Cloning and Characterization of PR5 Gene from Curcuma amada and Zingiber officinale in Response to Ralstonia solanacearum Infection

Article *in* Plant Cell Reports · May 2011

DOI: 10.1007/s00299-011-1087-x · Source: PubMed

5 authors, including:

D. Prasath
Indian Institute of Spices Research
57 PUBLICATIONS **107** CITATIONS
SEE PROFILE

Islam El-Sharkawy
University of Guelph
42 PUBLICATIONS **660** CITATIONS
SEE PROFILE

Sherif Sherif
University of Guelph
27 PUBLICATIONS **186** CITATIONS
SEE PROFILE

Plant Cell Rep (2011) 30:1799–1809
DOI 10.1007/s00299-011-1087-x

ORIGINAL PAPER

Cloning and characterization of PR5 gene from *Curcuma amada* and *Zingiber officinale* in response to *Ralstonia solanacearum* infection

D. Prasath · I. El-Sharkawy · S. Sherif · K. S. Tiwary · S. Jayasankar

Received: 12 January 2011 / Revised: 18 April 2011 / Accepted: 6 May 2011 / Published online: 19 May 2011

Abstract Ginger (*Zingiber officinale* Roscoe), is an important spice crop that is badly affected by *Ralstonia solanacearum* wilt. Ginger does not set seed and sexual recombination has never been reported. In spite of extensive search in its habitats, no resistance source to *Ralstonia* induced bacterial wilt, could be located in ginger. *Curcuma amada* Roxb. is a potential donor for bacterial wilt resistance to *Z. officinale*, if the exact mechanism of resistance is understood. Pathogenesis-related (PR)-5 proteins are a family of proteins that are induced by different phytopathogens in many plants and share significant sequence similarity with thaumatin. Two putative PR5 genes, *Ca*PR5 and *Zo*PR5, were amplified from *C. amada* and ginger, which encode precursor proteins of 227 and 224 amino acid residues, respectively, and share high homology with a number of other PR5 genes. The secondary and three-dimensional structure comparison did not reveal any striking differences between these two proteins. The expression of *Ca* and *Zo*PR5s under *R. solanacearum* inoculation was analyzed at different time points using quantitative real-time PCR (qRT-PCR). Our results reveal that *Ca*PR5 is readily induced by the bacterium in *C. amada*, while *Zo*PR5 induction was very weak and slow in ginger. These results suggest that the *Ca*PR5 could play a role in the molecular defense response of *C. amada* to pathogen attack. This is the first report of the isolation of PR5 gene from the *C. amada* and *Z. officinale*. Promoter analysis indicates the presence of a silencing element binding factor in *Zo*PR5-promoter, but not in *Ca*PR5. Prospective promoter elements, such as GT-1 box and TGTCA, implicated as being positive regulatory elements for expression of PR proteins, occur in the 5′-flanking sequences of the *Ca*PR5. Transient GUS expression study confirms its action with a weaker GUS expression in ginger, indicating that the PR5 expression may be controlled in the promoter.

Keywords Disease resistance · Ginger · Gene expression · Mango ginger · Pathogenesis-related proteins · Promoter

Communicated by P. Kumar.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-011-1087-x) contains supplementary material, which is available to authorized users.

D. Prasath · I. El-Sharkawy · S. Sherif · S. Jayasankar (✉)
Department of Plant Agriculture, University of Guelph, 4890 Victoria Ave. N., P.O. Box 7000, Vineland Station, ON L0R 2E0, Canada
e-mail: jsubrama@uoguelph.ca

D. Prasath
Indian Institute of Spices Research, Indian Council of Agricultural Research, Calicut, Kerala 673 012, India

I. El-Sharkawy
Vineland Research and Innovation Centre (VRIC), 4890 Victoria Ave. N., Box 4000, Vineland Station, ON L0R 2E0, Canada

S. Sherif · K. S. Tiwary
Department of Plant Agriculture, University of Guelph, Guelph, ON N1G 2W1, Canada

Introduction

Ginger (*Zingiber officinale* Roscoe; family Zingiberaceae) is a widely used spice, flavoring agent, herbal medicine and is also employed in the perfume industry. Cultivated ginger is a sterile crop, originated in India or Southeast Asia (Ravindran et al. 1994). Today ginger is cultivated in many tropical and subtropical areas. The main producers are India, China, Indonesia, and Nigeria (FAOSTAT 2010).

Ginger is affected by many diseases. Among those, bacterial wilt disease of ginger is one of the most important production constraints in tropical, sub tropical, and warm temperature regions of the world (Kumar and Sarma 2004). It inflicts serious economic losses to small and marginal farmers who depend on this crop for their livelihood. Geographical distribution of the pathogen is expanding in recent years due to the unintentional transmission of the bacterium through infected rhizomes of ginger, which are the primary propagules (Kumar and Hayward 2005). None of the released varieties are resistant to bacterial wilt. This is due to lack of genetic variability among the accessions for disease resistance, which is one of the bottlenecks in ginger genetic improvement. The search for resistance has been extended to other closely related genera in the family, Zingiberaceae. Among the Zingiberaceae members such as *Curcuma amada, C. longa*, *C. zedoria, C. aromatica, Kaempferia galanga, Elettaria cardamomum, Zingiber zerumbet*, and *Z. officinale* evaluated for their reaction to *Ralstonia solanacearum* (causal agent of bacterial wilt) biovar 3 (ginger-specific strain), and *Pythium* species, the Indian Mango Ginger, *C. amada* Roxb., exhibited significant resistance to both pathogens (Kumar et al. 2006), while *Z. zerumbet* is resistant to *P. aphanidermatum* (Kavitha and Thomas 2008).

On pathogen infection, a number of antimicrobial compounds are elicited in the host plant, as a part of their defense mechanism. Among these are an important group of antimicrobial proteins, collectively referred to as 'pathogenesis-related proteins' (PRs) (Van loon et al. 2006). These PR proteins are both local and systemic and are associated with the development of systemic acquired resistance (SAR) against further infection by pathogenic microbes (Evans and Greenland 1998). There are now many lines of evidence indicating that proteins of the PR5 group from various plant species have in vitro antifungal activity against several classes of fungi and oomycetes (Jayasankar et al. 2003; van Loon et al. 2006; Asselbergh et al. 2007). Recently, a PR5 gene family in *Z. zerumbet* was reported to be significantly upregulated in response to infection by *P. aphanidermatum* (Nair et al. 2010).

Over expression of PR5 genes has been shown to enhance pathogen resistance in plants (Datta et al. 1999; Muthukrishnan et al. 2001; van Loon et al. 2006). Certain isoforms of PR5 have strong affinity and hydrolytic activity toward (1,3)-β-D-glucans (Trudel et al. 1998; Grenier et al. 1999; Osmond et al. 2001), the major cell-wall constituent of most oomycetes (Latijnhouwers et al. 2003). The high resolution crystal structures of PR5 proteins revealed the presence of a long, deep surface cleft, which is crucial to their antifungal activities (Koiwa et al. 1999; Osmond et al. 2001; Leone et al. 2006; Ghosh and Chakrabarti 2008).

