academicJournals

Vol. 13(8), pp. 962-972, 19 February, 2014
DOI: 10.5897/AJB12.2068
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Function of *VtPGIP* in pathogenic fungus resistance of *Vitis thunbergii*

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Accepted 12 November, 2013

In plants, polygalacturonase inhibitor proteins (PGIPs) are very important to inactivate polygalacturonases secreted by pathogens. *Vitis thunbergii* Sieb. et Zucc. polygalacturonase inhibitor proteins (*VtPGIP*) was first isolated from the wild grape *Vitis thunbergii* Sieb. et Zucc., which exhibits high resistance to disease. *VtPGIP* is sublocated in the plant cell wall, and this location is consistent with the function of PGIPs in the first line of host defense. The promoter of *VtPGIP* contains salicylic acid (SA), abscisic acid (ABA), and fungus infection response elements. Results from real-time quantitative reverse transcriptase (RT)-PCR analysis showed that *VtPGIP* expression was induced by SA, ABA, and fungi. The results indicated that *VtPGIP* may have important functions in defense-related responses of *V. thunbergii* against pathogenic fungi.

Key words: *PGIP*, disease resistance, pathogenic fungi, *Vitis thunbergii*.

INTRODUCTION

Epiphytes cause significant losses as destructive pathogens of many fruits and vegetables worldwide; such organisms particularly affect the post-harvest industry. The main methods used to control fungal diseases include field and equipment sanitation, crop rotation, soil fumigation (Wheeler et al., 1994), and fungicide application; however, fungicides are deleterious to the environment. Other strategies are more promising and have been shown to decrease the incidence of fungal diseases by enhancing natural plant defense capabilities (Kessmann et al., 1994). A very important aspect is to determine potential molecules that exhibit defense functions in plants, that is, the natural defense system, and the molecules that respond to induction.

Fungal disease is caused by the secretion of hydrolytic enzymes to pectin substrates (Fish, 2005). Pathogens can release several types of enzymes, such as exo-polygalacturonases and endopolygalacturonases (endo-PGs), to breach this barrier and function with pectin methyl and acetyl esterases (Prade et al., 1999) to degrade pectin. Fungal endo-PGs, the first enzymes secreted by fungal plant pathogens, have important functions during the early stages of plant pathogenesis (English et al., 1971) to separate and macerate host tissues, thereby facilitating pathogen penetration and colonization of plant tissues. Subsequently, the products of this degradation process are used as a nutrient source for fungal growth (Karr et al., 1970).

Polygalacturonase-inhibiting proteins (PGIPs) are basic proteins present in the cell walls of most plants; PGIPs

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Abbreviations: SA, Salicylic acid; **ABA,** abscisic acid; **PGIP,** polygalacturonase inhibitor protein; **PG,** polygalacturonase. ***VtPGIP*,** *Vitis thunbergii* Sieb. et Zucc. *PGIP*.

are specific, saturable, reversible, and high-affinity ‘receptor’ endo-PGs of fungi (Cervone et al., 1987, 1990, 1989). PGIPs can directly impede pathogen invasion by inactivating polygalacturonases (PGs) (Hegedus et al., 2008). The inhibition of PG activities by PGIPs has been proposed to prolong the accumulation of oligogalacturonides and improve defense response (Federici et al., 2008). For instance, PGIPs of *Pyrus communis* (Tamura et al., 2004) and *Gossypium hirsutum* (James et al., 2001) inhibit fungal PG activity. However, field trials with the pear PGIP-expressing tomato lines provide contradictting results of the functions of PGIP in resistance. Hence, PGIPs from different plants vary in terms of inhibitory activity; PGIPs from a single plant also inhibit PGs from different fungi or different PGs from the same fungus with various strengths (Desiderio et al., 1997). For instance, a PG from *Aspergillus niger* is inhibited by PvPGIP1 and PvPGIP2 (Leckie et al., 1999) but not by PvPGIP3 and PvPGIP4 (D’Ovidio et al., 2004). *Fusarium moniliforme* PG is inhibited only by PvPGIP2 (Leckie et al., 1999). Hence, plants have evolved PGIPs with different recognition capabilities to counteract numerous PGs secreted by pathogenic fungi. *Arabidopsis thaliana* possesses two *PGIP* genes, which function in response to *Botrytis cinerea* infection; however, *Arabidopsis* PGIPs cannot inhibit PGs produced by *F. moniliforme* and *A. niger*; by comparison, *Arabidopsis* PGIPs can inhibit PGs produced by *Colletotrichum gloesporioides*, *Stenocarpella maydis*, and *B. cinerea* (Ferrari et al., 2003).

Vitis thunbergii Sieb. et Zucc. (*V. thunbergii*) is native to China and grown throughout the central and southern parts of China as well as in Korea and Japan. *V. thunbergii* has a strong resistance to *Coniothyrium diplodiella*, *Glomerella cigulata*, and other disease-causing pathogens. In addition, the plant hormones salicylic acid (SA) and abscisic acid (ABA) have been implicated in various plant responses (Rao et al., 2000; Borsani et al., 2001; Turner et al., 2002; Xiong et al., 2002). The cloned *Vt*PGIP promoter sequence contains SA and ABA-related elements components and allows the study of the expression and regulation of *Vt*PGIP by the corresponding SA or ABA treatment. In this study, *VtPGIP* was characterized to determine whether or not the *PGIP* gene in *V. thunbergii* is responsible for disease resistance. We also described *VtPGIP* expression in response to applied SA, ABA, and fungal infection. *Vt*PGIP may have important functions in the disease resistance of *V. thunbergii*.

MATERIALS AND METHODS

Plant materials and growth conditions

V. thunbergii plantlets were grown at 25°C under a 16 h/8 h (day/night) photoperiod by tissue culture. The plantlets were cultivated in the medium of 3/4 MS supplemented with 0.35 mg·L^{-1} indole-3-butyric acid.

Plant treatments

The leaves of seven-week-old intact tissue culture plantlets were selected to examine the effect of exposure to different defense response activators (signaling molecules). SA (Sigma, St. Louis) and ABA (Sigma, St. Louis) were dissolved in water, and the aerial parts of the plants were sprayed with 5 mM SA (Ferrari et al., 2003) or 50 mM ABA (Yuasa et al., 2007). The plants of the same age and treated with distilled water were used as control plants. *VtPGIP* expression on the leaves of tissue culture plantlets was analyzed at 0, 2, 6, 16, 24, 48, and 72 h after ABA and SA treatments. After harvest, the leaves were immediately frozen in liquid nitrogen and stored at -70°C for subsequent RNA extraction.

