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; H2O2_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 chinesis* 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 nicotianae* 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 Rieseborg, 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. hystricis 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. hystricis enhanced the expression level of CsPR5 from C. sativus. The responses of this TLP gene to salicylic acid (SA), abscisic acid (ABA), hydrogen peroxide (H₂O₂), 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-SS 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 (Nicotiana tabacum), cotton (Gossypium spp.), strawberry (Fragaria x ananassa), grapevine (Vitis vinifera), and maize (Zea mays) (Table 1). Based on the CsPR5 gene sequence in IL5211S, its homologs were isolated from two parents, namely C. hystricis accession HH and C. sativus cultivar Beijjingjiaou. 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, CCC3 and Csa013260 were identical. During comparison with the CsPR5 homologue gene from C. hystricis, 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. Arg⁹, Gln²¹, Asp³⁸, Asp³⁴, and Asp¹⁴⁰) 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., 1997; 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. hystricis on the CsPR5 gene transcription levels**

In order to investigate whether or not alien DNA introgression from C. hystricis 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. hystricis), 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Deduced amino acid sequences</th>
<th>Accession numbers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>grapevine</td>
<td>201</td>
<td>64</td>
<td>75</td>
</tr>
<tr>
<td>cotton</td>
<td>203</td>
<td>62</td>
<td>75</td>
</tr>
<tr>
<td>maize</td>
<td>207</td>
<td>61</td>
<td>70</td>
</tr>
<tr>
<td>tobacco</td>
<td>200</td>
<td>61</td>
<td>69</td>
</tr>
<tr>
<td>strawberry</td>
<td>201</td>
<td>60</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 2. List of primers used in this paper.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-5S/PR-5AS</td>
<td>AACAAYTGGCCTRACACCGT</td>
<td>GGATCATCTTGGWGRTARCTATA</td>
</tr>
<tr>
<td>CspR-5S/CspR-5AS₁</td>
<td>CAGGAGGAGGAGGCTGAT</td>
<td>TGCCAGTCTCCTAAACACCTC</td>
</tr>
<tr>
<td>CspR-5S/CspR-5AS₂</td>
<td>CCCGCAAACAGGAAAGAATC</td>
<td>AGACTTGGCAGGGTGTGGT</td>
</tr>
<tr>
<td>Hsp70-8/Hsp70-AS</td>
<td>GTCAAATAATCTGGGAAGATC</td>
<td>TTTGAGGTAGGAAGTGAT</td>
</tr>
<tr>
<td>EF1α-S/EF1α-AS</td>
<td>ACTGTGTGTCCTCATATTGT</td>
<td>AGGGTGAAAGCAGAAGAGC</td>
</tr>
</tbody>
</table>

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 H₂O₂ 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 H₂O₂) 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 24 h, 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 H₂O₂ at different time points is shown in Fig. 7D. It appeared that CsPR5 was not regulated by H₂O₂ 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.
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 hemibiotrophic 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 SAR treatment (Uknes et al., 1992). In strawberry plants, the induction level of the OLP gene, FaOLP2, 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 stress (Wasternack and Strasburger, 2000).

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 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$_2$O$_2$ 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$_2$O$_2$ may be involved in ABA induced defense gene (CAT1) expression (Xing et al., 2008). In the current study, the expression level of CsPR5 was significantly induced by MeJA and H$_2$O$_2$, respectively, which occurred at 72 and 24 h. This rapid enhancement of CsPR5 expression in IL5211S by MeJA and H$_2$O$_2$, 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 65-78%. 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 inoculum, 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$_2$O$_2$ 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 abscissic acid (ABA, 100μM), and sampled 3 h afterwards (Wan et al., 2010b). For the H$_2$O$_2$ treatment, seedlings were sprayed with H$_2$O$_2$ (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. DQ235524), 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 IL52115 were harvested and immediately frozen in liquid nitrogen, then stored at -80°C. Genomic DNA was isolated using a commercial plant DNA extraction kit (Biotek, 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/HSP70-AS, Table 2), which was designed to amplify an intron sequence of a gene encoding HSP70 gene (GenBank Accession No. EF208125). The first strand cDNA synthesis was performed using oligo (dT)12 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 S1125

A pair of degenerate primers, i.e. PR-SSPR-5AS (Table 2), based on conserved regions of PR5 genes (Pflieger et al., 2001), was employed to amplify the genomic DNA of IL52115. A 50 μL PCR reaction contained 2U Taq DNA polymerase (TaKaRa, Japan), 1 × PCR buffer, 1.5 mM MgCl2, 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 reclamed 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 IM 10<sup>th</sup> 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-5S/CsPR-5AS, 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 (Lynnons 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., 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.

Relative expression levels of 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 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 Ct method, which mathematically (standard curve) transforms the threshold cycle (Ct) 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 H2O2 treatments, CsPR5 was more prominently induced by MeJA and ABA. Therefore, these results suggest that the CsPR5 gene may play a role in the
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.

Acknowledgments

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Fig 7. Relative quantities of CsPR5 mRNA at various time points after treatment with MeJA (A), ABA (B), SA (C), and H2O2 (D). Each plant was individually assayed in triplicate. Values represent the mean reading from three plants, and error bars indicate standard error of means.
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