Resistance breeding in ginger is restricted to germplasm screening as it is an obligatory asexual crop (Ravindran et al. 2005). In spite of extensive search, no resistance source to *Pythium* soft rot, *Fusarium* yellows, and *Ralstonia* induced bacterial wilt could be located in *Zingiber* genus. On the other hand, *C. amada* shows very high levels of resistance to Ralstonia wilt proving some promise for developing bacterial wilt resistant ginger. However, this can be done only if the mechanism of resistance in *C. amada* is understood. A thorough genetic analysis would unravel the factors (genes) governing the resistance in *C. amada–R. solanacearum* pathosystem (Kumar et al. 2006). Lack of genetic variability in ginger coupled with available resistance in a closely related genus makes the use of functional genomics an ideal choice to impart *R. solanacearum* resistance in ginger. As a first step towards that, we have cloned and investigated the expression pattern of two PR5 genes in *C. amada* and *Z. officinale*. Based on the proteins' 3-D structure, we have also discussed the likely function of the protein encoded by these genes. We have also characterized 5′-flanking region of respective PR5s and its role in disease regulation.

Materials and methods

Plant material and pathogen inoculation

Forty-five to 60 days old *C. amada* and *Z. officinale* plants grown in the green house of the University of Guelph (Vineland Station, ON, Canada) were used for the experiment. The virulent colonies of *R. Solanacearum* (*R4Bv4*), cultured on Casamino acid-Peptone-Glucose (CPG) medium (Kelman 1954), were multiplied in sucrose peptone broth (g l^{-1} sucrose, 20; peptone, 10; K_2HPO_4, 0.5; $MgSO_4$, 0.25; pH 7.2) for 2 days. The bacteria was pelleted at 10,000 g for 20 min at 4°C, resuspended in water and poured around the base of the 45-day-old plants, as water suspension at a concentration of 10^9 cells ml^{-1} of water. The inoculated plants were grown in growth chamber (28 ± 2°C, 12 h light, 65% RH) and were monitored for wilt disease.

RNA isolation

Total RNA was isolated from *C. amada* and *Z. officinale* at 1, 4, 8, 16, and 24 h after inoculation (hai) with *R. solanacearum* and just before inoculation (0 hai) using the methods described by Boss et al. (1996). All RNA extracts were treated with DNase I (Promega) then cleaned up with RNeasy mini kit (Qiagen).

Isolation and in silico analysis of PR5 cDNA sequence

For the isolation of *C. amada* and *Z. officinale* homologs of PR5 cDNAs, first-strand cDNA synthesis was carried out using 20 µg of total DNase-treated RNA (pooled total RNA obtained from different time intervals) in a 50 µl aliquot. Based on the sequence similarity of various PR5 from different plant species, a pair of degenerate primer, PR5(F) and PR5(R) (primers# 1 and 2 in Table 1), was designed from the conserved regions to amplify the PR5 orthologues from *C. amada* and *Z. officinale*. A 1 µl aliquot of cDNA was used in a PCR with the appropriate degenerate primers. The isolated fragment was cloned in pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced and compared with database sequences using the BLAST program (Altschul et al. 1997). Extension of the partial cDNA clone was carried out using 3′ and 5′ RACE to complete the cDNA sequences (Clontech, Mountain View, CA, USA). Full length amplification of cDNA sequences designated as *Ca*PR5 and *Zo*PR5 was carried out using the Platinum Taq DNA Polymerase High Fidelity, following instruction provided by manufacturer (Invitrogen). Alignments of the predicted protein sequences were performed with ClustalX (Jeanmougin et al. 1998) and GeneDoc (Nicholas et al. 1997). The SignalP 3.0 server was used to predict the presence and location of signal peptide cleavage sites in the amino acid sequences (Bendtsen et al. 2004).

Molecular modeling

The Secondary structures of PR5 sequences were generated through Hierarchical Neural network (Garnier et al. 1996) and GOR 4 (Garnier Osugothorph Robson) secondary structure prediction tools (Chen et al. 2010). The aliphatic and Instability indexes were calculated using ProtParam (Gasteiger et al. 2005). Three dimensional structures of resistant and susceptible proteins were developed using SwissPDB viewer swissModel software (Schwede et al. 2003). The template used for homology modeling was 1Z3Q protein from banana. Modeled structures were saved in .pdb format. Model validation was done using WHATCHECK tool (Hooft et al. 1996). The aim of our comparative modeling was to generate the most probable structure of the *Ca*PR5, *Zo*PR5 protein through alignment with template sequences.

Table 1 The oligonucleotide sequences used to isolate PR5 from *C. amada* and *Z. officinale* and real-time quantitative PCR primers used for expression analysis

Primer name	Sequence 5′–3′
PR5-Forward (F)	GCCGCGCTCTGTTTCTTCCTCTTCC
PR5-Reverse (R)	ACTACAGTGTCACCTTCTGCCCTTG
Real-time quantitative PCR primers	
*Ca*PR5	
Forward (F)	CAACACCCTCGCCGAGTT
Reverse (R)	TTGGAGATGTCGAAGAAGTCGAA
*Zo*PR5	
Forward (F)	TCGCCCTCAACCAGTTCAA
Reverse (R)	GGCTGAAGTCTATACCCACGTTGT
Beta-Actin	
Forward (F)	CAACAGCAGAACGGGAAATTG
Forward (F)	CATAATCAAGGGCGACATATGC

Real time quantitative RT-PCR

DNase-treated RNA (5 µg) was reverse transcribed in a total volume of 50 µl using SuperScript III Reverse Transcriptase (Invitrogen, Burlington, ON, Canada). Real-time quantitative PCR was performed using 10 ng of total RNA in a 20 µl reaction volume with SYBR GREEN PCR MasterMix (Qiagen, Mississauga, ON, Canada) on a Mx4000® multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA). Mx4000® v 4.20 software (Stratagene, La Jolla, CA, USA) was used to design gene-specific primers (Table 1). For all the genes studied here, the optimal primer concentration was 200 nM. RT-PCR conditions were as follows: 95°C (15 min), then 45 cycles of 95°C (15 s) and 60°C (30 s). The products were further analysed by a dissociation curve program at 95–60°C (16 s). All RT-PCR experiments, for each gene, were run in triplicate with different cDNAs synthesized from three biological replicates. Each sample was run in three technical replicates on a 96-well plate. For each sample, a C_t (threshold constant) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative C_t method using the β-actin as an internal standard. To demonstrate that the efficiencies of the different gene primers were approximately equal, the absolute value of the slope of log input amount versus ΔC_t was calculated for *Ca*PR5, *Zo*PR5, and β-actin genes, and was determined to be <0.1. To determine relative fold differences for each sample in each experiment, the C_t value for the four studied genes was normalized to the C_t value for β-actin and was calculated relative to a calibrator using the formula $2^{\Delta\Delta C_t}$.

