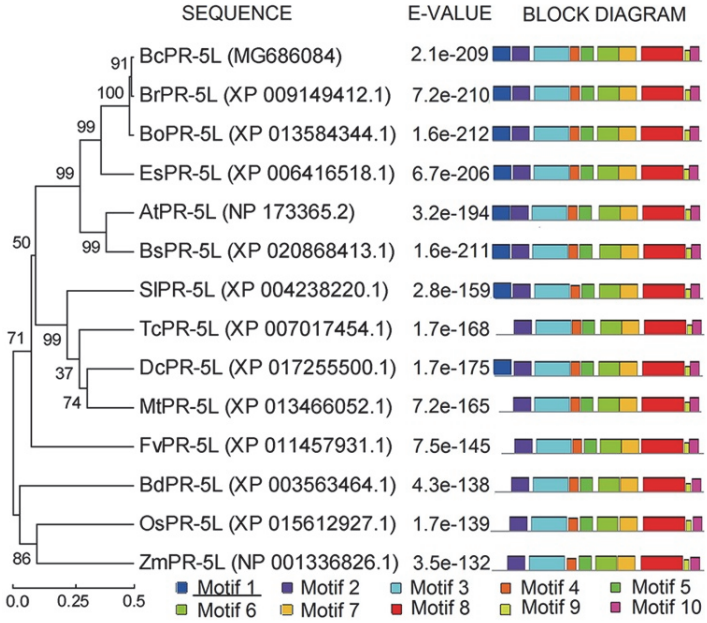

Fig. 2. Neighbor-joining phylogenetic tree of *PR-5L* genes in different plants species. Branches are labelled with plant species names (*Br - Brassica rapa*; *Bo - Brassica oleracea*; *Es - Eutrema salsugineum*; *At - Arabidopsis thaliana*; *Bs - Boechera stricta*; *Sl - Solanum lycopersicum*; *Mt - Medicago truncatula*; *Tc - Theobroma cacao*; *Dc - Daucus carota*; *Fv - Fragaria vesca;Bd - Brachypodium distachyon*; *Os - Oryza sativa*; *Zm - Zea mays*), followed by the conserved motifs of BcPR-5L protein.

Results

The *BcPR-5L* gene was isolated and cloned from non-heading Chinese cabbage (acc. No. MG686084). It was 747 bp in length and encoded protein with 248 amino-acid residues and the thaumatin family domain (Fig. 1). Analysis of physicochemical properties revealed a protein molecular mass of 25.78 kDa and an isoelectric point of 4.42. The schematic illustration of the 3-D structure for the BcPR-5L protein is shown in Fig. 1 Suppl. The protein contained disordered structure (23 %), alpha helix (16 %), beta strand (22 %) and TM helix (6 %) (Fig. 2 Suppl.).