The leaves of *V. thunbergii* tissue culture plantlets were inoculated with pathogenic fungi (*C. diplodiella, Erysiphe cichoracearumr*, *G. cigulata*, and *B. cinerea*). The plantlets of the same age and treated with water were used as control plants. After 3 d, the leaves treated with pathogenic fungi and water were harvested, immediately frozen in liquid nitrogen, and stored at -70°C for subsequent RNA extraction.

Isolating the *VtPGIP* gene

Genomic plant DNA was isolated using the CTAB extraction method described in a previous study (Chang et al., 1993). Total RNA was extracted by scaling down the experimental conditions previously described (Chang et al., 1993). Plant leaves (200 mg) were ground in liquid nitrogen to a fine powder by using mortar and pestle. Approximately, 900 µl of extraction buffer was added and the mixture was mixed thoroughly. The mixture was extracted with 900 µl of chloroform/isoamyl alcohol (24:1 v/v). The upper phase was transferred to a fresh tube, and the hydroxybenzene/chloroform/isoamylalcohol (25: 24:1 v/v/v) was added. The mixture was vortexed vigorously and centrifuged at 12,000 rpm for 10 min at 4°C. The clear supernatant was carefully transferred to a clean tube and extracted again with the chloroform/isoamylalcohol (24:1 v/v). The RNA was precipitated with 10 M LiCl for 8 h at -20°C, washed with 70% cold ethanol, air dried for 20 min, and resuspended RNA precipitate in 30 µl DEPC-treated ddH$_2$O. CDNA was synthesized using the ReverTra Ace-a-TM kit (ToYoBo, Osaka, Japan) according to the manufacturer’s instructions.

PCR was carried out in a 25 µl reaction volume containing 10× buffer, MgCl$_2$, dNTPs, primers, 50 to 100 ng of template DNA or cDNA, and 1 unit of *Taq* DNA polymerase using the following PCR profile: 4 min at 94°C; 38 cycles of 1 min at 94°C; 2 min at 54°C; 2 min at 72°C; final extension step of 72°C for 10 min. The gene primers [Pgip F: 5′-ATGGAGACTTCAAAACTTTTTCTTC-3′ (forward) and Pgip R: 5′-TCACTTGCAGCT CTGGAGTGG-3′ (reverse)] used were designed based on the sequencing of *V. vinifera* (Pinot noir PN40024).

The obtained amino acid sequence was aligned with related genes by using BioXM, and the alignment was analyzed using PROSITE (http://ca.expasy.org/prosite/). The three-dimensional structure of the VtPGIP protein and molecular modeling were analyzed using Swiss-Pdb Viewer 3.7.

Cloning of the *VtPGIP* promoter

The primers [F1: 5′-TGGGGAGGCTCTACCCAGGA-3′ (forward) and Q1: 5′-GGACGAGTGGCGAGTAAGAGTAGGAGG-3′ (reverse)] were designed based on the sequencing of *Vitis* (Pinot noir, PN40024). The promoter of the *PGIP* gene sequence was cloned by PCR. PCR was performed using genomic DNA extracted from *V. thunbergii* leaves with the primers p-pgip-F1/-Q1. A 36-cycle PCR was conducted (one cycle was performed under the following

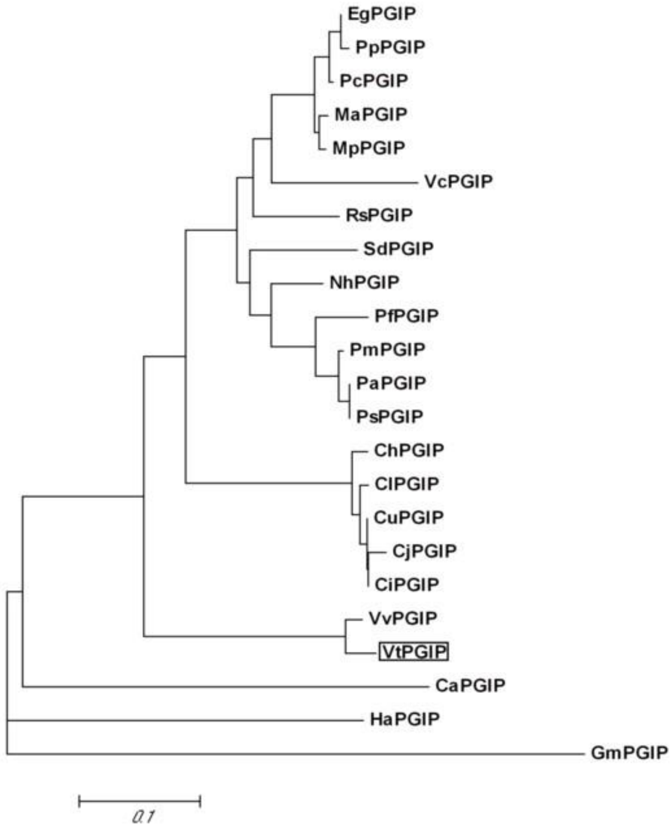


Figure 1. A phylogenetic tree of PGIPs from plants. In addition to the predicted proteins soybean, pear, and other PGIP used for phylogenrtic analysis include the PaPGIP(AAW57430), PmPGIP(AAF79181), EgPGIP(AAR15145), PcPGIP(AAP92913), MaPGIP(ABA26937), GmPGIP(CAI99394), VvPGIP(AAM74142), VtPGIP(ABU82741), ChPGIP(BAB85785), ClPGIP(BAB85784), CjPGIP(BAA28745), CuPGIP(BAA31841), CiPGIP(BAA31842), CaPGIP(ACB30360), MpPGIP(AFC95832), PpPGIP(AEO36938), PsPGIP(ACY41032), PfPGIP(ACY41031), HaPGIP(ABW89527), VcPGIP(AAK43471), SdPGIP(AAK43459), RsPGIP(AAK43455), NhPGIP(AAK43462). They were clustered using Clustal W2 and the unrooted phylogenetic tree was generated using Mega 3.1 with neighbor-joining method.

conditions: 1 min at 94°C; 2 min at 58°C; and 2 min at 72°C) with a final extension of 10 min at 72°C. The products were cloned into a pMD19-T vector (TaKaRa), and the two clones were sequenced.