Promoter isolation

Genomic DNA was extracted from leaves (*C. amada* and *Z. officinale*) using the DNeasy Plant Maxi Kit (Qiagen, Mississauga, ON, Canada). Promoter of PR5 (*Ca* and *Zo*) was cloned from *C. amada* and *Z. officinale* using Genome Walker™ Universal kit (Clontech, Mountain View, CA, USA). After cloning the ~800 bp promoter sequence of

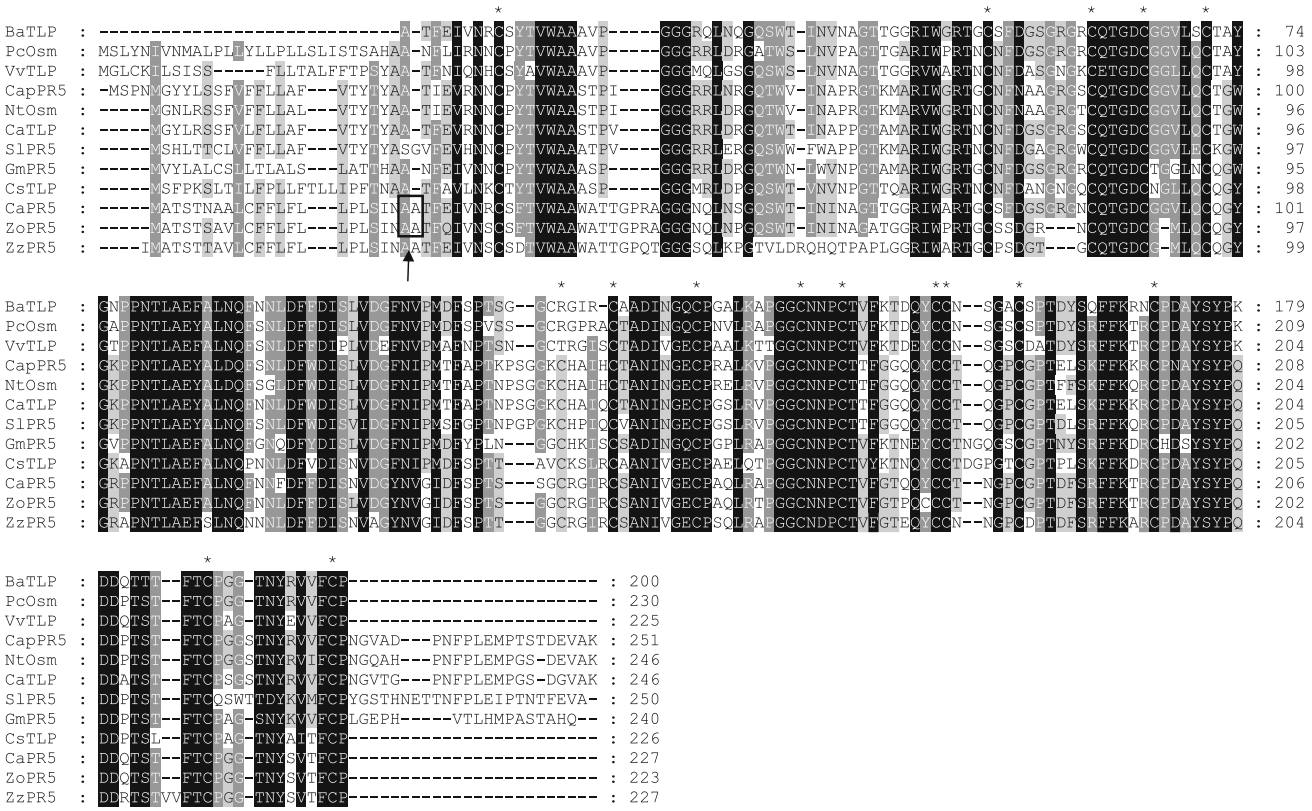

Fig. 1 Multiple amino acid sequence alignments of *Ca*PR5 and *Zo*PR5 with the closely related sequences using the ClustalW program. Conserved residues are shaded in *black*. *Asterisks* show the 16 conserved cysteine residues. The cleavage site between the signal peptide and mature protein is indicated by the *arrow*

PR5 from *C. amada* and *Z. officinale*, ClustalW was used for their comparison. PLANTCARE database available at (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to identify the motifs available in the promoter region (Lescot et al. 2002).

Construction of promoter fragment/GUSXX vector and GUS transient activity assay

The convenient GUSXX vector system developed by Pasquali et al. (1994) was used to construct promoter-GUSXX vector to study transactivation of the promoter. Transient GUS activity assay of *Ca*PR5 and *Zo*PR5 promoters was carried out in *Arabidopsis* mesophyll protoplasts (Yoo et al. 2007).

Results

Isolation and structural characterization of *Ca*PR5 and *Zo*PR5

PCR amplifications using degenerate oligonucleotide primers resulted in the isolation of partial cDNAs with the expected size 600 bp from *Curcuma amada*. The deduced nucleotide sequences exhibited strong similarity to the *Z. zerumbet* PR5 (*Zz*PR5), mRNA. Extension of the partial cDNA clones revealed that full-length cDNA was 681 bp in length. The predicted open reading frame (ORF) encodes a protein of 227 amino acids, and designated as *Ca*PR5 (Fig. 1). The predicted ORF was 80% identical in amino acid sequence to *Zz*PR5 (Supplementary Table S1). The predicted protein contained all the known characteristics of PR5 gene family, including the 16 conserved cysteine residues. To gain more information on PR5 signal transduction, a *Z. officinale* homolog of the PR5, has also been isolated from ginger. The full-length sequence was 669 bp in length with a predicted ORF encoding a protein of 223 amino acids, and was, therefore, designated as *Zo*PR5 (Fig. 1). The full-length predicted protein shared sequence identity ranging from 56 to 83% with closely related homologs and contained all the conserved domains (Supplementary Table S1). Comparison of *Ca* and *Zo*PR5 sequences revealed nine nucleotide Indels in two locations along the protein (Supplementary Fig. S1). Taken together, the results suggest that *Ca* and *Zo*PR5 are the members of thaumatin-like protein family (Liu et al. 2010).

The calculated molecular mass of *Ca* and *Zo*PR5 is 24.1 and 23.6 kDa, respectively. The instability indexes (II) of *Ca* and *Zo*PR5 proteins are computed to be 24.05 and 26.14, respectively. This classifies the proteins as stable. The aliphatic index, regarded as a positive factor for increased thermostability, was calculated as 50.35 and 53.86. In the deduced amino acid sequence of *Ca*PR5 protein, the total number of negatively charged residues (Asp + Glu) was 13, while the total number of positively charged residues (Arg + Lys) was 12. In case of *Zo*PR5 protein, the values were 12 and 11, respectively. The SignalP program (Bendtsen et al., 2004) predicted that the *Ca* and *Zo*PR5 N-terminus would be cleaved out at between Ala22 and Ala23 by translational processing. To classify *Ca* and *Zo*PR5 sequences among the various PR5 s, a phylogenetic tree was generated (Fig. 2). The dendrogram analysis defines that PR5 gene family could be divided into three main classes based upon sequence conservation. In the first group, *Ca*PR5 and *Zo*PR5 were placed in close proximity to a *Z. zerumbet* (*Zz*PR5; Nair et al. 2010). The second group encompassed the *Piper colubrinum* [*Pc*OSM(ABX71220)] and two TLPs from *Musa* spp. [*Ba*TLP (iZ3Q)] and *Vitis vinifera* [*Vv*TLP (ABD64682], respectively. Interestingly, in the third group *Solanum*, *Nicotiana* and *Capsicum* were grouped together. The close evolutionary relationship between PR5, Osmotin and TLP was previously observed (Liu et al. 2010).

