Characterization of the Gene Encoding Radish (*Raphanus sativus* L.) PG-inhibiting Protein

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Abstract. A radish (*Raphanus sativus* L.) polygalacturonase-inhibiting protein (PGIP) gene was cloned and compared to the PGIP gene (*BrPGIP2*) from Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) in order to gain more information on controlling a disease and improving produce quality. To clone the radish PGIP gene, primers were designed based on conserved sequences of two PGIP genes (*BnPGIP1* and *BnPGIP2*) from rape (*B. napus* L. ssp. *oleifera*), Chinese cabbage and *Arabidopsis thaliana*. PCR cloning was performed with cDNA from the stigma of radish 'Daejinyeoreum' as a template to confirm DNA fragments which were about 600 base pair in size. Sequence analysis revealed 84.1% homology with *BrPGIP2* and 70.1% with *BnPGIP1*. DNA walking was conducted to confirm the open reading frame of 972 bp, and the gene was named *RsPGIP1*. *RsPGIP1* consisting with 323 amino acids (aa) has a high leucine content (54/323) and contains 10 leucine-rich repeat domains, as do most *BrPGIPs* of Chinese cabbage. The gene expression of *RsPGIP1* was induced by abiotic stresses and methyl jasmonate. It showed enrichment in the stigma and the primary root than a leaf. Cloning *RsPGIP1* will aid to further apply practices on postharvest quality maintenance and disease control of the root.

Additional key words: PGIP, polygalacturonase, postharvest disease, produce quality, radish

Introduction

Radish roots are one of the most popular vegetables in Korea, where people consume in a number of different food recipes, including a main ingredient of 'Kimchi'. Radish roots produced in spring are less susceptible to low temperature, whereas fall season types are very sensitive.

It is widely accepted that the firmness of radish root is one of the most important quality criterion that is used to processing 'Radish Kimchi' as well as other food recipes made of radish root (Cheigh and Park, 1994). Several studies suggested that pectins in cell walls play an important role in maintaining tissue firmness during processing or cooking of the root (Fuchigami, 1987; York et al., 1985).

The plant cell wall is an important barrier to pathogen invasion. Most plant pathogens secrete a cocktail of cell wall degrading enzymes capable of depolymerizing the polysaccharides in the plant cell wall. In response, plants have evolved a

diverse battery of defense responses including proteinaceous inhibitors of pectin degrading enzymes such as PG (PGIP, PG inhibiting protein), pectinmethyl esterase (PMEI, PME inhibitor) and pectin lyase (PNLIP, PNL inhibitor protein), as well as hemicellulose degrading enzymes such as endoxylanase (XI, xylanase inhibitor), xylanase (XIP, X inhibitor protein) and xyloglucan endoglucanase (XEGIP, XEG inhibiting protein) (Juge et al., 2006). The majority of plant pathogenic fungi and bacteria secrete PG, which can degrade cell wall pectic components consisting of a rhamnogalacturonan main chain, and this seems to be involved in development of disease symptoms (Lebeda et al., 2001).

PGIPs that are present in plant cell wall have a leucine-rich repeat (LRR) domain, one of the features of plant resistance proteins, and directly reduce the aggressiveness of fungal PG (Liang et al., 2005). The existence of PGIPs has been confirmed in leek and onion, which are monocots, and also in a number of dicotyledons including soybean,

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apple, pear, orange, and kiwifruit. A variety of PGIP genes have been cloned from 103 plant species (De Lorenzo et al., 2001; NCBI gene bank). Recently, PGIP genes in rape, rice, strawberries and Chinese cabbage have been identified (Ahsan et al., 2005; Mehli et al., 2004). Most PGIP genes are composed of multi-gene families clustered in a particular chromosomal region. In the case of the two *Arabidopsis thaliana* PGIPs, they are located tandemly on chromosome 5 with a distance of 507 bp (Ferrari et al., 2003).

Numerous studies on PGIPs have investigated their expression in relation to invasion of fungal pathogens, the response to treatment with elicitors such as salicylic acid (SA), jasmonic acid (JA), and oligogalacturonides (OGs), and expression pattern after abiotic stress (De Lorenzo et al., 2001). In addition, the expression pattern of PGIP during developmental processes such as rice floral maturation and organ number has been reported (Jang et al., 2003). The expression of PGIPs of *Brassica napus* and *Arabidopsis* is regulated by some abiotic stresses (Ferrari et al., 2003).