The promoter of the *VtPGIP* gene was analyzed using promoter prediction software programs (Promoter Scan, Neural Network and CpG Island) and transcription factor prediction software programs (TF Search, Match 1.0-public and Plantcare).

Subcellular localization of the *Vt*PGIP protein

To determine the subcellular distribution of the *Vt*PGIP protein, we generated a plasmid that enabled the expression of *Vt*PGIP fused to EGFP. This plasmid was used as control and was transformed into onion epidermal cells. The entire coding region of the *VtPGIP* gene was cloned into the plasmid of Pyk2784-EGFP (enhanced green fluorescent protein), generating the p35S:*VtPGIP*-EGFP plasmid, which was designed to express the in-frame fusion protein of *VtPGIP*-EGFP. Onion epidermal cells were subsequently transformed with p35S: *VtPGIP*-EGFP or the control plasmid p35S:EGFP according to the particle bombardment method by using a particle delivery system (Bio-Rad Biolistic PDS-1000/He; Bio-Rad, Hercules, CA). The transformed onion epidermal cells were incubated at 25°C in the dark for 12 h, and *VtPGIP*-EGFP expression was then examined under a fluorescent microscope (Axio Imager A1; Carl Zeiss, Germany).

VtPGIP gene was induced by SA, ABA, and fungi

PCR was performed using 0.5 µg of cDNA to 1 µg of cDNA in a 20 µl reaction volume containing 10 µl of SYBR Green Master Mix (including *Taq* polymerase, dNTPs, $MgCl_2$, 10× buffer, SYBRgreen I; ToYoBo, Osaka, Japan), and two primers, using the following PCR profile: 95°C for 2 min; and 40 cycles of 94°C for 20 s, 59°C for 20 s, and 72°C for 20 s on a 7300 Real-time PCR System (Applied Biosystems). The following primers were used:

Target gene primer:

S1: 5′-TGTTCCAGTTTGATCTTTCGAG-3′
S2: 5′-CAAGCACCGATTGTGAAAGTAG-3′

Housekeeping gene primer:

actin1: 5′-TACAATTCCATCATGAAGTGTGATG-3′
actin2: 5′-TTAGAAGCACTTCCTGTCAACAATG-3′.

The PCR efficiency of each target mRNA was obtained; the absence of primer-dimer formation, which could interfere with the specific amplification, was checked in no template control sample. Each time point was determined as an average of the data obtained from triplicate trials. Relative gene expression was calculated using the following equation:

Relative expression = $[E_{target}^{\Delta CPtarget\ (control\ –\ sample)}]/[\ E_{reference}^{\Delta CPref(control\ –\ sample)}]$.

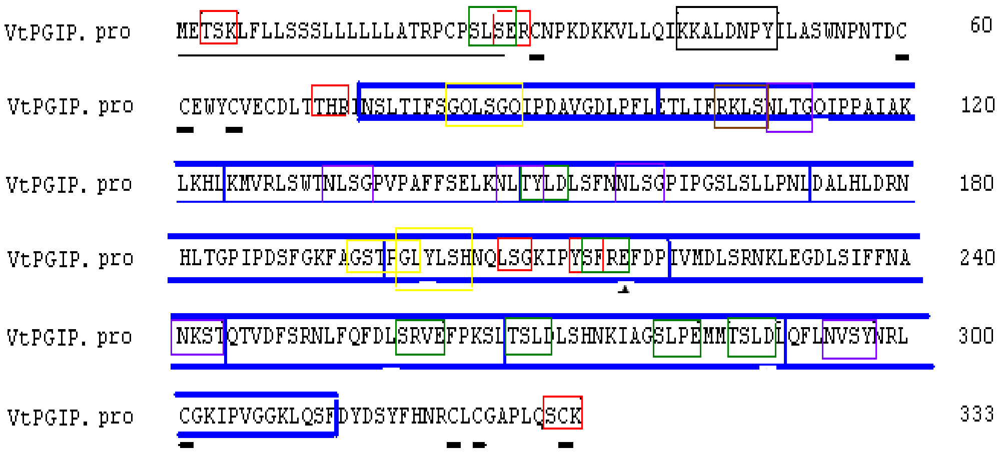
The relative levels of *VtPGIP* used to control *PGIP* mRNA were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The threshold cycle (Ct) value represents the PCR cycle in which the copy number passes the fixed threshold and can be first detected.

RESULTS

Analysis of the *VtPGIP* gene

A *PGIP* gene (EU037367) with a length of 1002 bp was isolated from *V. thunbergii VtPGIP* cDNA sequence was compared with the DNA sequence and showed no introns (unpublished). The amino acid sequences of some PGIPs were also compared and the results revealed sequence similarities as indicated by a phylogenetic tree of PGIPs from plants (Figure 1). Using sequence comparison data, we found that the species of the same genus were classified into the same group except *Eucalyptus grandis*, *Pyrus communis*, and *Malus pumila* because the PGIPs of these plants may have different evolutionary scenarios.

Similar to the majority of PGIPs, *Vt*PGIP showed unique characteristics and the encoded products were composed of 333 amino acid residues (Figure 2). The biological site was analyzed and a signal peptide of 27 amino acid residues of the *Vt*PGIP (Figure 3) was identified using Signal P and eight cysteine residues at



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VtPGIP. pro    METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC    60
VtPGIP. pro    CEWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK    120
VtPGIP. pro    LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLDALHLDRN    180
VtPGIP. pro    HLTGPIPDSFGKFAGSTFGLYLSHNQLSGKIPYSFREFDPIVMDLSRNKLEGDLSIFFNA    240
VtPGIP. pro    NKSTQTVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL    300
VtPGIP. pro    CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK                               333
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Figure 2. The deduced PGIP protein sequence obtained from *V. thunbergii*. The complete open reading frame of *VtPGIP*, the region underlined indicated the signal protein. The underlined symbol indicated the cysteine. The biological significance of the site of these 333 amino acids translated were analysed, whereas the regions in the protein sequence in the red frame indicate six protein kinase C phosphorylation sites (3-5，27-29，72-74，208-210，214-216，331-333). The regions in the green frame showed seven casein kinase II phosphorylation sites (25-28, 149-152, 214-217, 259-262, 268-271, 280-283, 286-289). The region in the black frame showed one tyrosine kinase phosphorylation site (42-49). The regions in the yellow frame showed three N-myristoylation sites (83-88, 195-200, 199-204). The region in the brown frame showed one cAMP- and cGMP-dependent protein kinase phosphorylation site (105-108), The regions in the purple frame showed six N-glycosylation sites (109-112, 133-136, 147-150,157-160, 241-244, 294-297), the framed regions in the protein sequence indicated ten repeat regions, and the consensus of the secondary structure of the LRR motif (xxLxLxx-NxL-t/sGxIPxxLxxL-xxL): any amino acid (x), leucine or an aliphatic amino acid (L), isoleucine (I), serine (S), threonine (T), Asparagine (N), Glycine (G), Proline (P).