The crystal structure of *Ca* and *Zo*PR5

The three-dimensional structure of *M. acuminata* TLP (1Z3Q) was used as a template for homology modeling of *Ca*PR5 and *Zo*PR5. The crystal models obtained for *Ca*PR5 and *Zo*PR5 (Fig. 3) comprise the characteristic thaumatin-like fold. Analysis of the secondary structure in *Ca*PR5 revealed the presence of six α-helices, 10 *β*-strands, and 15 turns, whereas *Zo*PR5 possessed seven α-helices, nine *β*-strands, and 13 turns. Three distinct domains are evident in the 3D model of above two PR5. This result is consistent with the previously characterized PR5 proteins (Kim et al. 2009). Domain I forms the central core of the molecule, which corresponds to the N terminus of the protein. It is made up of two bundles of antiparallel *β*-sheet connected by loops to form a flattened *β*-sandwich. Domain II comprises a main α-helix associated with shorter helical segments, and domain III consists of *β*-strands linked by a loop. A prominent deep cleft that transverses the protein surface is found at the interface of domains I and II. Molecular modeling revealed that *Ca*PR5 and *Zo*PR5 possess the 3D folds and core structures that are conserved in other PR5 proteins.

Expression analysis of *Ca*PR5 and *Zo*PR5

The expression patterns of the two PR5 genes were analyzed using the quantitative real-time-PCR (qRT-PCR) after infection with *R. solanacearum* in both species. The

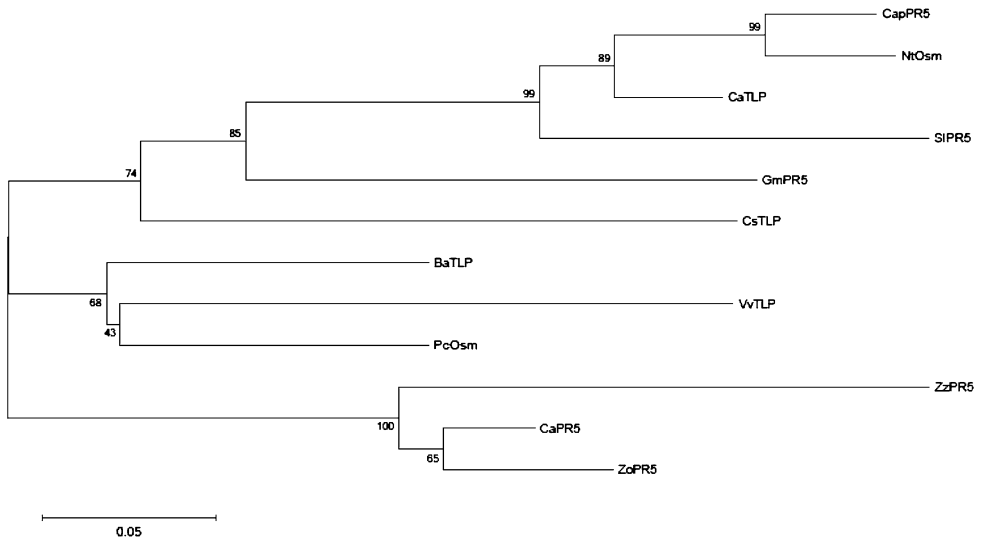

Fig. 2 Phylogenetic relationships between *Curcuma amada PR5* [*Ca*PR5]; *Zingiber officinale* [*Zo*PR5]; *Zingiber zerumbet* [*Zz*PR5 (ACL80664)]; *Musa spp.* [*Ba*TLP (iZ3Q)]; *Capsicum annuum* [*Ca*PR5 (ACB30367), *Ca*TLP (AAK97184)], *Glycine max* [*Gm*PR5 (BAH01715), *Vitis vinifera* [*Vv*TLP (ABD64682], *Piper colubrinum* [*Pc*OSM(ABX71220)], *Cammelia sinensis* [*Cs*TLP (ABE01396)], *Solanum lycopersicum* [*Sl*PR5(ACZ71038)] and *Nicotiana tobaccum* [*Nt*OSM(AAB224592)] based on amino acid sequences. Bootstrap values >50% are indicated at branch points

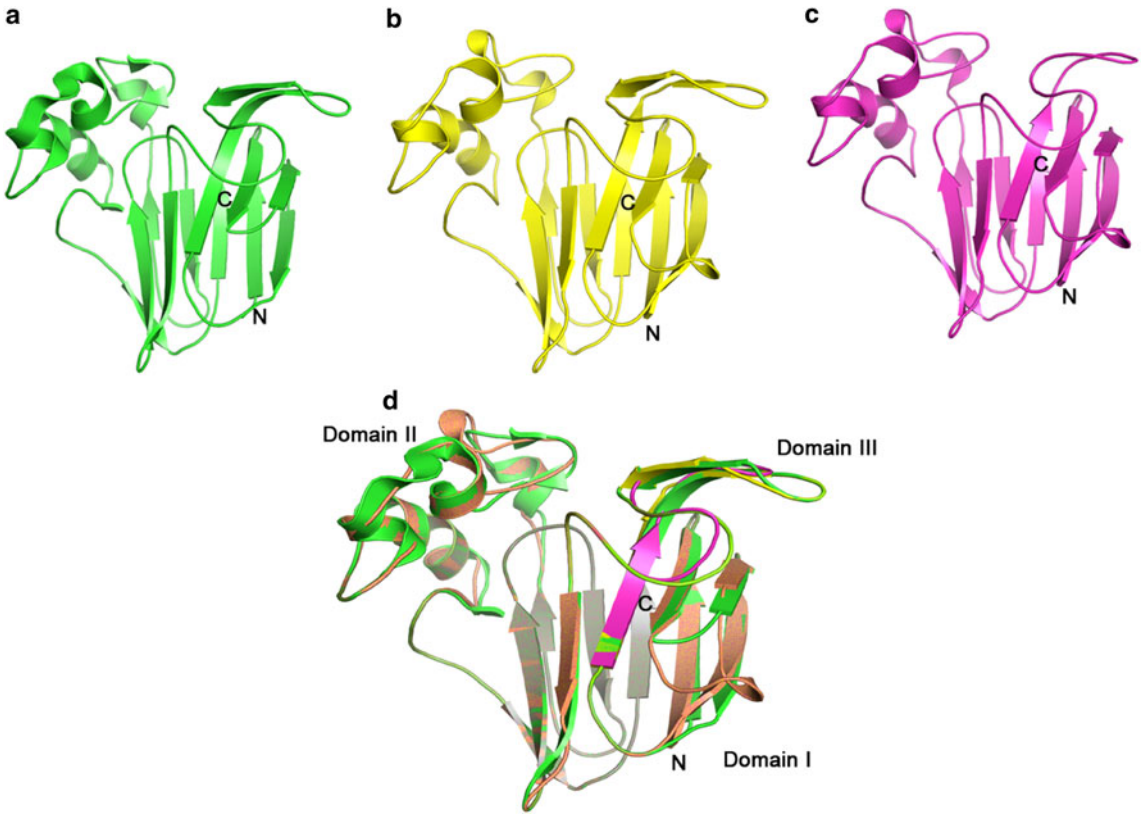

Fig. 3 Comparison of ribbon representation of PR5 [**a** (Banana, 1Z3Q); **b** (*Ca*PR5); **c** (*Zo*PR5); **d** (overlapping of three PR5)], showing the three domains. The cleft region lies at the interface of domains I and II. Three dimensional structures were developed using SwissPDB viewer swissModel software. The template used for homology modeling was 1Z3Q protein from banana

