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Enhanced expression of a thaumatin-like gene, involved in *Pseudoperonospora cubensis* and abiotic stresses, induced by DNA introgression from a wild relative, *Cucumis hystrix*

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Abstract

Introgression of alien DNA from wild relatives into cultivated plant genomes by means of interspecific hybridization is a method which has been widely used in breeding. However, the effects of introgression on the patterns of gene transcription of the host genome have rarely been studied. In the present study, in order to understand the molecular defense response of an introgressed line (IL5211S) with introgression from a wild cucumber, *Cucumis hystrix*, a thaumatin-like gene, referred to as *CsPR5*, was successfully isolated from IL5211S. The gene was 1504 bp in length with a putative open reading frame of 726 bp, encoding 241 amino acid residues. Open reading frame sequences of *CsPR5* from IL5211S and backcross parents were identified. It was also observed that the expression of the *CsPR5* gene was enhanced remarkably by DNA introgression of *C. hystrix* using real-time quantitative PCR (RT-qPCR). In addition, the expressions of *CsPR5* in response to *Pseudoperonospora cubensis* and four different abiotic stresses stimuli (namely salicylic acid, methyl jasmonate, abscisic acid, and hydrogen peroxide) were further analyzed at different time points. These stimuli triggered a significant induction of *CsPR5* within 72 h after treatment. These findings indicate that the expression of the *CsPR5* gene was enhanced by alien DNA introgression from *C. hystrix*. This may play a role in the molecular defense of IL5211S against pathogen invasion, and aid in protecting against environmental stresses.

Keyword: abiotic stress; cucumber; introgression line; PR5; qRT-PCR.
Abbreviations: ORF_open reading frame; SA_salicylic acid; MeJA_methyl jasmonate; ABA_abscisic acid; H_2O_2_hydrogen peroxide.

Introduction

Pathogen invasions and environmental stresses are the most limiting factors of agricultural productivity (Dubouzet et al., 2011; Mao et al., 2012). In addition to biotic stresses caused by plant pathogens or insects, a number of abiotic stresses also exist, such as extreme temperature, drought, heat, salinity, heavy metals, oxidation, and radiation, all of which have detrimental effects on crop growth and yield (Mao et al., 2012). However, certain plant species and ecotypes have developed various mechanisms to adapt to such stress conditions, among which one of the most important mechanisms is the accumulation of pathogenesis-related (PR) proteins (Datta et al., 1999; Stintzi et al., 1993). Based on structure and functional properties, thus far as many as 17 families of PR proteins have been identified in mono- and dicotyledonous plants (Christensen et al., 2002; Kitajima and Sato, 1999; Selitrennikoff, 2001; van Loon et al., 2006). The proteins of the PR-5 family share significant amino acid sequence homology with the sweet tasting protein in the fruits of the tropical plant *Thaumatococcus daniellii*, and have thus been named thaumatin-like proteins (TLPs) (Cornelissen et al., 1986). At present, TLPs may be subcategorized into either acidic, extracellular proteins, which are characterized by the presence of an N-terminal signal peptide, or basic proteins, with additional C-terminal extension targeted to vacuoles (Piggott et al., 2004). Previous studies have shown that some TLPs exhibit anti-fungal activity against many fungal pathogens in that they are able to degrade fungal cell walls (Zareie et al., 2002). For example, one PR5 protein which is generated during osmotic stress in tobacco exhibits antifungal activity against many fungi (Abad et al., 1996). Studies have demonstrated that the TLPs isolated from *Castanea sativa* and *Castanopsis chinensis* exhibit antifungal activity against *Trichoderma viride* and *Fusarium oxysporum* (Garcia-casado et al., 2000), as well as *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, and *Physalospora piricola* (Chu and Ng, 2003). It has also been shown that the TLP isolated from *Solanum nigrum* exhibits antifungal activity toward *Fusarium solani* f. sp.glycines, *Colletotrichum* spp. and *Macrophomina phaseolina* and *Phytophthora nicotiana* var. parasitica under in vitro conditions (Campos et al., 2008). In addition, over-expression of PR5 proteins in transgenic tobacco and potato enhanced resistance to *Phytophthora parasitica* var. *nicotianae* and *Macrophomina phaseolina* infection have been shown to delay the development of disease symptoms after fungal infection (Liu et al., 2012; Acharya et al., 2012). In addition to antifungal activities, it has also been demonstrated that plant TLPs have many other functions, including roles in development (Colas et al., 2012; Neale et al., 1990; Salzman et al., 1998) and freezing tolerance (Newton and Duman, 2000; Zhu et al., 1993). For example, two apoplastic TLPs and other PR proteins (i.e. glucanases and chitinases) have been bound directly to ice crystals via hydrophobic interaction, and were

shown to modify the freezing process by inhibiting intercellular ice growth and recrystallization in plants (Griffith et al., 2005). A beetle (*Dendroides canadensis*) TLP (TLP-Dcan1) was found to significantly enhance the thermal hysteresis of anti-freeze proteins (AFPs) through interaction with these proteins, despite the fact that TLP-Dcan1 itself lacks AFP activity (Wang and Duman, 2006). In addition, it has also been demonstrated that plant TLPs play important roles in protecting against osmotic stress (Zhu et al., 1995). Some studies have also reported that over-expression of the tobacco osmotin gene also improves plant tolerance to salinity and drought stresses in transgenic tobacco (Barthakur et al., 2001), salt tolerance in transgenic strawberry (Husaini and Abdin, 2008), and drought tolerance in transgenic cotton (Parkhi et al., 2009). Cucumber (*Cucumis sativus*. L.) is one of the most widely used vegetable crops worldwide. In the laboratory used for the present study, based on successful interspecific hybridization (Chen et al., 1997), massive interspecific hybridization introgression lines and several special germplasm resources, such as allotetraploid (Chen and Kirkbride, 2000), allotriploid (Chen et al., 2003), and monosomic alien addition lines (Chen et al., 2004), have been developed by means of backcrossing between interspecific hybrids and cultivated cucumbers, the phenotypic traits of which showed extensive segregation. Recently, the authors also identified an introgression line (2n=14) from the progenies of interspecific hybridization of *Cucumis* species (IL5211S), which is highly resistant to downy mildew (Wan et al., 2010a). Introgression refers to the stable integration of genetic material from one species into another by means of repeated back-crossing (Baack and Rieseberg, 2007). In plant breeding programs, the breeder typically pays close attention to the desired genes (traits) originating from the alien donor species, if they have been transferred to the recipient through introgressive hybridization. On the other hand, in many cases little emphasis is placed on the effects of alien DNA introgression on the changes of the recipient genome, including chromosomal rearrangements, genome expansion, differential gene expression, gene silencing and epigenetic genetic (e.g. DNA methylation patterns) (Adams and Wendel, 2005). In this study, in order to examine the effects of alien DNA introgression from the wild relative, *C. hystrix* on expression of *CsPR5* gene, the *CsPR5* gene was isolated and characterized, by encoding a protein homologous to the TLPs of the PR5 protein family from IL5211S. Real-time PCR analysis showed that the introgression of *C. hystrix* enhanced the expression level of *CsPR5* from *C. sativus.* The responses of this TLP gene to salicylic acid (SA), abscisic acid (ABA), hydrogen peroxide (H_2O_2), and methyl jasmonic acid (MeJA) were then observed, all of which have previously been identified as signal molecules implicated in plant response to abiotic stresses and pathogen invasion (Skriver and Mundy, 1990; Gaffney et al., 1993).