conserved positions (Figure 2). *Vt*PGIP was typical of extracellular leucine-rich repeat (LRR) proteins; each protein with 10 repeats exhibits variations in the xxLxLxx-NxLt/sGxIPxxLxxLxxL (De Matteo et al., 2006) 24 amino acid consensus. However, the xxLxLxx motif of the LRR regions is considered as the main site of PG interaction (De Matteo et al., 2006). The computational and mutational analyses identified the residues within and near this region that can account for the specificity after PG-PGIP interactions (Figure 2).

Considering the structure of PGIP from *Phaseolus vulgaris* (De Matteo et al., 2006), we found that the N-terminal regions of the *Vt*PGIP contained disulfide bridges and the C-terminal regions contain disulphide bonds (Figure 4). The N-terminal regions of the *Vt*PGIP consist of an α-helix residue and a short β-strand that forms H-bonds with the residues of sheet B1. *Vt*PGIP structure, which contains seven α-helix motifs, were analyzed using Swiss-Pdb Viewer 3.7. In the *Vt*PGIP structure, the C-terminal region comprises the last two 3_{10}-helices, the last strand of sheet B2, and a short loop (Figure 4).

Analysis of the *VtPGIP* promoter

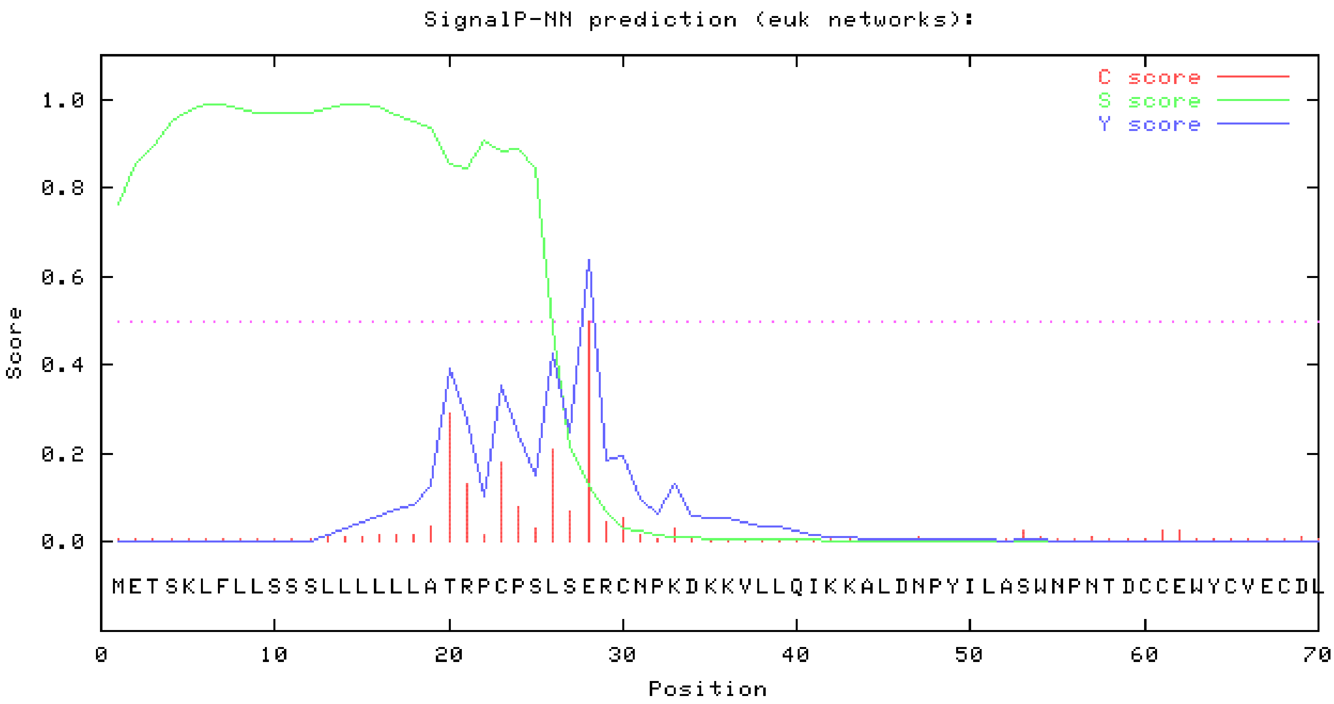
The promoter sequence of *VtPGIP*, with a length of 1650 bp (Figure 5), was obtained (JF832390) and the promoter sequence was analyzed (Figure 5 and Table 1a,b). The promoter of the *VtPGIP* gene contained *cis*-acting elements involved in the responses to ABA (ABRE), SA (TCA-element), and fungal elicitor responsive element (Box-W1). The promoter also contains transcription factor binding sites, such as Nkx2-5 and c-Rel.

Analysis of the subcellular distribution of the *Vt*PGIP protein

The control proteins were uniformly distributed in the cells (Figure 6A, 6B, and 6C). The *Vt*PGIP-EGFP fusion proteins were predominately located in the plant cell wall (Figure 6D, 6E, 6F). The signal peptide sequence of VtPGIP was analyzed online and the result indicated that the sequences should have accordant distribution located in the plant cell wall.