aim of this study was to gain insights into transcriptional changes that occur during defense responses triggered by *R. solanacearum*. Bacterial infection did influence the PR5 gene expression in *C. amada* (Fig. 4). *Ca*PR5 mRNA level was significantly elevated (greater than fourfold of the basal level) within 1 hai. Peaked at 4 hai and gradually declined after 8, 16, and 24 hai. Interestingly, no difference in the induction of *Zo*PR5 in ginger was observed, suggesting that the signal for systemic activation of this gene was not there when compared to *Ca*PR5 in *C. amada*. Although expression pattern of *Zo*PR5 in *Z. officinale* was modulated upon pathogen inoculation, transcript levels were approximately 5 and 15-fold lower than *Ca*PR5 in *C. amada* after 1 hai and 4 hai (Fig. 4).

The rapid and robust expression levels of *Ca*PR5 in *C. amada* within the first 4 h of inoculation (0–4 hai) could reflect a quick reinforcement of defense machinery at the attempted site of pathogen ingress, perhaps by sensing the physical force exerted by the pathogen. Such induction of pathogenesis-related genes is a critical factor deciding the host's effectiveness in suppressing an invading pathogen. Thus, the very early accumulation of PR5 transcripts further indicates a potentially crucial role for this gene in the *R. Solanacearum* resistance machinery of *C. amada*.

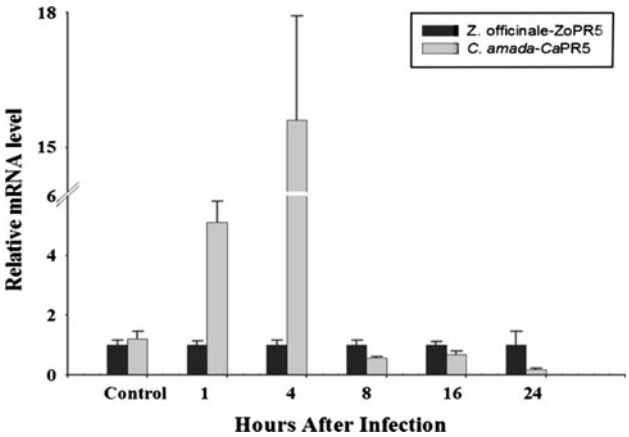

Fig. 4 Time course of the expression levels of the *Ca* and *Zo*PR5 genes in *C. amada* and *Z. officinale* determined by qRT-PCR after *R. Solanacearum* inoculation. The relative expression is the mean of three technical replicates. *Error bars* denote standard errors

In silico analysis of the *Ca and Zo*PR5 promoter sequence

The upstream flanking regions of *Ca*PR5 and *Zo*PR5 were isolated using Genome Walker™ Universal kit (Clontech,

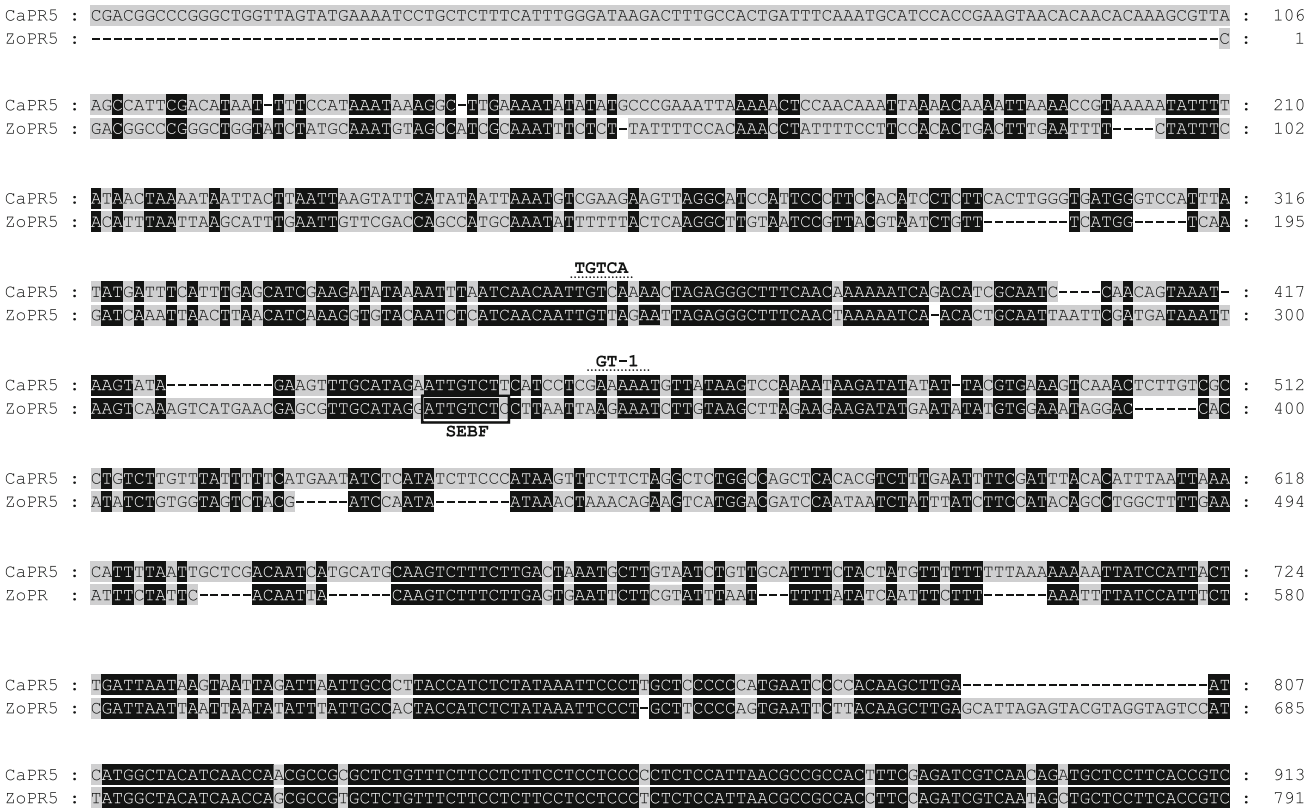

Fig. 5 Comparison of PR5 5′ flanking region of *C. amada* and *Z. officinale*. Conserved residues are shaded in *black*. The location of the putative *cis*-elements of interest identified using PLANTCARE is indicated. The potential SEBF is marked as *rectangle*. The GT-1 and W-box consensus sequences are highlighted with *dotted line* and *labeled*