Results

Isolation and sequence analysis of CsPR5 from IL5211S

Using degenerate primers PR-5S and PR-5AS, a band with a predicted size of ~530 bp was produced by PCR amplification from introgression line IL5211S. The band was recovered, cloned and sequenced. A BLASTx search in GenBank of this sequence revealed that this cloned fragment was part of the sequence encoding PR5 gene. Through the genome walking procedure, the entire length of a putative homologue *PR5* gene, *CsPR5*, was successfully isolated from IL5211S. This *CsPR5* gene was 1504 bp in length, and contained a putative open reading frame (ORF) of 726 bp, encoding 241 amino acid residues and a 687 bp nucleotide upstream sequence with a 91 bp nucleotide downstream sequence (Fig. 1).

Sequence alignment and phylogenetic analysis

A GenBank BLASTP search conducted with the putative protein sequences revealed high similarity with other identified TLPs. *CsPR5* showed the highest sequence homology with black pepper (*Piper colubrinum*) osmotin protein (GenBank accession ABX71220), with 68% identity and 78% similarity. In addition, the *CsPR5* gene was also shown to share a relatively high homology with TLPs and related antifungal PR-5 proteins, such as osmotins and zeamatin from tobacco (*Nicofiana tabacum*), cotton (*Gossypium spp*.), strawberry (*Fragaria* × *ananassa*), grapevine (*Vitis raparia*), and maize (*Zea mays*) (Table 1). Based on the *CsPR5* gene sequence in IL5211S, its homologs were isolated from two parents, namely *C. hystrix* accession HH and *C. sativus* cultivar Beijingjietou. Additionally, *CsPR5* homolog gene (Csa013260) was obtained from the cucumber genome database (http://cucumber.genomics.org.cn/page/cucumber/index.jsp). Sequence alignments showed that the *CsPR5* gene sequences from IL5211S, CC3 and Csa013260 were identical. During comparison with the *CsPR5* homologue gene from *C. hystrix*, it was found that five amino acid mutations existed, indicating that this gene was isolated from IL5211S and not derived from the backcross parent CC3. The deduced amino acid sequences of the *CsPR5* gene and TLPs from other species were further aligned using DNAMAN6.0 software. Multiple alignments of amino acid sequences revealed that five amino acid residues (i.e. Arg^{85}, Glu^{121}, Asp^{134}, Asp^{139}, and Asp^{222}), reported to be involved in formation of an acidic cleft in the three-dimensional structure of zeamatin, were conserved in *CsPR5*, as shown in Fig. 2. This suggests that these amino acids may play a similar role in the formation of eight potential disulfide bonds. In addition, the *CsPR5* protein was also shown to consist of 16 invariant cysteine residues, which stabilized the three-dimensional structures of the PR5 proteins (Batalia et al., 1996; Nelson et al., 1992; Hu and Reddy, 1997). In order to analyze the phylogenetic relationship between *CsPR5* and TLPs from other plants, a neighbor-joining (NJ) phylogenetic tree was constructed based on amino acid sequences of *CsPR5* and those from 13 other representative plants (Fig. 3), and the results showed that these TLPs were separated into five clades. Among them, *CsPR5* was most closely related to *Vitis vinifera* TLP (AF003007), as they were clustered into the same clade, and was most distantly related to *Atriplex nummularia* TLP (M84467).

Differential expression levels of CsPR5 in different tissues of IL5211S

The expression patterns of the *CsPR5* gene in different tissues were performed using real-time PCR. As may be seen from Fig. 4, differential expression levels were found in the roots, stems and leaves of IL5211S. Of these tissues, the highest expression levels of *CsPR5* were observed in the leaves; a moderate level of *CsPR5* was observed in the stems; and the lowest levels occurred in the roots.

Effects of introgression from C. hystrix on the CsPR5 gene transcription levels

In order to investigate whether or not alien DNA introgression from *C. hystrix* induced the expression of the *CsPR5* gene, qRT-PCR was used to analyze the expression levels of the *CsPR5* gene among a wild relative (*C. hystrix*), a backcross

Table 1. Comparison of the deduced amino acid sequences of the *CsPR5*genes from IL5211S with the known related protein sequences from other species.

Species	Deduced amino acid sequences			Accession numbers	References
	Length	Identity (%)	Similarity (%)		
grapevine	201	64	75	AF178653	Unpublished
cotton	203	62	75	AY303690	Unpublished
maize	207	61	70	P33679	Malehorn et al., (1994)
tobacco	200	61	69	P14170	Nelson et al., (1992)
strawberry	201	60	74	DQ325524	Zhang and Shih (2007)

Table 2. List of primers used in this paper.