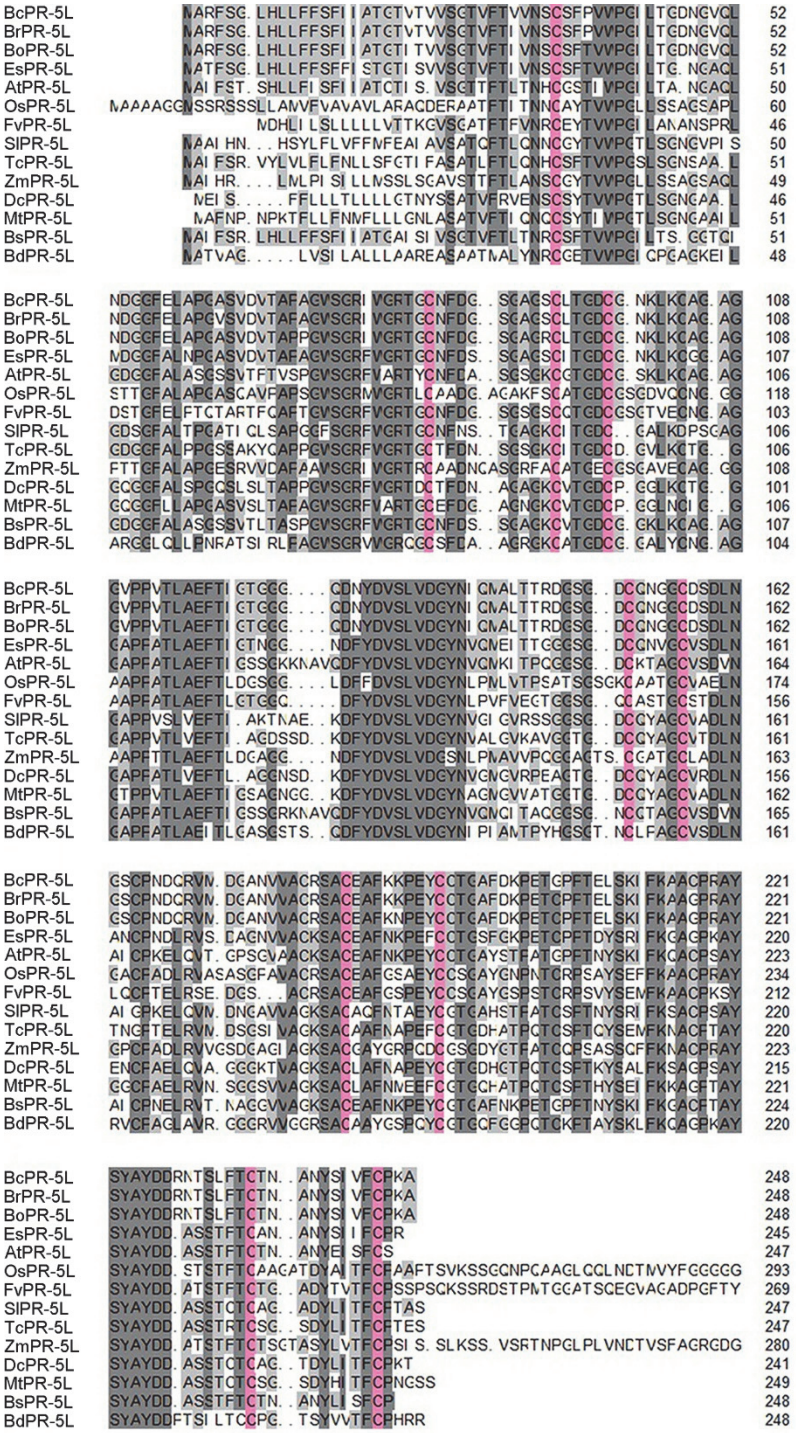

Fig. 3. Multiple sequences alignment of BcPR-5L protein and other homologous PR-5L proteins. Ten conserved cysteine residues are indicated with *lilac colour*.

The phylogenetic tree showed that BcPR-5L had a close relationship with BrPR-5L (98 %) from *Brassica rapa*, and its homology with other species was also high. All PR-5L species had 10 motifs, and the motif MAIFSGLHLLFFSFIIATGTI in the N-terminal (*SignalP 3.0* software) predicted that it was a signal peptide (Fig. 2). Using *DNAMAN6.0* multiple sequence alignment, 10 highly conserved cysteine residues were detected (Fig. 3).

To establish the localization of BcPR-5L protein, a fusion expression vector, *pEZS-NL-GFP-BcPR-5L*, was constructed and introduced into onion epidermal cells using a gene gun. An empty *pEZS-NL-GFP* vector was utilized as a control. After incubation for 16 h in the dark, green fluorescent protein (GFP) was detected by confocal laser microscopy. In the control, GFP fluorescence was detected not only along the cell membrane, but also in the nucleus and inside the cytosol (Fig. 4*A-C*), however, the BcPR-5L-GFP fluorescence was found only in the cell membrane (Fig. 4*D-F*); its locakization was further confirmed after plasmolysis (Fig. 4*G-I*).