Effect of the treatments on *VtPGIP* gene expression

Studies on plant defense signaling have revealed that plants adopt a network of signal transduction pathways via different kinds of signaling molecules. Gene products were verified after the respective PCR products were sequenced. *VtPGIP* expression was analyzed in the treated



Measure	Position	Value	Cut-off point	Signal peptide?
max. C	28	0.498	0.32	YES
max. Y	28	0.636	0.33	YES
max. S	6	0.990	0.87	YES
mean S	1-27	0.888	0.48	YES
D	1-27	0.762	0.43	YES

Figure 3. The signal peptides were identified using Signal P, and the Signal P-NN. The most likely cleavage site is between positions 27 and 28: SLS-ER.

leaves and then compared with that in the untreated control leaves. The result confirmed that *VtPGIP* expression was induced after ABA and SA treatment (Figures 7A and 7B). The highest transcription level was found at 2 h after SA treatment compared with the control group (0 h), and the highest *VtPGIP* expression was increased 57.68 times compared with that of the control group (Figure 7B). For the ABA treatment, the highest *VtPGIP* expression was found at 6 h, and this expression was 10 times greater than that of the control group (Figure 7A). The results confirmed that the *VtPGIP* gene was strongly induced by ABA treatment and consisted of the promoter sequence containing *cis*-acting elements involved in the responses to ABA and SA.

The relative *VtPGIP* expressions from the leaves inoculated with four different pathogens were monitored. The results showed that *VtPGIP* expression from the leaves infected with fungi was higher than that in the control group (Figure 8). After the plants were infected with *B. cinerea*, *VtPGIP* expression increased 100 times higher than that of the control group. Similarly, after *C. diplodiella* vaccination, *VtPGIP* expression relative to that of the control group was also approximately 100 times higher. After *E. cichoracearum* and *G. cigulata* were inoculated, *VtPGIP* expression increased approximately 10 times compared with that of the control group. These results suggested that *VtPGIP* was involved in plant disease resistance.

DISCUSSION

The structural data and the close homology with other *PGIP*s confirmed that the sequence was *PGIP*. *VtPGIP* was previously isolated and showed a high degree of similarity to *PGIP* genes from other plants. For the amino acid sequence, the species of the same genus were classified into the same group, but *E. grandis*, *P. communis*, and *M. pumila* may indicate that PGIPs have various evolutionary histories. In all of the PGIPs studied, the LRR domain is conserved, which reveals that protein-protein interactions are involved in immune functions and

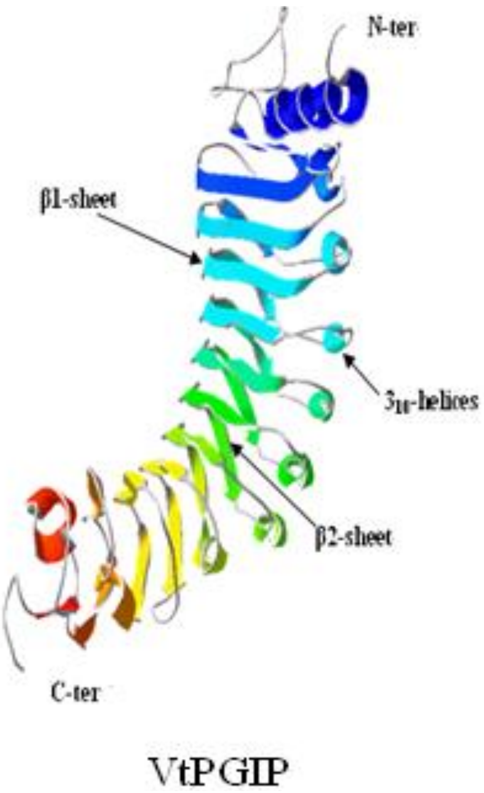


Figure 4. Ribbon representation of the crystal structure of the VtPGIP. As with the ribbon representation of the crystal structure of PGIP2 from *Phaseolus vulgaris* (De Matteo et al., 2006), the figure showed that the *VtPGIP* had a right-handed superhelical fold typical of LRR proteins. The fold of the VtPGIP consists of a central LRR domain flanked by the N- and C-terminal cysteine-rich regions and, also like the crystal structure of PGIP2 from *Phaseolus vulgaris*, the secondary structure of the LRR motif showed seven α-helices located on the convex side of the VtPGIP protein. Meanwhile, the majority of the LRR proteins had two B sheets connected with the helices on the convex side by loops or b-turns were analysed by Swiss-Pdb Viewer 3.7, parallel to the B2 sheet. B2 is distorted because of the twisted shape of the molecule and the variable length of the β-strands (Hegedus et al., 2008). From this figure, it showed the VtPGIP had twenty-two B -sheets. Sheets B1 and B2 are xLx and xxLxLxx regions, respectively, and the helix is the xLt/sGx region.

recognition of non-self molecules in plants (Jones, 2001). The *Vt*PGIP protein may inhibit PG activity because the sequence 'function-sites' of LRR did not change during evolutionary development.

The three-dimensional structure of the bean PGIP has been investigated (Penninck et al., 1996), and results showed that the two extended B sheets in the LRR region are highly conserved; these B sheets are also involved in the interactions with pathogen PGs (De Matteo et al., 2003). The consensus LRR domain of the *Vt*PGIP showed similarities to the LRR regions of other PGIPs and PGIP-like plant proteins. The three-dimensional structures of *Vt*PGIP were also analyzed (Figure 4). In the structural view, *Vt*PGIP showed numerous sites similar to *P. vulgris* PGIP (*Pv*PGIP); hence, it may also contain PG-binding sites. The sequences of the amino acid residue of *Vt*PGIP were analyzed and four cysteine residues were identified at the flanks of the LRR regions within the N- and C-terminal domains; this result is consistent with that of the PGIP of bean (De Matteo et al., 2006). The results suggested that the type of N-terminal extension affects substrate specificity and may determine the functional diversity of these enzymes. The N- and C-terminal cysteine-rich regions then form disulphide bridges, which are considered to be important in maintaining and providing additional stability of the secondary and tertiary structures. In the functional view, glycosylation possibly provides higher stability and increased protease resistance. This characteristic has been observed in many fungal PGs and may be crucial for their enzymatic activity. The six N-linked glycosylation sites were found in the LRR domains in *Vt*PGIP (Figure 2). The positions and numbers of the N-linked glycosylation sites were not highly conserved among plant PGIPs (Mattei et al., 2001).