Name	Forward primer (5'-3')	Reverse primer (5'-3')
PR-5S/ PR-5AS	AACAAYTGYCCRTACACCGT	GGATCATCTTGWGGRTARCTATA
CsPR-5S$_1$/ CsPR-5AS$_1$	CAGGAGGAGGAAGCGTGAT	TGCCAAGTCTCCAAAACCTC
CsPR-5S$_2$/ CsPR-5AS$_2$	CCCGCACAACAGGAAGAATC	AGACTTGGCAGGGGTTGTTG
HSP70-S/HSP70-AS	GTCAAAATACTGGGAAGATC	TTTGAGGTAGGAAGTGTAGT
EF1α-S/ EF1α-AS	ACTGTGCTGTCCTCATTATTG	AGGGTGAAAGCAAGAAGAGC

cultivation parent (CC3, i.e. 'Beijingjietou'), and an introgression line (IL5211S). From the results presented in Fig. 5, it is clear that the *CsPR5* gene exhibited significant changes in transcription levels. Among them, the expression level of the *CsPR5* gene was highest in IL5211S, which was about twice and three times higher than those in CC3 and HH, respectively.

Induction of the CsPR5 gene in response to P. cubensis

In order to understand the response of *CsPR5* to biotic stresses, IL5211S plants were treated using *P. cubensis*. The expression pattern of *CsPR5* at different time points after treatment is shown in Fig. 6. It was observed that a significant induction clearly occurred during the early stages after treatment. At 6 h after treatment, the expression level of the *CsPR5* gene increased to 15.4-fold. Then, the expression level continued to increase until the 48 h time point, reaching 26.5-fold at 12 h, 40.2-fold at 24 h and 56.9-fold at 48 h. After that, the expression level decreased, but was still 31.8-fold higher than that of *CsPR5* at 0 h.

Effects of SA, ABA, MeJA and H_2O_2 on the CsPR5 gene expression

The expression patterns of the *CsPR5* gene in response to abiotic stresses were examined using real-time PCR analysis. In this study, four different abiotic stimuli (i.e. MeJA, ABA, SA, and H_2O_2) were used to treat the IL5211S plants, and the results are shown in Fig.**7**. The figure shows the expression pattern of the *CsPR5* gene at the different time points in response to MeJA. It was observed that exogenous application of MeJA clearly caused a rapid induction of the *CsPR5* gene to 66.2-fold at 6 h post-treatment. The expression levels continued to increase until the 24 h time point, reaching 77.2-fold at 12 h and 85.4-fold and 24 h, and remained at approximately the same level (82.7-fold) at 48 h. The expression level then decreased to 60.5-fold at 72 h post-treatment. In response to the ABA treatment, two expression peaks of the *CsPR5* gene occurred at 72 h post-treatment. A significant induction (93.2-fold) of *CsPR5* was observed at 6 h post-treatment. The expression level then decreased to 53.4-fold at 12 h, increased to 83.6-fold at 24h, and peaked at 48 h post-treatment. Afterwards, the expression level decreased to 64.9-fold at 72 h post-treatment (Fig. 7B). During exposure to SA treatment, compared with the untreated plants, the expression level of the *CsPR5* gene continued to increase until the 48 h time point, reaching 1.4-fold at 6 h, 1.5-fold at 12 h, 2.2-fold at 24 h, and 3.1-fold at 48 h post-treatment. Then, the expression level decreased to 1.4-fold at 72 h, and remained at a similar level at 6 h post-treatment (Fig. 7C). The expression pattern of *CsPR5* in response to H_2O_2 at different time points is shown in Fig. 7D. It appeared that *CsPR5* was not regulated by H_2O_2 during the early stages after treatment. The expression level of the gene increased only to 1.4-fold at 6 h. The expression level then increased rapidly 29.3-fold at 12 h, and peaked at 24 h post-treatment (39.6-fold). Afterwards, the expression of the gene decreased steadily, 15.1-fold at 48 h, and 13.0-fold at 72 h post-treatment.

Discussion

Over the past decade, many studies have examined the genomic changes in introgressive hybridization progenies and synthesized polyploids, including the changes of DNA methylation and gene expression. Adams and Wendel (2005) observed that some crosses resulted in repeatable reciprocal tissue-specific expression of alternative alleles, and that these changes were not due to gene deletions. The results also document that repeatable changes in gene expression occur as a result of hybridization. Liu et al. (2004) found that marked changes in DNA methylation and transcription in several cellular genes and transposon (TE)-related segments occurred in two stable rice lines with introgression from wild rice (*Zizania latifolia*), compared with the rice parent. In the present study, a PR5 homologous gene of plant proteins, *CsPR5*, was successfully isolated from IL5211S. The results showed that alien DNA introgression from *C. hystrix* induced significant change of transcription of the *CsPR5* gene. In this study, it is shown that the presence of a high degree of identity and similarity between *CsPR5* and TLP from other species and conserved residues (five amino acid and sixteen cysteine residues) may ensure the normal function of the *CsPR5* protein from IL5211S. In addition, the phylogenetic relationship analysis based on amino acid sequences from *CsPR5* and TLPs demonstrated that among the tested plant species *CsPR5* was most closely related to *Vitis vinifera* TLP (AF003007), and most distantly related to *Atriplex nummularia* TLP (M84467). Therefore, it is predicted that *CsPR5* may also possess a similar role in antifungal activity and defense response against abiotic stresses. In addition, the expression level of *CsPR5* was significantly induced after infection with *P. cubensis*, suggesting an association between the activation of this gene and resistance to *P. cubensis*, and thus it is believed that *CsPR5* may be involved in *P. cubensis*-induced defense response. Moreover, the expression patterns of *CsPR5* in response to *P. cubensis* infection and exogenous SA treatment were observed