Leaves are the most susceptible organs to *H. parasitica* infection. After infection with *H. parasitica*, the leaves of AJH began to turn yellow, point-like and discontinuous necrosis appeared; the plants eventually wilted. By comparing SZQ and AJH after inoculation, we found that SZQ was more resistant than AJH at the same time (Fig. 5). The expression pattern of *BcPR-5L* gene in the leaves was analyzed by real time qPCR. The results showed that the expression of *BcPR-5L* gene in SZQ initially increased, reaching maximum at 48 h and decreased at 72 h. In AJH, a similar expression pattern was observed, a peak also appeared at 48 h. But the overall *BcPR-5L* gene expression was lower than that in SZQ (Fig. 6). Therefore, we believe that expression of the *BcPR-5L* gene was positively correlated with the cultivar resistance to *H. parasitica* infection.

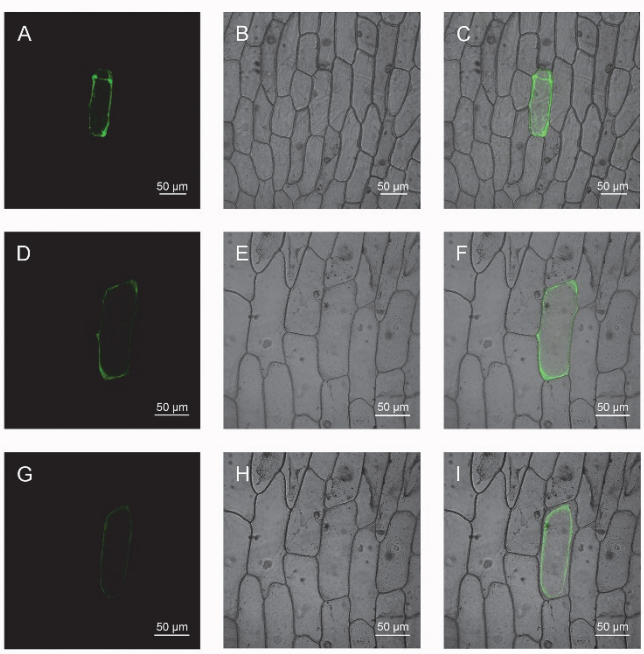

Fig. 4. Subcellular localization of the BcPR-5L protein. Green fluorescent protein fluorescence of control GFP (*A-C*), BcPR-5L-GFP (*D-F*), and after plasmolysis (*G-I*). *C*, *F*, *I* are merged images of *A+B*, *D+E,* and *G+H*, respectively.

Discussion

Batalia *et al*. (1996) reported that PR-5 proteins have electrostatically polarized surface which is considered to be the binding site of the fungal plasma membrane receptor and can be the key factor determining antifungal activity. In our study, we cloned *BcPR-5L* from non-heading Chinese cabbage. Analysis of amino-acid sequence alignment and neighbor-joining phylogenetic tree showed that BcPR-5L protein was highly homologous to BrPR-5L and had also high homology with other proteins, indicating that PR-5L protein was highly conserved in the evolution of different species. The BcPR-5L protein contained 10 conserved cysteine residues, which play an important role in reducing enzyme digestion caused by pathogens and in inhibiting fungal infection. The sulfhydryl groups in the cysteine residues tend to form disulfide bonds, which plays a very important role in maintaining the structure and function of the protein (Frendo *et al*. 1992, Shi 2002).

In this study, *BcPR-5L* gene expression was relatively low at 24 h, which implies that *BcPR-5L* gene did not function at earlier periods of the infection (Wang *et al*. 2010). The increase of the expression of *BcPR-5L* gene at 48 h suggests that the *BcPR-5L* gene might be involved in the defense reaction after the hypersensitive response (Kang *et al.* 2002). The decrease in *BcPR-5L* gene expression at 72 h may be due to the destruction of the defense system. The lowest expression of the *BcPR-5L* gene at 96 h was due to leaf death. The cDNA sequences of *BcPR-5L* in SZQ and AJH were the same, but the overall expression of *BcPR-5L* gene in SZQ was higher than that in AJH, which might be the reason why SZQ is resistant to the infection. The higher expression in the resistant cultivar SZQ might be the result of different transcription regulation efficiency in the resistant and susceptible cultivars. So, future work will be focused on the difference in the transcription regulation of the gene in both cultivars.