The subcellular localization of the transiently expressed PGIP protein was determined. The result showed that *Vt*PGIP was localized in the plant cell wall, and this finding is consistent with that in previous studies on the localization of many other PGIPs (De Lorenzo et al., 2001). The plant cell wall provides structural stability (Shanmugam, 2005) and functions as the first barrier to counteract pathogens; PGs are also the first enzymes to be secreted when pathogens encounter plant cell walls (De Lorenzo and Ferrari, 2002). PGIPs are located in the plant cell wall and limit fungal invasion by counteracting the action of PGs.

Plants may have evolved mechanisms to respond to pathogens and other stress-related molecules (Ramonell et al., 2002). PGIPs may have an important function in the overall resistance to biotic and abiotic stresses (Li et al., 2003). The characteristics of *PGIP* promoter sequence was analyzed using promoter prediction software and transcription factor prediction software because the regulation of gene expression only covers a small part of the non-coding region; experimental studies are often difficult to conduct without the specific software. The promoter sequences were analyzed online to detect the presence of putative *cis*-acting regulatory elements in the promoter regions of *VtPGIP* (Table 1a,b). Several elements, including ABRE, Box-4, G-box, W1-box, HSE, TC-rich repeats, and TCA-element, were detected. *VtPGIP* expression was regulated by ABA, SA, and fungi. Such hormones and pathogens functioned as positive regulators of *VtPGIP* expression in the present experiment. In addition, several elements containing a binding site for Nkx2-5 and c-Rel transcription factors were