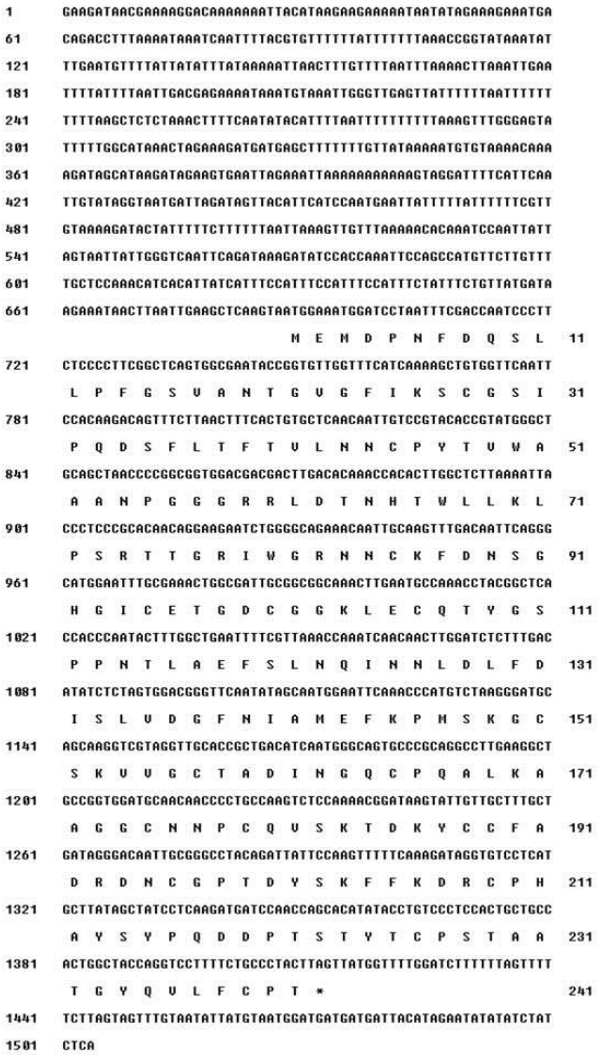

Fig 1. Nucleotide sequence and deduced protein sequence of the *CsPR5* gene (GenBank Accession No. GU208825). The start codon letters ATG are framed, and the stop codon letters TAG are marked by dots at the bottom. Numbers on the left represent nucleotide positions, while numbers on the right represent amino acid positions. Transcriptional regulatory signals are underlined. The predicted N-terminal signal sequence is showed by closed squares.

to be quite similar, which suggests that *CsPR5* may mainly be involved in the active defense response to *P. cubensis* through SA signaling pathways. In addition to pathogens, it is well known that SA plays a vital role in plant defense and is generally involved in the activation of defense responses against hemi-biotrophic and biotrophic pathogens, as well as the development of systemic acquired resistance (SAR) (Grant and Lamb, 2006). In Arabidopsis plants of previous experiments, the induction of PR5 gene increased to 20-fold within one day after SA treatment (Uknes et al., 1992). In strawberry plants, the induction level of the OLP gene, FaOLP2, increased to approximately 30-fold at 48 h after SA treatment (Zhang and Shih, 2007). In apple plants, the TLP gene, *Md*TL1*a*, was also shown to be induced by SA (Kim et al., 2003). In the current study, it was also observed that the exogenous application of SA triggered an induction of *CsPR5* and increased to its maximum, 3-fold, at 48 h after SA treatment (Fig. 7C). The results indicated that *CsPR5* could be an active member in SAR. In addition, it was further observed that despite the fact that *CsPR5* and *FaOLP2* belong to TLP genes, *CsPR5* was less induced by SA in IL5211S than *FaOLP2* in strawberry, which suggested that the degree of induction of the TLP genes in response to SA may vary among different plant species. Multiple studies have shown that the phytohormone ABA acts under conditions such as cold temperature, drought tolerance, and adaptive responses to environmental stresses (Wasilewskaa et al., 2008). In this study, the *CsPR5* gene was induced significantly by the exogenous application of ABA (Fig. 7B). Moreover, time-point analysis demonstrated that *CsPR5* was prominently enhanced at 6 h after ABA treatment, which is concordant with the previous experimental results for strawberry (Zhang and Shih, 2007). This suggests that *CsPR5* may also be involved in the response of cucumber to environmental stresses. However, a clear difference was shown in the expression patterns of TLPs from IL5211S and strawberry exposed to the same ABA. In this study, the induction of *CsPR5* reached its maximum at 6 h and 48 h after treatment. In strawberry, the induction peak of FaOLP2 occurred at only 6 h after treatment. The exact difference remains unclear. In recent years, in addition to ABA and SA, MeJA and H_2O_2 have also been considered as signal molecules used during plant defense response against pathogen invasions and environmental stresses (Lorenzo and Solano, 2005; Wasternack, 2007). Jasmonate insensitive 1/MYC2 (JIN1/MYC2) encodes a transcription factor involved in the transcriptional regulation of several JA responsive gene expressions (Lorenzo et al., 2004). In addition, some studies have reported that H_2O_2 may be involved in ABA induced defense gene (CAT1) expression (Xing et al., 2008). In the current study, the expression level of *CsPR5* was induced significantly by MeJA and H_2O_2, the respective peaks of which occurred at 72 and 24 h. This rapid enhancement of *CsPR5* expression in IL5211S by MeJA and H_2O_2, together with the identification of homologous MYB/MYC recognition sites, indicated that *CsPR5* may be involved in the defensive response of cucumber to environmental stresses.

Materials and methods

Plant materials, P. cubensis infection and hormone treatments

"IL5211S", an introgression line of *C. hystrix* / *C. sativus*, was studied. In the spring of 2009, seeds of IL5211S were germinated and grown in a growth chamber for 12 h with light at 25°C, and for 12 h without light at 18°C. The relative humidity (RH) was kept at 65-75%. For pathogen infection treatments, two true-leaf stage seedlings were treated with *P. cubensis*. The inoculation procedure was performed as follows: the second true-leaf from each seedling was inoculated with a single drop (approximately 0.05 mL) of inoculums, containing 1.2×10^5 sporangia per mL. The seedlings were then placed inside plastic boxes and incubated at 20°C with approximately 90% RH without light for 24 h, then placed in a growth chamber (24-30 °C) with a 16 h d^{-1} photoperiod and 65-75% RH.

Hormone and H_2O_2 treatments were performed as follows: seedlings at the second true-leaf stage were sprayed with solutions of salicylic acid (SA, 100μM), methyl jasmonic acid (MeJA, 100μM) or absciscic acid (ABA, 100μM), and sampled 3 h afterwards (Wan et al., 2010b). For the H_2O_2 treatment, seedlings were sprayed with H_2O_2 (10 μM) of sterile water. Control plants were sprayed with sterile water only.