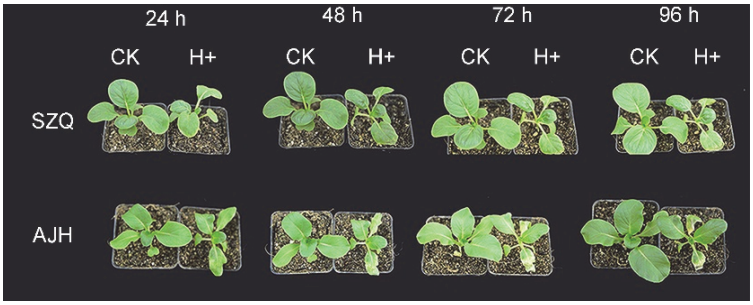

Fig. 5. Seedlings of SZQ and AJH at 24 h, 48 h, 72 h, and 96 h after inoculation with *Hyaloperenospora parasitica*.

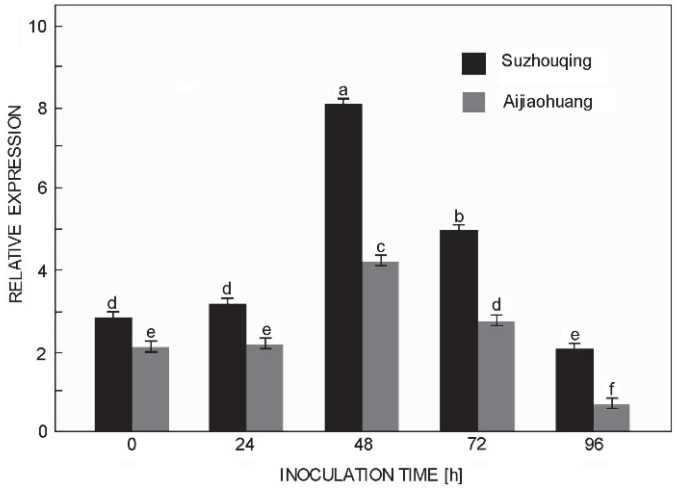

Fig. 6. Real time qPCR analysis of the expression profile of *BcPR-5L* in SZQ and AJH leaves after inoculation with *H. parasitica*. Means ± SDs, $n = 3$. Statistically significant differences at $P < 0.05$ are indicated with different letters.

Many PR-5 members, such as *TaPR5* (Wang *et al*. 2010) and *HvPR-5c* (Osmond *et al*. 2010), have been shown to possess defensive antifungal activity through interference with plasma membrane permeability. In the present study, the transient expression assay with the onion epidermal cells confirmed that BcPR-5L was a membrane protein (Fig. 4). The result might also indicate that BcPR-5L, similarly to other PR-5 family members, plays a substantial role in the resistance of the cell membrane to the invasion of *H. parasitica*.

The specific function of *BcPR-5L* gene in non-heading Chinese cabbage has not been completely elucidated. However, the studies of Bormann *et al.* (1999) revealed that the binding of PR-5 proteins to nascent (1,3)-β-D-glucans prevented proper fungal growth. In addition, Velazhahan and Muthukrishnan (2003) reported that transgenic tobacco overexpressing a PR-5 protein has an enhanced plant resistance to *Alternaria alternata*. Furthermore, overexpression of PR-5 proteins from *Arabidopsis thaliana* was inhibitory to *Verticillium dahliae* (Hu *et al*. 1997). Thus, *BcPR-5L* could be considered a potential target gene in non-heading Chinese cabbage for resistance against *H. parasitica* infection. In our future studies, we will use transgenic methodology to verify the *BcPR-5L* gene function and study the signal pathway of *BcPR-5L* gene in the resistance of non-heading Chinese cabbage to downy mildew.

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