The effect of apple PGIP was tested on a well characterized cell wall destructing enzyme, PG from a postharvest soft rot pathogen, *Colletotrichum acutatum* (Gregori et al., 2008). Recently, the genes encoding PGIPs were cloned and characterized in Chinese cabbage which is a main crop belonging to Brassicaceae family same as radish (Hwang et al., 2010).

Bacterial soft rot in radish root, caused by *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) is a soil epidemic disease. *Pcc* is a facultatively gram-negative bacterium and attacks plant tissue through the secretion of a cocktail of cell wall degrading enzymes mainly including PG during high temperature and high humidity like rainy spell in summer. Therefore, the study on inhibiting PG activity of *Pcc* is necessary.

In this paper, we cloned cDNA using RT-PCR after confirming the genomic sequence of *RsPGIP* gene from radish through PCR cloning and DNA walking, and investigated *RsPGIP* expression pattern in relation to environment stress and signaling molecules to find a useful way of applications for managing radish quality and diseases in postharvest.

Materials and Methods

Plant Material and Sample Preparation

Raphanus sativus L. 'Daejinyeorum' (Monsanto-Korea, Seoul, Korea) radish seed was immersed in water in dark conditions at 28°C for 36 h, sowed in sterilized soil, and cultivated in a greenhouse for 4 weeks with a day temperature of 18 to 20°C and a night temperature 10 to 12°C. For preparation of genomic DNA, 4-weeks-old radish leaves were extracted and stored at -70°C after being frozen in liquid N₂. We attempted to clone the PGIP gene from

radish stigma through cDNA library screening based on the method used for the isolation of Chinese cabbage PGIP from stigma (Hwang et al., 2010). Ten young plants were placed at 4°C with 16 h day and 8 h night for 30 days to induce flower. After vernalization, plants were transplanted into 5 L pots and cultivated for 6 weeks in the greenhouse. When plants were in full bloom, stigmas were excised with a scalpel, immediately frozen in liquid N₂, and stored at -70°C for total RNA extraction.

For analysis of the expression patterns of *RsPGIP1* in various plant tissues, the 4-week-old radish plants were placed at 4°C for 30 days under 16 h light conditions to induce flowering and then grown in the greenhouse until flowering. Stigma, leaf blade, and primary root tissues from flowering plants were harvested, frozen in liquid N₂, and stored at -70°C for later use.

To investigate the expression pattern after treatments with low temperature, salt stress, mechanical wounding, and defense response activators of *RsPGIP1*, radish seeds were germinated at 28°C for 36 h, and seedlings were grown in the greenhouse for 4 weeks. After selecting young uniform plants, they were conditioned for 5 days at 23°C with 16 h light and 8 h dark. RT-PCR was performed after those treatments.

PCR Cloning and Sequence Analysis

For PCR cloning, a primer CL (PCR cloning 5' primer) and CR (PCR cloning 3' primer) were designed based on the conservative base sequences of PGIP genes from *Arabidopsis*, rape and Chinese cabbage. PCR primer sequences for all experiments are given in Table 1. Total RNA was extracted with an RNeasy plant mini kit (Qiagen, Valencia, CA, USA) from the stigma of blooming flowers of radish and quantified with a spectrophotometer (Beckman Coulter DU 650, Indianapolis, IN, USA). The synthesis of 1st strand cDNA was completed using a Superscript 1st-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) from 5 µg total RNA according to the manufacturer's recommendation. Genomic DNA was extracted from young leaves of radish using a DNeasy Plant Mini Kit (Qiagen) and quantified with a spectrophotometer. The PCR template used was a 1:20 dilution of the 1st strand cDNA product (1 µL). PCR was conducted using primer 5' CL and 3' CR. The same primer set was used for the PCR with genomic DNA. PCR amplifications were carried out in a final volume of 20 µL containing 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 50 mM KCl, 0.25 mM each dNTP, 0.2 µM each primer, 20 ng template DNA and 1 unit of Taq DNA polymerase using the following: 94°C for 5 min, then 35 cycles of (94°C for 1 min, 58°C for 1 min, 72°C for 1 min), with final extension step of 72°C for 10 min. PCR products were analyzed on a 1.2% agarose gel

Table 1. Primer sequences used for PCR and RT-PCR analyses.