Mountain View, CA, USA), sequenced (Fig. 5). *Cis*-acting regulatory elements are important molecular switches involved in the transcriptional regulation of a dynamic network of gene activities controlling various biological processes, such as response to hormones and abiotic stress and developmental processes. In silico analysis has been used with success in the identification of putative regulatory elements in plant promoters (Wei-Min et al. 2005; Pujade-Renaud et al. 2005). To identify conserved plant *cis*-acting regulatory elements, the *Ca*PR5 (−808 bp) and *Zo*PR5 (−686 bp) promoter sequences were analyzed using PlantCARE database. Several putative regulatory *cis*-elements were identified within the amplified fragment, including a number of elements associated with stress-related responses. As given in Table 2, a typical W-box element containing the consensus sequence TGAC was identified within the *Ca* and *Zo*PR5 5′-flanking region. W-boxes are binding sites for WRKY plant-specific transcription factors involved in stress-induced gene expression (Eulgem et al. 1999; Dong et al. 2003). The results revealed the presence of a sequence motif, in *Zo*PR5 and not in the *Ca*PR5 promoter sequence that shows 88.9% identity with a regulatory element found in the promoter of *SEBF* (Boyle and Brisson 2001). The results presented in Fig. 5 indicate that the *SEBF* binding site is located within the sequence of GACTGTCAC. The consensus binding site of *SEBF*, pyTGTCNC, is present in a number of PR genes and shows striking similarity to the auxin response element (Boyle and Brisson 2001). Two other putative cis-regulatory elements (GT-1 box and TGTCA) previously described as being involved in the activation of defense-related genes were also identified in *Ca*PR5. The sequence TGTCA is a motif recognized by a plant homeodomain transcription factor associated with disease resistance responses in rice (Luo et al. 2005). The GT-1 element (GAAAAA) is involved in the regulation of a gene activated by pathogen infection and salt stress (Park et al. 2004).

Promoter activity

To compare the activity of *Ca*PR5 and *Zo*PR5 promoters, the GUS reporter assay was used. *Ca*PR5 promoter construct (promoter without SEBF region) had strong GUS activity, twofold higher than in *Zo*PR5 promoter (construct with SEBF region). As expected GUS activity was weak in empty vector and was strong in 35S CaMV promoter (Fig. 6).

Table 2 Putative *cis*-elements present in the upstream sequence of *Ca and ZoPR5* gene

Putative *cis*-element/ consensus	Function/response	Location	
		*Ca*PR5	*Zo*PR5
ABRE, ACGTG	Early responsive to dehydration	−489, −582	–
ACGTATERD1, ACGT		–	−174, −671
BIHD1OS, TGTCA	Transcriptional factor, in disease resistance responses	−362	–
GT1CONSENSUS, GRWAAW	GT-1 binding sites in the PR-1A promoter influences the level of inducible gene expression in vivo	−24, −121, −142, −454, −525, −593, −683, −712	−52, −53, −67, −68, −91, −293, −387, −466
GT1GMSCAM4, GAAAAA	Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor	−454, −525	–
IBOXCORE, GATAA	Conserved sequence upstream of light-regulated genes	−47, −713	−293, −467, −569
MYBCORE, CNGTTR	Involved in regulation of genes that are responsive to water stress	−407, −676	−169
SEBFPR10A, YTGTCWC	Repression of the defense gene PR-10a by the single-stranded DNA binding protein SEBF	–	−333
WBOXATNPR1, TTGAC	WRKY DNA binding proteins in the regulation of NPR1 gene expression	−363, −497, −657	−191, −303
WBOXNTERF3, TGACY	Involved in activation of ERF3 gene	−496, −658	−82, −190, −302, −308, −442
WBOXNTCHN48, CTGACY	Possibly involved in elicitor-respsonsive transcription of defense genes in tobacco	–	−81
WRKY71OS, TGAC	Parsley WRKY proteins bind specifically to TGAC-containing W box elements within the PR-10 genes	−363, −497, −658	−82, −191, −303, −309, −443

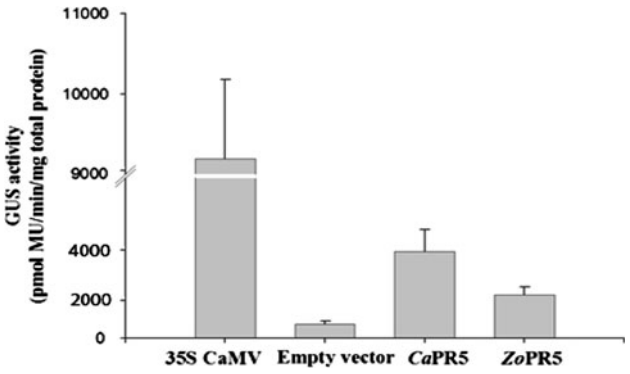

Fig. 6 Expression of *Ca*PR5-GUS and *Zo*PR5-GUS. Transient GUS activity (mean and standard errors) in Arabidopsis mesophyll with 35S CaMV, empty vector, *Ca*PR5-GUS and *Zo*PR5-GUS. *Error* bars denote standard errors

Discussion

Identification of PR genes and the determination of their expression patterns in response to various stresses have enhanced our understanding of the role of PR genes in stress adaptation in higher plants. To date, various PR5 genes have been cloned from different plants, such as PR5 from cherry tomato (Ren et al. 2010), *Pg*PR5 from ginseng (Kim et al. 2009), *Ph*OSM from Petunia (Kim et al. 2002) and *Zz*PR5 from *Z. zerumbet* (Nair et al. 2010). In this study, we describe the isolation and characterization of two PR5 genes from *C. amada* and *Z. officinale*. Searches of the NCBI database suggested that *Ca*PR5 and *Zo*PR5 genes belong to large group of PR5 genes. A comparison of the deduced amino acid sequences of *Ca*PR5 and *Zo*PR5 with other PR5s reveals expected similarities confirming them to be PR5 genes belonging to the 'thaumatin-like' sub group. As would be expected, *Ca* and *Zo* PR5s coding sequences have the highest identities (>90%) with each other, and grape PR5 sequences have moderate identities (more than 60%) with each other. Most PR5s, including TLPs possess 16 conserved cysteine residues; *Ca*PR5 and *Zo*PR5 also have 16 cysteines (Fig. 1). These 16 cysteines form eight disulfide bonds, representing a very thermostable and pH-stable compound (Breiteneder 2004). The disulfide bridges formed by these conserved cysteines help stabilize the molecule and allow for correct folding and high stability under extreme thermal and pH conditions (Fierens et al. 2009), as well as for resistance to protease degradation (Smole et al. 2008). *Ca*PR5 is predicted to encode a precursor protein of 227 amino acid residues with an isoelectric point of 5.25, whereas *Zo*PR5 to encode precursor protein of 223 amino acid with 5.18 isoelectric

point. The instability index (II) and aliphatic index that determine the stability of the proteins were similar, confirming that both proteins are stable. Previous studies reported that the extracellular PR5s tend to be acidic, whereas vacuolar PR5s tend to be basic (Stintzi et al. 1993). Both PR5s (*Ca* and *Zo*) contain an N-terminal signal peptide targeting mature proteins into the secretory pathway. A putative N-terminal elongation is known as a signal peptide sequence used for secretary transport (Sato et al. 1995). All of these observations suggest that *Ca*PR5 and *Zo*PR5 may also possess the antifungal activity toward some fungal species. The four additional amino acids in *Ca*PR5 have any role for the activity of this protein remains to be seen.