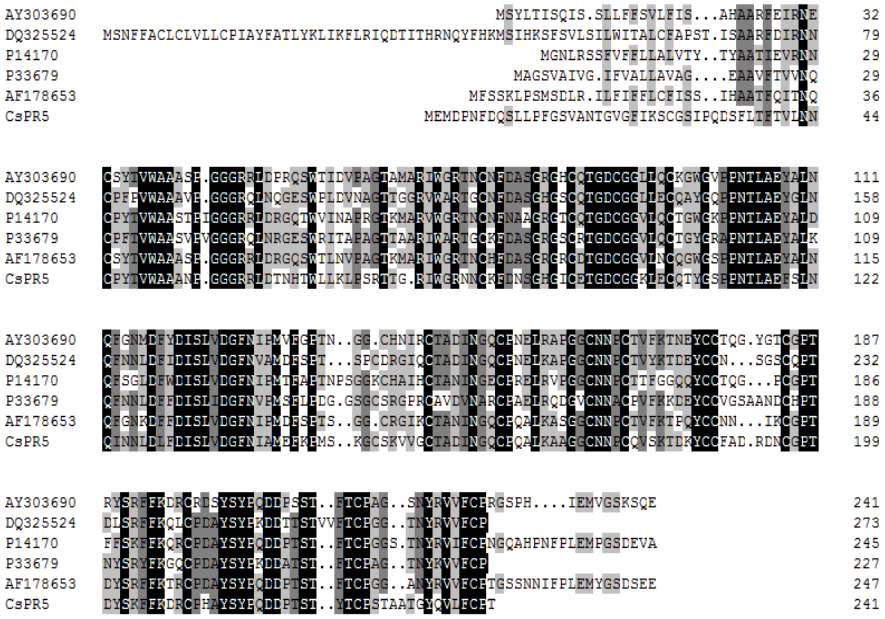

Fig 2. Amino acid sequence alignment of CsPR5 and related PR5 proteins from other species. Amino acids shaded in black indicate identical residues in all sequences in the alignment. The sequences listed represent CsPR5 (GenBank Accession No. GU208825), tobacco Osmotin (GenBank Accession No. P14170), maize zeamatin (GenBank Accession No. P33679), strawberry FaOLP2 (GenBank Accession No. DQ325524), grapevine Vrosmotin (GenBank Accession No. AF178653), and cotton OSMI (GenBank Accession No. AY303690). Circles indicate the 16 conserved cysteine residues. Arrows indicate the position of acidic side chains contained in the acidic cleft region of maize zeamatin.

DNA / RNA isolation and cDNA preparation

Leaves of IL5211S were harvested and immediately frozen in liquid nitrogen, then stored at -80°C. Genomic DNA was isolated using a commercial plant DNA extraction kit (Bioteke, China).

Total RNA was isolated from the leaves using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Promega, Madison, WI, USA) to remove any traces of genomic DNA, according to the manufacturer's instructions. Successful removal of DNA contamination was confirmed by the absence of PCR amplification product using a primer pair (HSP70-S/HSP70-AS, Table 2), which was designed to amplify an intron sequence of a gene encoding HSP70 gene (GenBank accession number EF208125). The first strand cDNA synthesis was performed using oligo $(dT)_{15}$ primers and 200 units of Maloney Marine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) for 1 h at 42°C. RNA extraction and cDNA synthesis from all samples were performed for two biological replicates.

Isolation of the thaumatin-like gene, CsPR5, from introgression line 5112S

A pair of degenerate primers, i.e. PR-5S/PR-5AS (Table 2), based on conserved regions of *PR5* genes (Pflieger et al., 2001), was employed to amplify the genomic DNA of IL5211S. A 50 µL PCR reaction contained 2U *Taq* DNA polymerase (Takara, Japan), 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.5 µM each of forward and reverse primers, 0.4 mM dNTP, and a 100 ng template DNA. PCR was carried out in a PTC-200 thermocycler (BioRad, Hercules, CA, USA) programmed for an initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 1 min, and a final extension of 7 min at 72 °C. DNA fragment of expected size (~533bp) was reclaimed from agarose gel and purified using a MinElute gel extraction kit (Qiagen, Germany). The DNA was then cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and transformed into competent cells of the *Escherichia coli* JM 10^9 strain, according to manufacturer's instructions. Single clone was re-amplified using universal forward and reverse primers. Cloned DNA fragments were then sequenced commercially. Based on the sequence of the 533 bp fragment, nucleotide sequences from both the upstream and downstream regions of the 533 bp fragment were obtained by applying a genomic walking procedure using the universal Genome Walker Kit (Clontech, Palo Alto, CA, USA). These additional sequences allowed the design of a set of primers ($CsPR\text{-}5S_1$/$CsPR\text{-}5AS_1$, Table 2) to isolate the complete coding region of the *CsPR5* gene. The amplification conditions were the same as described above for obtained the 533 bp fragment, with the exception that 35 cycles were performed at an annealing temperature of 55 °C.

Sequence and phylogenetic analysis

DNA sequences were compared with translated sequences using BLASTx (Altschul et al., 1997) in GenBank (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Multiple sequence alignments were performed with the software DNAMAN (Lynnon Biosoft, Quebec, Canada). Various transcription regulatory elements were identified using the PLACE database (Higo et al., 1999). A phylogenetic tree was constructed based on the bootstrap neighbor-joining (NJ) method with a Kimura 2-parameter model using MEGA v4.0 (Tamura et al., 2007). The stability of the internal nodes was assessed via bootstrap analysis with 1,000 replicates.

Quantitative real-time PCR and data analysis

The relative expression levels of the *CsPR5* gene in leaf samples collected at different time points after biotic and

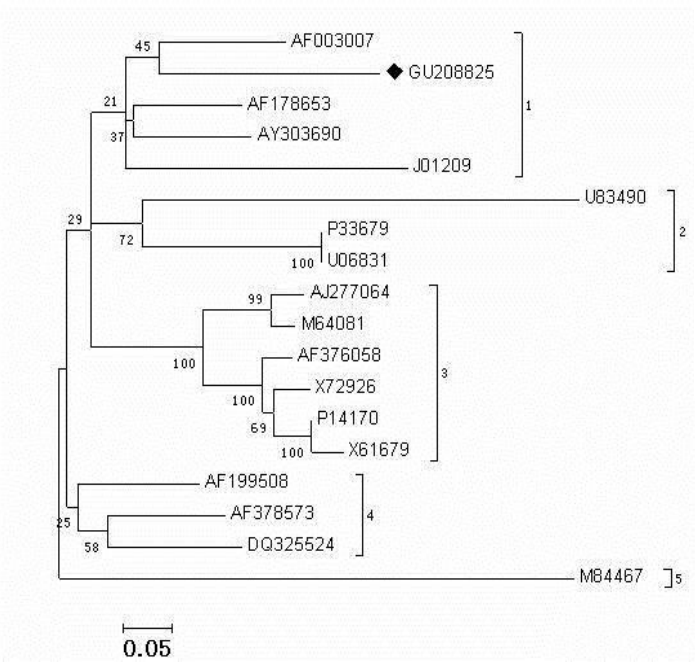

Fig 3. A phylogenetic tree constructed based on the amino acid sequences of *CsPR5* (GU208825), *V. vinifera* (AF003007), *V. riparia* (AF178653), *Gossypium* spp., (AY303690), *T. daniellii* (J01209), *Arabidopsis thaliana* (U83490), *Z. mays* (P33679), *Z. mays* (U06831), *Lycopersicon esculentum* (AJ277064), *N. tabacum* (M64081), *Petunia hybrida* (AF376058), *Solanum commersonii* (X72926), *N. tabacum* (P14170), *N. tabacum* (X61679), *F. ananassa* (AF199508), *Sambucus nigra* (AF378573), *F. ananassa* (DQ325524), and *A. nummularia* (M84467).