Gene	Primer	Sequence (5' → 3')
PCR cloning	CL (PCR cloning 5' primer)	CGCCAAGCTCAAGTATCTCC
	CR (PCR cloning 3' primer)	TCGAATTCCTGAAGTGTCCC
DNA walking	5' TSP1	AAGCATCGCCTCCGAGCTT
	5' TSP2	GTTTTCGGTATATAACCGGAGAGCTGG
	5' TSP3	TGGTTGTGTGATAGGTAAAGGTCAGGTCA
	5' TSP4	GGGATCTGACCGGTGAGGTTA
	5' TSP5	GTGAGTTTGCGGAAGACAAGAGTCTGTAG
	5' TSP6	AAGTCACCGACTTCAGGCGGGAT
	3' TSP1	GATCACAGGGAATATCCCGGTT
	3' TSP2	TGGACCGAAGCTCCTCTCCAGTT
	3' TSP3	TGTGTGGACACATCCCCACAGGA
<i>RsPGIP1</i>	RsPGIP-FW	ATAGGGATCCCCATGTGTGGTAAACAACGACA
	RsPGIP-RV	AGTCGTCGACTCACTTGCAACTATCAAGAGGTGC
<i>RsACTIN</i>	RsACTIN-FW	ATGACATGGAGAAGATCTGG
	RsACTIN-RV	TGAGCTTGTTTTGGAAGTCT

(Agarose M, Biosesang, Seongnam, South Korea) containing EtBr (Sigma, St. Louis, MO, USA) and the corresponding DNA bands were recovered and cloned into pGEM-T Easy Vectors (Promega, Madison, WI, USA) for sequencing.

DNA sequences of the insert (about 600 bp) were determined by Genotech Co. Ltd. (South Korea). Sequence analysis was performed using the software BioEdit ver 7.0.5 (www.mbio.ncsu.edu/BioEdit/) and Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) in the GenBank database (www.ncbi.nlm.nih.gov).

DNA Walking Experiments

Two walks were performed in the 5' direction and also in the 3' direction for a total of three DNA walking experiments using a primer designed based on the confirmed base sequence of RsPGIP. The first experiment in the 5' direction was carried out by using a DNA walking *SpeedUp*TM Premix Kit (Seegene, Seoul, South Korea) after designing primers 5' target-specific primer (TSP) 1, 5' TSP2, and 5' TSP3. Since a region that includes the start codon of RsPGIP was not acquired through the first experiment, primers 5' TSP4, 5' TSP5, and 5' TSP6 were designed using the sequence of the acquired 5' region; the second experiment in the 5' direction was then performed. Walking the 3' direction was done using primers 3' TSP1, 3' TSP2, and 3' TSP3, providing sequence data to confirm the gene as RsPGIP.

Sequence Analysis

The NCBI website was used for the database search and

the analysis of the deduced amino acid sequence similarity. A multiple sequence alignment was done with the ClustalW program (www.ebi.ac.uk/clustalw/; Larkin et al., 2007). A phylogenetic tree was generated using the neighbor-joining method by Mega4 (<http://www.megasoftware.net/>; Kumar et al., 2008). Signal peptide prediction and motif searches were performed using SignalP ver 3.0 (www.cbs.dtu.dk/services/SignalP/; Bendtsen et al., 2004) and PROSITE (www.expasy.org/prosite/; Hulo et al., 2006), respectively.

Stress Treatments

To induce wound, leaves were mechanically damaged by crushing the boundary point at the mid-rib with sterile forceps. For cold treatment, 4-week old plants were kept at 4°C under 16 h of daylight for 3 days. For salt treatment, plants were watered, and then their leaves were sprayed with 0.5 M NaCl every 12 h for 3 days. For drought treatment, watering was withheld for 4 days, at which time plants began to wilt. For all the treatments, samples were collected from 4-week-old plants at 0, 0.5, 1, 2, 4, 8, 16, 24, 48, and 72 h after treatment. The same age plants treated with Methyl-jasmonic acid (MeJA) were used as positive control, and those without any treatment were used as negative control. After each treatment, leaves were collected and frozen immediately in liquid N₂ from three individual plants and stored at -70°C for RNA extraction.