Thus far, crystal structures have been determined for seven plant TLPs (Liu et al. 2010), all show similar 3D structures with three domains and a cleft structure between domains I and II (Ghosh and Chakrabarti 2008). As shown in Fig. 3, the crystal structure of *Ca*PR5 and *Zo*PR5 has no striking differences, except for an extra loop in domain II.

The cleft between domain I and II is acidic in both the PRs because of five highly conserved amino acids (arginine, glutamic acid, and three aspartic acid residues). This acidic cleft is assumed to be relevant to their specific receptor binding for an antifungal activity (Koiwa et al. 1999; Min et al. 2004).

The family of PR5 is highly redundant in plants and their expression levels can vary from organ to organ and in response to different stimuli (Tachi et al. 2009). The reason why plants produce a large number of diverse PRs is not clear. However, it can be speculated that during evolution, plants have evolved many PR genes and isoforms to protect themselves against various types and races of pathogens. Indeed, different PR5 genes are known to be activated by different signals (Campos et al. 2002). This perhaps explains the presence of *Zo*PR5 in *Z. officinale* and *Ca*PR5 in *C. amada*. Therefore, the characterization of various PR5 isoforms is needed to better understand their roles and functions. The quick and robust induction of PR genes is another factor deciding the host's effectiveness in suppressing an invading pathogen (Pritsch et al. 2000; Ge et al. 2007). Thus, the very early and robust accumulation of *Ca*PR5 transcripts further indicates a crucial role for this gene in the *R. solanacearum* resistance machinery in *C. amada*. The same pathogenic bacteria did not result in significant induction of either *Ca*PR5 or *Zo*PR5 transcripts at 24 h (Fig. 4). The lack of timely and significant expression of PR5 in *Z. officinale* may be related to the lower sensitivity of *Zo*PR5 to *R. solanacearum* when compared with *C. amada*. The different expression patterns of PR5 proteins upon *R. solanacearum* treatment indicate the complexity of the PR families in higher plants.

The promoter sequences obtained (Table 2) have several previously recognized regulatory sequence motifs that are expected to be involved in the temporal and spatial control of PR5 expression. Accordingly, the 0.81 kb of *Ca*PR5's 5′-flanking region directed transient GUS expression was studied in *Arabidopsis* protoplasts. Consistent with earlier results, higher expression pattern was recorded for *Ca*PR5. In contrast, the GUS expression was weak with *Zo*PR5 promoter. This indicates that either the *cis*-elements present in the *Ca*PR5 or the silencing element (SE) in the *Zo*PR5 promoter region is essential for the regulation of these PR5 genes. The silencing elements present in the *Zo*PR5 promoter (SEBF and a GACTGTCAC motif) might be responsible for non activation of *Zo*PR5 promoter in *Z. officinale*. In potato, removal of the silencing element, which is located between −52 and −27, leads to further activation, suggesting that this element participates with the elicitor response element (ERE) in the regulation of *PR-10a* (Matton et al. 1993; Després et al. 1995; Boyle and Brisson 2001). The lack of extensive developmental expression and the insensitivity of *Zo*PR5-GUS promoter to *R. solanacearum* are likely to make this a good candidate promoter for studies requiring inducible gene expression.

In conclusion, our study identified a potential component of the basal defense of *C. amada* against *R. solanacearum*. Further studies on the analyses of *Ca*PR5 and its promoter functions using transgenic plants will reveal the exact roles and functions of *Ca*PR5 protein involved in the bacterial defense in *C. amada*.

Acknowledgments DP gratefully acknowledges the Department of Science and Technology, (DST), Government of India for awarding prestigious BOYSCAST fellowship and the Indian Council of Agricultural Research (ICAR), Government of India for the permission to undertake this research work. Funding from CFI and OIT to SJ are gratefully acknowledged.

References

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 programs. Nucleic Acids Res 25:3389–3402

Asselbergh B, Curvers K, Franca SC, Audenaert K, Vuylsteke M, Breusegem FV, Hofte M (2007) Resistance to *Botrytis cinerea* in *sitiens*, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. Plant Physiol 144:1863–1877

Bendtsen JD, Nielsen H, von Heijine G, Brunak S (2004) Improved prediction of signal peptides: SingalP 3.0. J Mol Biol 340:783–795

Boss PK, Davies C, Robinson SP (1996) Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiol 111:1059–1066

Boyle B, Brisson N (2001) Repression of the defense gene *PR-10a* by the single-stranded DNA binding protein SEBF. Plant Cell 13:2525–2537

Breiteneder H (2004) Thaumatin-like proteins-a new family of pollen and fruit allergens. Allergy 59:479–481

Campos MA, Ribeiro SG, Rigden DJ, Monte DC, Grossidesa MF (2002) Putative pathogenesis-related genes within *Solanum nigrum* L. var. *americanum* genome: isolation of two genes coding for PR5-like proteins, phylogenetic and sequence analysis. Physiol Mol Plant Pathol 61:205–216

Chen S, Li X, Cai Q, Hu N, He H, Yao YD, Mitola J (2010) Classification and control of cognitive radios using Hierarchical Neural Network. Adv Neural Netw Res Appl 67(4):347–353

Datta K, Velazhahan R, Oliva N, Ona I, Mew T, Khush GS, Muthukrishnan S, Datta SK (1999) Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease. Theor Appl Genet 98:1138–1145

Després C, Subramaniam R, Matton DP, Brisson N (1995) The activation of the potato *PR-10a* gene requires the phosphorylation of the nuclear factor PBF-1. Plant Cell 7:589–598

Dong J, Chen C, Chen Z (2003) Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Mol Biol 51:21–37

Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE (1999) Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. EMBO J 18:4689–4699

Evans IJ, Greenland A (1998) Trangenic approaches to disease protection: Applications of antifungal proteins. Pestic Sci 54:353–359

FAOSTAT (2010) Food and Agricultural commodities production. http://faostat.fao.org/site/339/default.aspx. Accessed 31 Aug 2010

Fierens E, Gebruers K, Voet AR, De Maeyer M, Courtin CM, Delcour JA (2009) Biochemical and structural characterization of TLXI, the *Triticum aestivum* L. thaumatin-like xylanase inhibitor. J Enzyme Inhib Med Chem 24:646–654

Garnier J, Gibrat JF, Robson B (1996) GOR method for predicting protein secondary structure from amino acid sequence. Methods Enzymol 266:540–553

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) The proteomics protocols handbook. Humana Press, Totowa, pp 571–607

Ge X, Li GJ, Wang SB, ZhuH ZhuT, Wang X, Xia Y (2007) AtNUDT7, a negative regulator of basal immunity in *Arabidopsis*, modulates two distinct defense response pathways and is involved in maintaining redox homeostasis. Plant Physiol 145:204–215

Ghosh R, Chakrabarti C (2008) Crystal structure analysis of NP24-I: a thaumatin-like protein. Planta 228:883–890

Grenier J, Potvin C, Trudel J, Asselin A (1999) Some thaumatin-like proteins hydrolyse polymeric β-1, 3-glucans. Plant J 19:473–480

Hooft RWW, Vriend G, Sander C, Abola EE (1996) Errors in protein structures. Nature 381:272