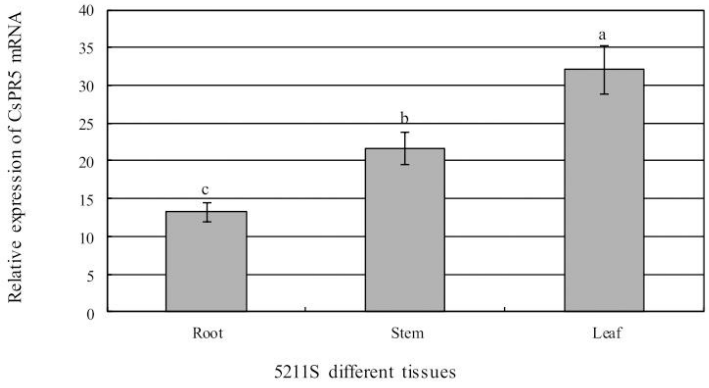

Fig 4. Relative expression levels of *CsPR5* mRNA in different tissues of IL5211S. Each tissue sample was individually assayed in triplicate. Values represent the mean reading from three plants, and error bars indicate standard error of the means.

abiotic stimuli treatments were determined by means of real-time quantitative PCR. Cucumber elongation factor 1-alpha (EF1α), a housekeeping gene, was used as the reference gene (Wan et al., 2010b). The primer (EF1α-S/EF1α-AS) sequence is listed in Table 2. The *CsPR5* sequence was used for designing PCR primers with Beacon Designer 2.06 (Primer Biosoft International, Palo Alto, California, USA). Real-time PCR reactions were performed in a total volume of 25 μL containing 12.5 μL 2 × SYBRGreen PCR MasterMix (Applied Biosystems, Carlsbad, CA), 1 μL (10

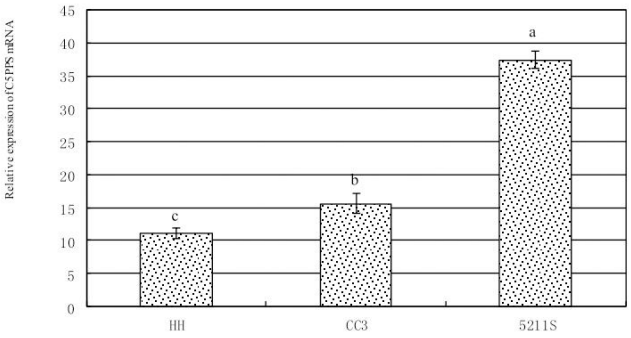

Fig 5. Relative quantitative analysis of the *CSPR5* protein gene within HH (*C. hystrix*), backcross parent (CC3), and the introgression line of *C. hystrix* / *C. sativus* (IL5211S). Values represent the mean reading from three plants, and error bars indicate standard error of the means.

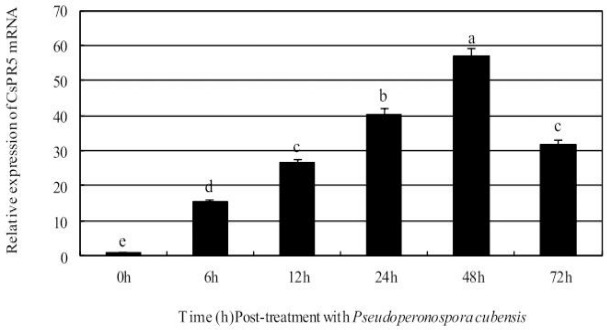

Fig 6. Relative expression levels of *CsPR5* mRNA at various time points after *P. cubensis* treatment. Each plant sample was individually assayed in triplicate. Values represent the mean reading from three plants, and error bars indicate standard error of the means.

pmoles) of each primer, 25 ng template DNA (15 × diluted cDNA from samples), and 9.5 μl sterile distilled water. The thermal conditions for the real-time PCR were 95°C for 10 min (denaturation), followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate in 96-well reaction plates using the iQ5 machine (Bio-Rad, Hercules, CA). Quantification analysis was performed using the comparative C_T method, which mathematically (standard curve) transforms the threshold cycle (C_t) into the relative expression level of genes. Standard deviations were calculated using qBase software v1.3.5 (Hellemans et al., 2007) on a standard curve generated using a five-fold dilution series of one sample, over at least six dilution points measured in triplicate. The data for two biological replicates were assayed in triplicate.

Conclusions

In conclusion, in addition to *P. cubensis*, the four abiotic stresses stimuli used in this study triggered a significant induction of *CsPR5* at 72 h after treatment. Furthermore, compared with the SA and H_2O_2 treatments, *CsPR5* was more prominently induced by MeJA and ABA. Therefore, these results suggest that the *CsPR5* gene may play a role in the