Treatments of Different Defense Response Activators

The biggest leaves of 4-week-old plants were selected to

examine the effects of different defense response activators. MeJA (100 μ M, Sigma, St. Louis, USA) and salicylic acid (50 mM, SA; Sigma, St. Louis, USA) were sprayed on the aerial parts of plants. Plants sprayed with distilled water were used as a control. Leaves were harvested at 0, 0.5, 1, 2, 4, 8, 16, 24, 48, and 72 h after treatment.

RNA Purification and RT-PCR Analysis

For RT-PCR analysis, the total RNA was purified from the frozen sample using the RNeasy Plant Mini Kit (Qiagen) and according to the manufacturer's instructions. cDNA synthesis was performed with the same method of PCR cloning. RT-PCR was carried out using redesigned RsPGIP-FW and RsPGIP-RV primers.

Results

Isolation of the RsPGIP Gene

In this study, PCR cloning primers prepared based on the conserved regions of PGIP genes from *Arabidopsis*, rape, and Chinese cabbage were used to amplify DNA fragments from radish stigma cDNA and genomic DNA. DNA product with an expected size of about 600 bp was amplified by PCR (Fig. 1B). The DNA fragment of 676 bp amplified from genomic DNA was approximately 100 bp longer than

that amplified from stigma cDNA. Cloning and sequencing of the two products revealed that the 88 bp DNA fragment was an intron of the radish PGIP gene. The intron has good 5' and 3' splice site consensus CAG-GT and ACAG-GT respectively (Fig. 1A). High similarities in amino acid sequences were found between radish *PGIP* cDNA and PGIP from Chinese cabbage, rape, *Arabidopsis*. The *PGIP* gene from radish was named *RsPGIP1* (*Raphanus sativus* L. PGIP gene copy No. 1).

The full length ORF sequence of *RsPGIP1* was confirmed by performing 5' DNA walking twice and 3' DNA walking once. The first 5' DNA walking experiment was performed using primers 5' TSP1, 5' TSP2, and 5' TSP3, constructed based on the sequence of partial *RsPGIP1* gene and confirmed by PCR cloning. The band with the most distinct 0.5 kilo base (kb) size was confirmed in the lane of DW (DNA Walking) -ACP (Annealing Control Primer) 2. The sequence of this band included the intron (88 bp) area from the location of 5' TSP3 (587) to 165, and was confirmed as being 510 bp in size (Fig. 1C). Since the translation start site in the DNA walk experiment of the first 5' DNA walk was not confirmed, primers 5' TSP4, 5' TSP5, and 5' TSP6 were used in the second 5' DNA walking experiment. They were designed after analysis of the first 5' DNA walk. As a result of performing the second 5' DNA walk, clear bands of 0.3, 0.6, 1.6, and 2.0 kb size were detected in the lane of DW-ACP1. Among them, fragments of 0.3 kb and 1.6 kb size were cloned in the pGEMT-easy vector and their sequence analyzed. These two bands represented the *RsPGIP1* gene sequence and the sequence of 1631 bp including the *RsPGIP1* promoter region from 5' TSP6 (258) to -1373 (Fig. 1D).

DNA walking from the 3' direction was performed using primers 3' TSP1, 3' TSP2, and 3' TSP3, constructed based on the DNA sequence of partial *RsPGIP1* and confirmed with PCR cloning. A variety of clear bands were detected in the DW-ACP1 lane as well as other lanes, but in the DW-ACP1 lane, bands of 5.5 kb and 1.3 kb size were detected. Both bands were from the *RsPGIP1* gene sequence and from the location of 3' TSP6 (868) to 2160, the sequence of 1292 bp was confirmed (Fig. 1E).