Jayasankar S, Li Z, Gray DJ (2003) Constitutive expression of *Vitis vinifera* thaumatin like protein after in vitro selection and its role in anthracnose resistance. Funct Plant Biol 30:1105–1115

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trends Biochem Sci 23:403–405

Kavitha PG, Thomas G (2008) Population genetic structure of the clonal plant *Zingiber zerumbet* (L.) Smith (Zingiberaceae), a wild relative of cultivated ginger, and its response to *Pythium aphanidermatum*. Euphytica 160:89–100

Kelman A (1954) The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693–695

Kim H, Mun JH, Byun BH, Hwang HJ, Kwon YM, Kim SG (2002) Molecular cloning and characterization of the gene encoding osmotin protein in Petunia hybrid. Plant Sci 162:745–752

Kim YJ, Lee JH, Jung DY, Gayathri S, Shim JS, In JG, Yang DC (2009) Isolation and characterization of pathogenesis-related protein 5 (PgPR5) gene from *Panax ginseng*. Plant Pathol J 25(4):400–407

Koiwa H, Kato H, Nakatsu T, Oda J, Yamada Y, Sato F (1999) Crystal structure of tobacco PR-5d protein at 1.8Å resolution reveals a conserved acidic cleft structure in antifungal thaumatin-like proteins. J Mol Biol 286:1137–1145

Kumar A, Hayward AC (2005) Bacterial diseases of ginger and their control. In: Ravindran PN, Babu KN (eds) Monograph on ginger. CRC Press, Boca Raton, pp 341–366

Kumar A, Sarma YR (2004) Characterization of *Ralstonia solanacearum* causing bacterial wilt of ginger in India. Indian Phytopathol 57:12–17

Kumar A, Bhai RS, Sasikumar B, Anandaraj M, Parthasarathy VA (2006) *Curcuma amada* Roxb. a bacterial wilt evading species in Zingiberaceae—A potential source of valuable genes for bacterial wilt resistance. In: The 4th International Bacterial wilt symposium, 17–20th July 2006. The Lakeside Conference Centre, Central Science Laboratory, York, UK

Latijnhouwers M, de Wit PJGM, Govers F (2003) Oomycetes and fungi: similar weaponry to attack plants. Trends Microbiol 11:462–469

Leone P, Menu-Bouaouiche L, Peumans WJ, Payan F, Barre A, Roussel A, Van Damme EJM, Rouge P (2006) Resolution of the structure of the allergenic and antifungal banana fruit thaumatin-like protein at 1.7-A. J Biochimie 88:45–52

Lescot M et al (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30:325–327

Liu JJ, Zamani A, Ekramoddoullah AKM (2010) Expression profiling of a complex thaumatin-like protein family in western white pine. Planta 231:637–651

Luo HL, Song FM, Goodman RM, Zheng Z (2005) Up-regulation of OsBIHD1, a rice gene encoding BELL homeodomain transcriptional factor, in disease resistance responses. Plant Biol 7:459–468

Matton DP, Prescott G, Bertrand C, Camirand A, Brisson N (1993) Identification of *cis*-acting elements involved in the regulation of the pathogenesis-related gene *STH-2* in potato. Plant Mol Biol 22:279–291

Min K, Ha SC, Hasegawa PM, Bressan RA, Yun D-J, Kim KK (2004) Crystal structure of osmotin, a plant antifungal protein. Proteins Struct Funct Bioinform 54:170–173

Muthukrishnan S, Liang GH, Trick HN, Gill BS (2001) Pathogenesis-related proteins and their genes in cereals. Plant Cell Tissue Organ Cult 64:93–114

Nair RA, Kiran AG, Sivakumar KC, Thomas G (2010) Molecular characterization of an oomycete-responsive PR-5 protein gene from *Zingiber zerumbet*. Plant Mol Biol Rep 28:128–135

Nicholas KB, Nicholas HB, Deerfield DW (1997) GeneDoc: analysis and visualization of genetic variation, EMBnet News 4:14

Osmond RI, Hrmova M, Fontaine F, Imberty A, Fincher GB (2001) Binding interactions between barley thaumatin-like proteins and (1,3)-β-D-glucans. Kinetics, specificity, structural analysis and biological implications. Eur J Biochem 268:4190–4199

Park HC, Kim ML, Kang YH, Jeon JM, Yoo JH, Kim MC, Park CY, Jeong JC, Moon BC, Lee JH, Yoon HW, Lee SH, Chung WS, Lim CO, Lee SY, Hong JC, Cho MJ (2004) Pathogen- and NaClinduced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. Plant Physiol 135:2150–2161

Pasquali G, Ouwerkerk PB, Memelink J (1994) Versatile transformation vectors to assay the promoter activity of DNA elements in plants. Gene 149:373–374

Pritsch C, Muehlbauer GJ, Bushnell WR, Somers DA, Vance CP (2000) Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum*. Mol Plant Microbe Interact 13:159–169

Pujade-Renaud V, Sanier C, Cambillau L, Pappusamy A et al (2005) Molecular characterization of new members of the *Hevea brasiliensis* hevein multigene family and analysis of their promoter region in rice. Biochim Biophys Acta 1727:151–161

Ravindran PN, Sasikumar B, Johnson KG, Ratnambal MJ, Babu KN, Zachariah JT, Nair RR (1994) Genetic resources of ginger (*Zingiber officinale* Rosc.) and its conservation in India. Plant Genet Resour Newsl 98:1–4

Ravindran PN, Babu KN, Shiva KN (2005) Botany and crop improvement of ginger. In: Ravindran PN, Babu KN (eds) Monograph on ginger. CRC Press, Boca Raton, pp 15–85

Ren X, Kong Q, Wang P, Jiang F, Wang H, Yu T, Zheng X (2010) Molecular cloning of a PR-5 like protein gene from cherry tomato and analysis of the response of this gene to abiotic stresses. Mol Biol Rep. doi:10.1007/s11033-010-0169-0

Sato F, Koiwa H, Saki Y, Kato N, Yamada Y (1995) Synthesis and secretion of tobacco neutral PR-5 protein by transgenic tobacco and yeast. Biochem Biophys Res Commun 211:909–913

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 3:3381–3385

Smole U, Bublin M, Radauer C, Ebner C, Breiteneder H (2008) Mal d2, the thaumatin-like allergen from apple, is highly resistant to gastrointestinal digestion and thermal processing. Int Arch Allergy Immunol 147:289–298

Stintzi A, Heitz T, Prasad V, Kauffmann WMS, Geoffroy P, Legrand B, Fritig B (1993) Plant "Pathogenesisrelated" proteins and their role in defense against pathogens. Biochimie 75:687–706

Tachi H, Fukuda-Yamada K, Kojima T, Shiraiwa M, Rakahara H (2009) Molecular characterization of a novel soybean gene encoding a neutral PR-5 protein induced by high salt stress. Plant Physiol Biochem 47:73–79

Trudel J, Grenier J, Potvin C, Asselin A (1998) Several thaumatin-like proteins bind to β-1,3-glucans. Plant Physiol 118:1431–1438

van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol 44:135–162

Wei-Min L, Zhi-Xing W, Xin-Wu P, Shi-Rong J (2005) Cloning and characterization of the light-inducible *Gacab* promoter from *Gossypium arboreum*. Agric Biotechnol 2:17–22

Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572