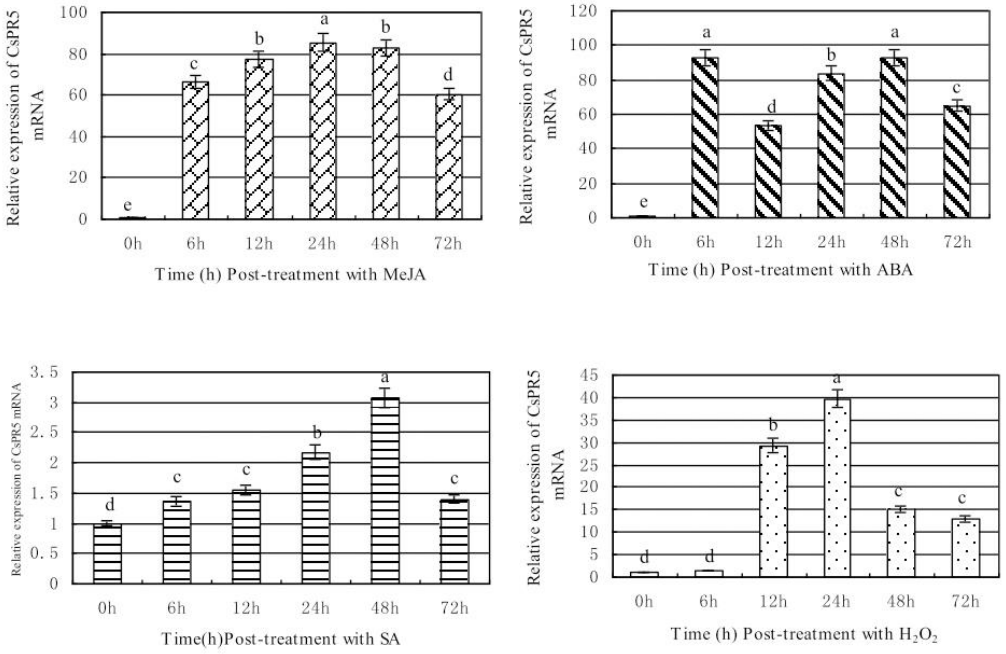

Fig 7. Relative quantities of *CsPR5* mRNA at various time points after treatment with MeJA (A), ABA (B), SA (C), and H_2O_2 (D). Each plant was individually assayed in triplicate. Values represent the mean reading from three plants, and error bars indicate standard error of means.

molecular defense of IL5211S to pathogen invasions. Moreover, the results also showed that the expression level of the *CsPR5* gene was enhanced by introgression of *C. hystrix*. This increased expression level may be involved in the defense response to *P. cubensis* and abiotic stimuli in IL5211S.

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References

Abad LR, D'Urzo MP, Liu D, Narasimhan ML, Reuveni M, Zhu JK (1996) Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Sci. 118: 11-23

Acharya K, Pal AK, Gulati A, Kumar S, Singh AK, Ahuja PS (2012) Overexpression of Camellia sinensis thaumatin-like protein, *CsTLP* in potato confers enhanced resistance to Macrophomina phaseolina and Phytophthora infestans infection. Mol Biotechnol. DOI: 10.1007/s12033-012-9603-y

Adams KL, Wendel JF (2005) Allele-specific, bidirectional silencing of an alcohol dehydrogenase gene in different organs of interspecific diploid cotton hybrids. Genetics 171: 2139-2142

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programmes. Nucl Acids Res. 25: 3389-3402

Baack EJ, Rieseberg LH (2007) A genomic view of introgression and hybrid speciation. Curr Opin Genet Dev. 17: 513-518

Barthakur S, Babu V, Bansal KC (2001) Overexpression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. J Plant Biochem Biot. 10: 31-37

Batalia MA, Monzingo AF, Ernst S, Roberts W, Robertus JD (1996) The crystal structure of the antifungal protein zeamatin, a member of the thaumatinlike, PR-5 protein family. Nat Struct Biol. 3: 19-23

Campos MA, Silva MS, Magalhães CP, Ribeiro SG, Sarto RPD, Vieira EA, Grossi de Sá MF (2008) Expression in *Escherichia coli*, purification, refolding and antifungal activity of an osmotin from *Solanum nigrum*. Microb Cell Fact 7:7

Chen JF, Staub JE, Tashiro Y, Isshiki S, Miyazaki S (1997) Successful interspecific hybridization between *Cucumis sativus* L. and *C. hystrix* Chakr. Euphytica 96: 413-419

Chen JF, Kirkbride JH (2000) A new synthetic species of *Cucumis* (Cucurbitaceae) from interspecific hybridization and chromosome doubling. Bittonia 52: 315-319

Chen JF, Staub JE, Qian CT, Jiang J, Luo XD, Zhuang FY (2003) Reproduction and cyogenetic characterization of interspecific hybrids derived from *Cucumis hystrix* Chakr.× *C. sativus* L. Theor Appl Genet. 106: 688-695

Chen JF, Luo XD, Qian CT, Jahn MM, Staub JE, Zhuang FY, Lou QF, Ren G (2004) *Cucumis* monosomic alien addition lines: morphological, cytological, and genotypic analyses. Theor Appl Genet. 108: 1343-1348

Christensen AB, Cho BH, Næsby M,Gregersen PL, Brandt J, Madriz-Ordeѭana K,Collinge DB, Thordal-Christensen H (2002) The molecular characterization of Pathogen invasions and environmental stresses represent the most limiting factors for agricultural productivity the two barley proteins establishes the novel PR-17 family of pathogenesis-related