Primers RsPGIP-FW and RsPGIP-RV were designed based on the sequence of the full-length ORF of *RsPGIP1*. cDNA from the *RsPGIP1* gene was cloned by PCR using the prepared primers and stigma cDNA. The DNA sequence was analyzed by cloning the cDNA of *RsPGIP1* into pGEMT-easy. The *RsPGIP1* gene sequence was confirmed from cDNA and exactly matched the sequence of *RsPGIP1* confirmed from genomic DNA. The *RsPGIP1* gene was deposited in GenBank database with accession no. EF392661.

Fig. 1. Gene structure and amplification of the *RsPGIP1* gene region. (A) Gene structure of *RsPGIP1*. CL, PCR cloning 5' primer; CR, PCR cloning 3' primer; TSP, target specific primer; ACP, annealing controlled primer. (B) PCR cloning product. (C) PCR products of 1st 5' region DNA walking experiment, Lane 1: ACP1, Lane 2: ACP2. (D) PCR products of 2nd 5' region DNA walking experiment, Lane 1: ACP1, Lane 2: ACP2. (E) PCR products of 3' region DNA walking experiment, Lane 1: ACP1, Lane 2: ACP2. DNA fragments for sequencing are indicated with an arrow. M, wide range DNA logical marker (100 bp-10 kb).

Structural Analysis of *RsPGIP1*

The promoter region of *RsPGIP* (*pRsPGIP1*) was analyzed for the presence of putative *cis*-acting regulatory elements (Table 2). An element containing the type II MYB consensus

sequence (MBSII), a binding site for Myb-related transcription factors, was present at the -588 position. Additional putative *cis*-acting regulatory elements are present in *pRsPGIP1*. A sequence at position -799 showed high similarity to the

Fig. 2. Alignment of predicted amino acid sequences of PGIPs. The signal peptide of *RsPGIP1* is underlined, and the cysteines and glycosylation sites are indicated with a star and upward arrow. The amino acid sequences shown are for *AtPGIP1*, *Arabidopsis thaliana AtPGIP1* (accession no. NM120769); *AtPGIP2*, *Arabidopsis thaliana AtPGIP2* (accession no. NM120770); *BnPGIP1*, *Brassica napus BnPGIP1* (accession no. AF529691); *BnPGIP2*, *Brassica napus BnPGIP2* (accession no. AF529693); *RsPGIP1*, *Raphanus sativus RsPGIP1* (accession no. EF392661).

Table 2. Notable putative *cis*-acting elements in the *RsPGIP1* promoter.

Category	<i>cis</i> Element	<i>Rs PGIP1</i>	
		sequence ^z	position ^y
Myb binding site	MBS II [a(a/c)c(a/t)a(a/c)c]	aaccaac	-588/-581
pathogen response	LS4	ttgact	-444/-438
			-324/-318
	HSRE	taaaatntng	-194/ -186/ -170/
Wound induction	PINIHK	aagcgtaagt	-799

^zSequence is indicated from the 5' to the 3' end.

^yPosition of the *cis*-element with respect to the translation start (5' end/3' end).

sequence 5'-AAGCGTAAGT-3' found at position -165 of potato proteinase inhibitor II K promoter that is required for wound-induced expression (Palm et al., 1990). Three putative HSRE elements (-194, -186, -170), which are AT-rich motifs conserved in the promoters of several tobacco pathogen-induced genes (Pontier et al., 2001), also are present in *pRsPGIP1*. In addition, two sequences identical to the LS4 elements (-444, -324), TTGACT, which acts as a negative regulator of *Arabidopsis PRI* expression and as an elicitor-responsive element (W box) in parsley *PRI* (Ferrari et al., 2003).