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-1650  TGGGGAGGCTCTACCCAGGAAAGGGCGATGGTTCCCAGGGTCACTTGGTGAAGCTCCATG
-1590  TAGTGTGAGTGGCAGTGTAAGAACGTGGCCCACAAATCCTATGGATTTACATCACGAAGT
                            ABRE
-1530  CAACAAGATCAGCCGCACGCAGTTAATATTAATACCAAGGGGCCTCCATTATTGACTTTG
         circdian
-1470  ACATCCATGCGAAGCGCTCAACCCACAACACTCCTCGACCTTAGGCCACGTTTGCGGGAC
                                                        G-Box
-1410  AAATGCGTGTAGAAATAAAAATTAATCAAAATTTCATAGATTTTTAAATATTTATCTTTA
-1350  TATATTTTATAATTTTATTAAAGTAATTTATTGTTACGAGATTATATCAAATTAATATAA
-1290  AAGTTTGCAAATAATTATTTCTTAGCATATTTTAAAAAATATATATAAATACATATTGAA
-1230  TTTATTCTTAAAAAACAAGCCTGTTTTTAAAATGTTATTTTTAATCAAATATATTTTCAA
                                                             Box 1
-1170  AAATTTTAAAAATATTCTGAATTCTAAAGAATAAAATATATTGTAAATAAATTCTCTACA
-1110  TATTTTTTATGGTGGGAATAGATTTGAAAGCCAACGATCGTCCAACCAGAAGAATCTACA
-1050  CGGTAACCAGCTAATGATACTGGTCCAAGAGATTGATCGGACCCAGAGAATTCGCTGAAA
                                                        HSE
-990   GTCAAAGTTTTGACTTTCTTGTTTTAAGCAACGGATTCAGATCCATGAGGCTTGACCCAC
                                  CCAAT-box               Box-W1
-930   TGAAATCAAATATAAAATATCTAGTTGTGAGAGTTGTGGCAAAACTACGCTAAATTTAGG
-870   TTATGCTCACGAGAATTTCCTTGGGGTACGTTTGGTTTAGGAAAATTTGGAGGAAAATGT
                                         ARE
-810   AAAGTAAGAAAATACAAAGAAAAAAAAATAATAATAAATTTAAAATTAATAAATTATTTTT
-750   ATATGTTATTTTAAATTTATTTATTTATTTTAATCTATGATTAAATCATTTGAGAATGTA
-690   TAAATATTTTATTAATTTTGATTATATTTTATTTTTTTTTAAGACAATCAAATGATAGAA
-630   TAATTTTTTAAAATATTTTTCGATAACCAAATATAACTTTAATATAGAGAAAATGATGGT
                                                          As-2-box
-570   TGAATTTATATTTTTAGAATTTTTTTAATAATATAGAAAAAAAAGGTAAGGTTATCTAAT
-510   TTGTGGTATTAATGGAAATAATTATTTTTTAATAAAGTTGTTGAGTTTATCTATTTCTAA
                             AT1-motif
-450   ATTAGTATTTTGAAAATGTCTTTTAAATTAAAAATAATTTTTAAGATTTTAAATTTTTGA
-390   TCCCTATTTTTTATTTTTAAAAACTAAAAAGTAGTAAATCCTATATTCTGATTTATTTTT
-330   TTATGAAAATATTATATATTTATATTTTTAATAAAAACAATTTTTAGTATTTTTCATTTT
                                                          Tc-rich repeats
-270   TTTCAATTATTTCTCATAATGTTTAAAAAAAATATTAAACCATTTTAAATTTATTTTAAA
-210   AAATAAAATATTTTTAAAATAAATTTTCAGAAAATATTTTTAATCCAAATTTGTAAAATA
-150   TATTTTACACTAAAGAAACAAAAATGGAAGCCCTCAAGGCCGCCCTTAGCTGGAAGCCCA
                  AE-box
-90    TTTAACATATTCGTGTTCAATTTGGCCACGCCAGCAAGTGTCTATAAATTCAAGATTTGG
-30    TCTAGGCATTACGTAGCCAAATTCAGAGAAATGGAGACTTCAAAACTTTTTCTACTCTCC
                                      M  E  T  S  K  L  F  L  L  S
       TCCTCTCTCCTCCTACTCTTACTCGCACTCGTCCAATCGTCGACCTGCAGGCATGCA
       S  S  L  L  L  L  L  L  A  L  V  Q  S  S  T  C  R  H  A
```

Figure 5. The promoter sequence analysed by Plantcare, it showed that it had ABRE (cis-acting element involved in the abscisic acid responsiveness), Box-4, G-box, W1-box (fungal elicitor responsive element), HSE, TC-rich repeats and other transcription factor-binding sites on the positive strand. A TCA-element (cis-acting element involved in salicylic acid responsiveness) was on the negative strand. The expression of *PGIP* might be correlated with abscisic acid, salicylic acid, light, the fungal elicitor, heat stress and defence and stress responsiveness.

present in the PGIP promoter. Such factors possibly regulated the transcription of several plant genes in response to a wide range of environmental cues.

An accumulation of *VtPGIP* transcripts was observed in the seven-week-old tissue culture seedlings, and *VtPGIP* expression was induced by SA and ABA (Figures 7A and

Table 1a. The structure of the promoter according to promoter prediction software.

The *PGIP* gene promoter	**Promoter prediction software**		
	Promoter scan	**Neural network promoter prediction**	**CpG Island prediction**
yy	http://bimas.dcrt.nih.gov/molbio/proscan	http://www.fruitfly.org/seq_tools/ promoter.html	www.itba.mi.cnr.it/sun/webgene
	Promoter region predicted on reverse strand from 1550 to 1300 Promoter Score: 53.33 (Promoter Cut-off point= 53.000000) TATA found at 1346, Est.TSS = 1314	Start End Score 1593 1643 0.93	CPGPLOT islands of unusual CG composition yy from 1 to 1650 Observed/Expected ratio > 0.60 Percent C + Percent G > 50.00 Length > 200

The structure of the promoter was analysed according to three different online software programs. Bioinformatics software was used to analyse the promoter. Some transcription factors and their positions were obtained in the TF Search, but the positions of unknown or new transcription factors could not be analysed; The promoter of the *VtPGIP* gene contain cis-acting elements involved in the responses to abscisic acid (ABRE), salicylic acid (TCA-element) and fungal elicitor responsive element (Box-W1) and it contain some transcription factor binding sites, such as Nkx2-5;c-Rel.

Table 1b. Structure of the promoter according to transcription factor prediction software.

The *PGIP* gene promoter	**Transcription factor prediction software**		
	TF Search	**Match1.0-public**	**Plantcare**
yy	http://www.cbrc.jp/research/db/TFSEARCH.html	http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi?	http://bioinformatics.psb.ugent.be/webtools/plantcare/html/（Fig.5）
	Total of 7 high-scoring sites found. Max score: 89.0 points, Min score: 86.8 points.	Total sequence length=480. Frequency of sites per nucleotide=0.008. Total number of sites found=4.Nkx2-5; NF-kappaB (p65);c-Rel; NF-kappaB	Fungal elicitor responsive element; abscisic acid elements; salicylic acid elements.