protein. Mol Plant Pathol. 3: 135–44
Chu KT, Ng TB (2003) Isolation of a large thaumatin-like antifungal protein from seeds of the Kweilin chestnut Castanopsis chinensis. Biochem Biophys Res Comm. 301: 364-370
Cornelissen BJC, Hooft van Huijuijnen RAM, Bol JF (1986) A tobacco mosaic virus induced tobacco protein is homologous to the sweet tasting protein thaumatin. Nature 321: 531-532
Datta K, Muthukrishnan S, Datta SK (1999) Expression and function of PR-protein genes in transgenic plants. In: Datta, SK, Muthukrishnan S, editors, Pathogenesis-Related Proteins in Plants, New York: CRC Press; p. 261-277.
Dubouzet JG, Maeda S, Sugano S, Ohtake M, Hayashi N, Ichikawa T, Kondou Y, Kuroda H, Horii Y, Matsui M, Oda K, Hirochika H, Takatsuji H, Mori M (2011) Screening for resistance against *Pseudomonas syringae* in rice-FOX Arabidopsis lines identified a putative receptor-like cytoplasmic kinase gene that confers resistance to major bacterial and pathogens in Arabidopsis and rice. Plant Biotechnol J. 9: 466-485
Gaffney T, Friedrich L, Vernooij B, Nwgrotto D, Nye D, Uknes S (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261: 754-756
Grant M, Lamb C (2006) Systemic immunity. Curr Opin Plant Biol. 9: 414-420
Garcia-casado G, Collada C, Allona I, Soto A, Casado R, Rodriguezcerezo E, Gomez L, Aragoncillo C (2000) Characterization of an apoplastic basic thaumatin-like protein from recalcitrant chestnut seeds. Physiol Plantarum 110: 172–180
Griffith M, Lumb C, Wiseman SB, Wisniewski M, Johnson RW, Marangoni AG (2005) Antifreeze proteins modify the freezing process in planta. Plant Physiol. 138: 330-340
Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8: R19
Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res. 27: 297-300
Hu X, Reddy ASN (1997) Cloning and expression of a PR5-like protein from Arabidopsis: inhibition of fungal growth by bacterially expressed protein. Plant Mol Biol. 34: 949-959
Husaini AM, Abdin MZ (2008) Development of transgenic strawberry (*Fragaria x ananassa* Dutch.) plants tolerant to salt stress. Plant Sci. 174: 446-455
Kim SH, Lee J, Kim S-R (2003) Molecular characterization of a fruit-preferential thaumatin-like gene from apple (*Malus domestica* cv. Fuji). J Plant Biol. 46: 52-58
Kitajima S, Sato F (1999) Plant pathogenesis-related proteins: molecular mechanisms of gene expression and protein function. J Biochem. 125:1-8
Liu DQ, He X, Li WX, Chen CY, Ge F (2012) Molecular cloning of a thaumatin-like protein gene from Pyrus pyrifolia and overexpression of this gene in tobacco increased resistance to pathogenic fungi. Plant Cell Tiss Org Cult. 111: 29-39
Liu ZL, Wang YM, Shen Y, Guo WL, Hao S, Liu B (2004) Extensive alterations in DNA methylation and transcription in rice caused by introgression from *Zizania latifolia*. Plant Mol Biol. 54: 571-582
Lorenzo O, Solano R (2005) Molecular players regulating the jasmonate signalling network. Curr Opin Plant Biol. 8: 532-540
Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defence responses in Arabidopsis. Plant Cell 16: 1938-1950
Malehorn DE, Borgmeyer JR, Smith, CE, Shah DM (1994) Characterization and expression of an antifungal zeamatin-like protein (*Zlp*) gene from *Zea mays*. Plant Physiol. 106: 1471-1481
Mao XG, Zhang HY, Qian XY, Li A, Zhao GY, Jing RL (2012) TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in Arabidopsis. J Exp Bot. 63: 2933-2946
Neale AD, Wahleithner JA, Lund M, Bonnett HT, Kelly A, Meeks-Wagner DR, Peacock WJ, Dennis ES (1990) Chitinase, β-1, 3 glucanase, osmotin, and extension are expressed in tobacco explants during flower formation. Plant Cell 2: 673-684
Nelson DE, Raghothama KG, Singh NK, Hasegawa PM, Bressan RA (1992) Analysis of structure and transcriptional activation of an osmotin gene. Plant Mol Biol. 19: 577-588
Newton SS, Duman JG (2000) An osmotin-like cryoprotective protein from the bittersweet nightshade *Solanum dulcamara*. Plant Mol Biol. 44: 581-589
Parkhi V, Kumar V, Sunilkumar G, Campbell LM, Singh NK, Rathore KS (2009) Expression of apoplastically secreted tobacco osmotin in cotton confers drought tolerance. Mol Breeding 23: 625-639
Pflieger S, Palloix A, Caranta C, Blattes A, Lefebvre V (2001) Defense response genes co-localize with quantitative disease resistance loci in pepper. Theor Appl Genet. 103: 920-929
Piggott N, Ekramoddoullah AKM, Liu JJ, Yu X (2004) Gene cloning of a thaumatin-like (PR-5) protein of western white pine (*Pinus monticola* D. Don) and expression studies of members of the PR-5 group. Physiol Mol Plant Pathol. 64: 1-8
Salzman RA, Tikhonova I, Bordelon BP, Hasegawa PM, Bressan RA (1998) Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. Plant Physiol. 117: 465-472
Selitrennikoff CP (2001) Antifungal proteins. Appl Environ Microbiol. 67: 2883-2894
Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2: 503-512
Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie 75: 687-706
Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 24: 1596-1599
Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in *Arabidopsis*. Plant Cell 4: 645-656
Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An *Arabidopsis* MYB homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5: 1529-1539
van Loon LC, Rep M, Pietersen CMJ (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol. 44:135–162
Wan HJ, Zhao ZG, Malik AA, Qian CT, Chen JF (2010a) Identification and characterization of potential NBS-encoding resistance genes and induction kinetics of a putative candidate gene associated with downy mildew resistance in *Cucumis*. BMC Plant Biol. 10: 186

Wan HJ, Zhao ZG, Qian CT, Sui YH, Malik AA, Chen JF (2010b) Selection of appropriate reference genes for gene expression studies by quantitative real-time PCR in cucumber. Anal Biochem. 399: 257-261

Wang L, Duman JG (2006) A thaumatin-like protein from larvae of the beetle Dendroides canadensis enhances the activity of antifreeze proteins. Biochemistry 45, 1278-1284.

Wasilewskaa A, Vlad F, Sirichandra C, Redko Y, Jammes F, Valon C, dit Fery NF, Leung J (2008) An update on abscisic acid signaling in plants and more. Mol Plant 1: 198-217

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot. 100:681-697

Xing Y, Jia W, Zhang J (2008) AtMKK1 mediates ABA-induced CAT1 expression and H_2O_2 production via AtMPK6 coupled signaling in Arabidopsis. Plant J. 54: 440-451

Zareie R, Melanson DL, Murphy PL (2002) Isolation of fungal cell wall degrading proteins from barley (*Hordeum vulgare* L.) leaves infected with *Rhynchosporium secalis*. Mol Plant-Microbe Interact. 15: 1031-1039

Zhang YH, Shih DS (2007) Isolation of an osmotin-like protein gene from strawberry and analysis of the response of this gene to abiotic stresses. J Plant Physiol. 164: 68-77

Zhu BL, Chen THH, Li PH (1995) Expression of 3 osmotin-like protein genes in response to osmotic stress and fungal infection in potato. Plant Mol Biol. 28: 17-26

Zhu BL, Chen THH, Li PH (1993) Expression of an ABA-responsive osmotin-like protein gene during the induction of freezing tolerance in *Solanum commersonii*. Plant Mol Biol. 21: 729-735