Sequence analysis revealed that the ORF of *RsPGIP1* is 972 bp in length. Domain analysis revealed that the full-length *RsPGIP1* gene contains an ORF encoding a protein of 323 amino acids and has a conserved *N*-terminal sequence motif and an LRR region (Figs. 2 and 3). The deduced amino acid sequence of the ORF of *RsPGIP1* has a high degree of similarity with previously isolated PGIPs and contains features characteristic of PGIPs found in other plants. The ORF of *RsPGIP1* gene encodes for a polypeptide that shows 86, 84, 75, 63, 63, 61, 61, 61, 59, 58, 56, 56, 56, and 43% similarity with PGIPs from Chinese cabbage, rape, *Arabidopsis*, apricot, peach, pear, apple, cotton, citrus, grape, potato, tomato, and bean, respectively. Fig. 4 shows the phylogenetic relationship of amino acid sequences between radish *RsPGIP1* and other PGIPs. The encoded polypeptide has common features of other PGIP proteins. The ORF of *RsPGIP1* showed 88.1, 84.0 and 76.3% similarity with PGIPs from Chinese cabbage (*BrPGIP2*, NCBI accession no. FJ771027), rape (*BnPGIP2*, NCBI accession no. AF529693), and *Arabidopsis* (*AtPGIP1*, NCBI accession no. NM120769), respectively.

The predicted molecular weight of the *RsPGIP1* deduced from the amino acid sequences is 36 kDa and the isoelectric point 8.31 [Peptide Mass]. The *RsPGIP1* gene product has high leucine content (54 of 323 aa) including 39 aa that are highly conserved. The region spanning nearly 74% of

the mature peptide (from amino acid 64 to 301) contains ten conserved LRR motifs (xLxxLdLSxNxLTGxIPxxLxxLx) with an average size of 24 aa (Fig. 3), where 'L' indicates leucine residue or any aliphatic amino acid residue, 'd' did the acidic amino acid residue and 'x' can be any amino acid. The LRR domain, which has a conserved region, can form a β -sheet/ β -turn motif, assumed to be an Avirulence (Avr) protein recognition site that is an important part of common cloned plant R genes (Di Matteo et al., 2006; Federici et al., 2006). LRR domains are suggested to be involved in defense signal activation by interacting with a signaling component or binding to a ligand (Worrall et al., 1998).

Analysis using the ExPASy proteomics server of the Swiss Institute of Bioinformatics (SIB) revealed that radish *RsPGIP1* has five potential *N*-glycosylation sites (NXS/T) found in conserved positions and also a number of potential phosphorylation sites including the presence of eight highly conserved cysteine residues, four in the first 65 aa and four in the final 32 aa of the mature peptide. Based on SignalP-HMM (Hidden Markov Models) and SignalP-N, N (neural networks) prediction to *N*-terminal peptides, as determined by amino acid sequencing, radish *RsPGIP1* has an *N*-terminal signal peptide of 22 amino acids (Fig. 2). The putative cleavage site is between

			LRR
RVIS-	LAIN-	IDVSGQIPPEVGDLS	1
YLQT-	LVFRKL	TNLTGQIPRTITKLK	2
YLR-	LRLSW-	TNLTGPVPGFLSELK	3
NLQF-	VDLSF-	NDLTGSIPSSLSLLP	4
NLLS-	LDLSR-	NKLTGSIPESFGSFP	5
AKVPD	LYLSH-	NQLSGYIPKTLGNLD	6
FNR--	IDFSR-	NKLGGDASM-LFGAN	7
KTTWY	VDLSR-	NMF-QFDLSRVVIPK	8
TLGI-	LDLNH-	NGITGNIPVQWTEA	9
PLQF-	LNVSY-	NRLCGHIPTG-GKLQ	10
xLxx-	LdLSx-	NxLTGxIPxxLxxLx	Consensus

Fig. 3. Ten putative leucine-rich repeats (LRRs) in *RsPGIP1*. Consensus: x, any amino acid; L, leucine or aliphatic amino acid; I, isoleucine; T, serine/threonine; d, acidic residue.

Fig. 4. Phylogenetic tree of *RsPGIP1* and the other PGIPs. The tree was constructed from amino acid sequences by the neighbor-joining method of ClustalW and Mega4 (<http://www.megasoftware.net/>). Numbers at the nodes are bootstrap values from 100 replications and represent weighted amino acid substitutions, with the scale bar represents 10% weighted sequence divergence. At, *Arabidopsis thaliana*; Br, *Brassica rapa*; Pv, *Phaseolus vulgaris*. *AtPGIP1* (accession no. NM120769); *AtPGIP2* (accession no. NM120770); *BrPGIP1* (accession no. FJ898288); *BrPGIP2* (accession no. FJ771027); *BrPGIP3* (accession no. FJ898286); *BrPGIP4* (accession no. FJ898289); *BrPGIP5* (accession no. FJ898287); *PvPGIP2* (accession no. FM253103); *PvPGIP4* (accession no. AJ786411).

the 22nd and 23rd aa (SLS-KD) as shown with an underline, and the 22nd aa was confirmed to be serine, also well conserved in other PGIPs.