The structure of the promoter was analysed according to three different online software programs. Bioinformatics software was used to analyse the promoter. Some transcription factors and their positions were obtained in the TF Search, but the positions of unknown or new transcription factors could not be analysed; The promoter of the *VtPGIP* gene contain cis-acting elements involved in the responses to abscisic acid(ABRE), salicylic acid(TCA-element) and fungal elicitor responsive element(Box-W1). and it contain some transcription factor binding sites, such as Nkx2-5;c-Rel.

7B). *VtPGIP* expression in the untreated control leaves was analyzed and positively confirmed the relative expression of *VtPGIP* after ABA and SA treatment. On the basis of signaling molecules, plant defense genes were activated and regulated by different signal transduction pathways. SA, a product of the phenylpropanoid pathway, is involved in the expression of localized hypersensitivity reactions and systemic acquired resistance (Raskin, 1992). Studies have shown that SA has a critical function in the defense signaling pathway. In many plant species, SA levels increase as PR gene expression and disease resistance are activated (Johnson et al., 2003). The phytohormone ABA is involved in plant abiotic stress response and regulation of various biotic stress responses. Enhanced resistance against necrotrophic pathogens was demonstrated;

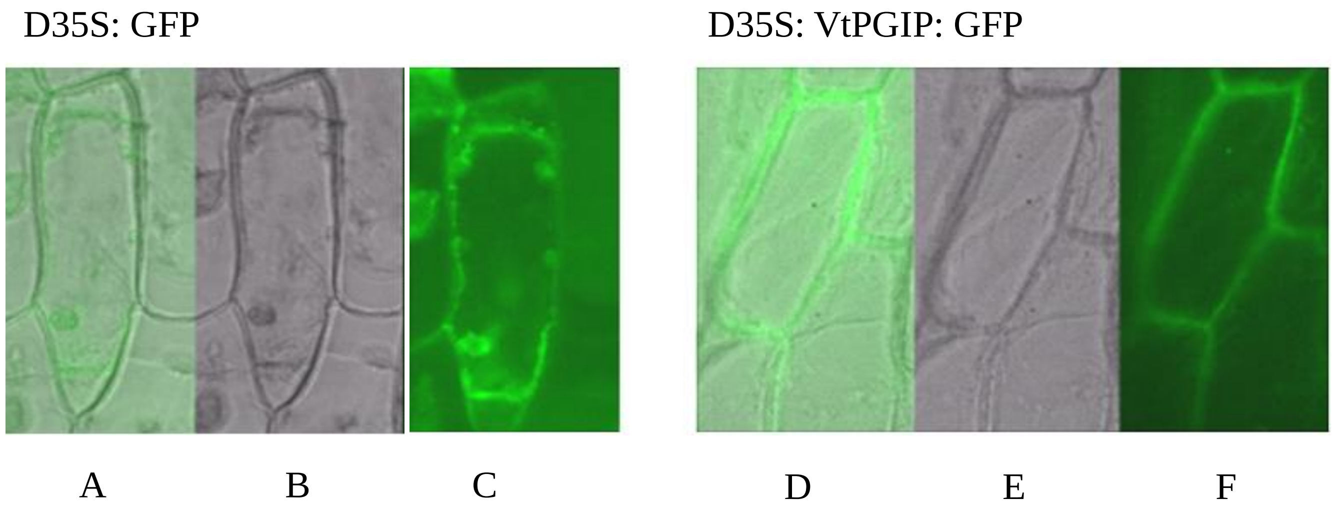

Figure 6 Subcellular localization of *Vt*PGIP in onion epidermal cells. The onion epidermal cells were transformed with p35S:VtPGIP-EGFP(D-F). The expression and subcellular distribution of the proteins were examined under a fluorescent microscope (C, F) and a light microscope (B, E) and then merged (A, D). The data shown were representative of three independent experiments. EGFP: Enhanced green fluorescent proteins. It was found that this protein was localized in the plant cell wall.

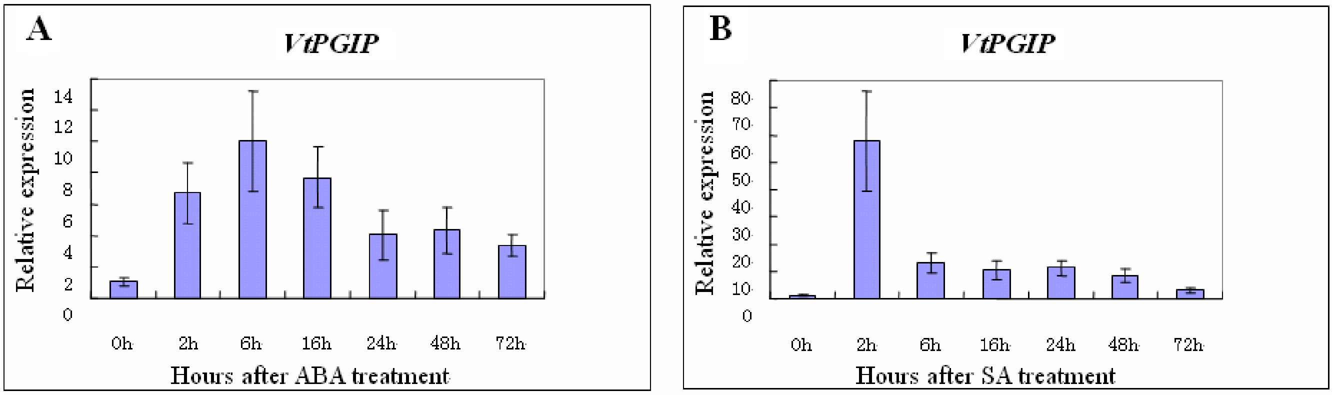

Figure 7. Time course of the changes in mRNA levels of *PGIP* gene in leaves after ABA (A) and SA (B) treatments. The leaves were harvested 0, 2, 6, 16, 24, 48, and 72 h after the ABA (A) and SA (B) treatments. Analysis was performed by real-time RT-PCR (see Materials and methods). The results of this study suggested that the *PGIP* gene may be involved in SA and ABA-regulated defence responses, as the expression of *VtPGIP* strongly increased after treatment. They showed the maximum expression level of induction of *VtPGIP* was observed at 6 h and 2 h by ABA and SA treatments, respectively. Then expression levels of the *VtPGIP* transcripts decreased. After a longer period of time, it would return to basal levels of expression.

this procedure was based on primed callose accumulation controlled by an ABA-dependent defense pathway (Ton and Mauch-Mani, 2004). Plants secrete different defense proteins to protect themselves from pathogen invasion. These kinds of defense proteins are also induced by various signal molecules, such as SA and ABA. Thus, many important families of defense proteins are expressed and regulated by different signal molecules via different transduction pathways. Mechanical wounding or damage possibly occurs in the infection site where defense-related genes may be activated against pathogens (Cheong et al., 2002). Microarray studies have demonstrated that mechanical wounding and insect feeding account for distinct and overlapping sets of gene activation (Reymond et al., 2000). PGIPs are regulated during development and after wounding and pathogen infection or treatments with elicitors, SA, and cold temperature (De Lorenzo et al., 2001; Ferrari et al., 2003; Li et al., 2003).

PGIPs are also induced in many plant tissues under various environmental conditions, and PGIPs have been isolated in many plants. Other signaling molecules or defense response activators can induce *PGIP* gene expression (Ferrari et al., 2003). In the present study, *VtPGIP* gene expression was regulated by SA and ABA signal transduction pathways.

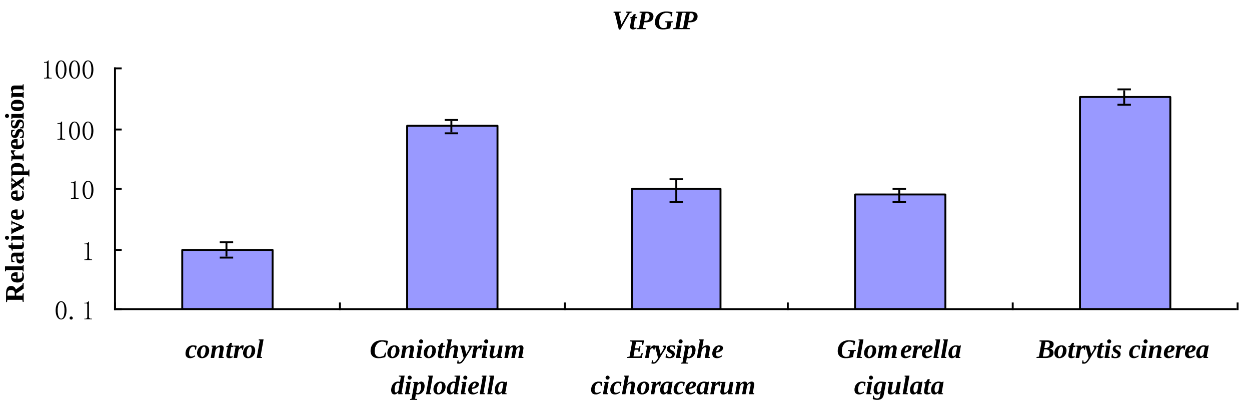

Figure 8. Expression pattern of *VtPGIP* from leaves was in response to different pathogen fungi (*Coniothyrium diplodiella, Erysiphe cichoracearumr*, *Glomerella cigulata* and *Botrytis cinerea*) respectively.

The importance of PGIPs in plant defense has been elucidated by a series of studies. For instance, the overexpression of the *PGIP* gene in *Arabidopsis* reduces symptoms and colonization by *B. cinerea* (Ferrari et al., 2003). In the present study, the *VtPGIP* expression level from pathogen-infected leaves (*C. diplodiella, E. cichoracearumr*, *G. cigulata*, and *B. cinerea*) was higher than that of the control leaves. This phenomenon is more evident in *B. cinerea* and *C. diplodiella* than in other species.

In conclusion, PGIP has an important function in plant disease resistance. The *PGIP* gene expression levels were enhanced in response to applied SA and ABA. This result may be used as a basis to increase plant resistance to pathogen as induced by SA or ABA treatment in *V. thunbergii*. Hence, appropriate plant hormones should be applied in resistant engineering of other plants. *PGIP* expression in treated plants is possibly upregulated compared with untreated plants. As a result, plants may exhibit greater resistance to pathogens after treatments with appropriate plant hormones.

ACKNOWLEDGMENTS

This work was supported by the Innovation Fund of Jiangsu Province CX(10) (Project No.110) and the national 98 project (2011-G28)

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