Organ Specificity in *RsPGIP1* Expression

We examined tissue-specific expression of *RsPGIP1*. The expression of *RsPGIP1* was observed in three organs of *Raphanus sativus*, roots, leaves, and stigma. However there was a quantitative difference in terms of expression level. Less expression was observed in the leaves than in stigma or primary root from the flowering plants (Fig. 5). Also, the enrichment of *RsPGIP1* transcripts in stigma showed similar pattern as the case of *BrPGIPs* in Chinese cabbage (Hwang et al., 2010).

Effect of Different Stresses on the Transcript Levels of the *RsPGIP1* Gene

When *Raphanus sativus* plants were treated with different

Fig. 5. RT-PCR analysis of *RsPGIP1* gene expression in plant organs. S, stigma; L, leaf blade; R, primary root. Total RNA was extracted from tissues of radish plants when in full-bloom.

Fig. 6. Effect of mechanical wounding, low temperature, salt, and jasmonic acid treatments on the *RsPGIP1* gene induction. RT-PCR reaction with primers for actin was performed to determine quantitative equality of the RNA templates used for samples. W, mechanical wounding; C, low temperature; S, salt; J, jasmonic acid.

environmental stresses such as mechanical wounding, cold temperature, high salt, and dehydration (Fig. 6), RT-PCR revealed that the *RsPGIP1* gene was induced rapidly in response to mechanical wounding, in which a peak was shown 2 to 4 h after treatment. Also, the gene was induced in plants treated with low temperature and salt. Transcript levels of *RsPGIP1* increased gradually between 1 and 72 h after salt treatment, whereas, in the case of dehydration, induction levels did not change (Data was not shown). In all the stress treatments, accumulation of transcripts was low, and bands were not detected after 30 cycles of RT-PCR. However, after 35 cycles induction was confirmed. In all experiments, the 4-week-old leaves without any treatment were used as a control. In the control, transcript of *RsPGIP1* was low and was only detected by RT-PCR after 35 cycles.

We observed tissue-specific expression of *RsPGIP1* at the flowering stage (Fig. 5). Interestingly, transcript level of *RsPGIP1* in the leaves from the flowering plants was higher than 4-week-old leaves (Figs. 5 and 6). This suggests that the expression of *RsPGIP1* is differentially regulated by developmental stage.

Activities of Different Defense Response Activators in the *RsPGIP* Gene Expression

When treated with MeJA and SA, only MeJA induced the *RsPGIP1* gene in leaves of 4-week-old *R. sativus* in response to defense response activators. Expression was rapidly induced after MeJA treatment at 1, 2, and 4 h, and then steadily decreased. Transcription was highest from 1 to 4 h after treatment (Fig. 6).

Discussion

Cloned a *Raphanus sativus* L. PGIP gene using PCR cloning and DNA walking was named as *RsPGIP1*. The gene has similar domain structures and sequence homology to other PGIPs, including the Brassicaceae family; the highest match in deduced amino acid sequence among them was 86%.

PGIPs are reported to be induced in many plant tissues under various environmental conditions and have been found in many plant species. In our study, the constitutive expression of the *RsPGIP1* was confirmed in the stigma. To a lesser extent, accumulation of transcripts was also observed in leaves of flowering radish plants (Fig. 5). Conversely, leaves from 4-week-old plants showed relatively low expression of *RsPGIP1* in the absence of applied stress or any defense response activator treatments (Fig. 6). These results are similar to the expression pattern of the *BnPGIPs* (Li et al., 2003) and *BcPGIP* (Ahsan et al., 2005). The expression of *RsPGIP1*

is also observed by mechanical wounding as reported in PGIPs from *Arabidopsis* (Li et al., 2003), Chinese cabbage (Ahsan et al., 2005), strawberry (Mehli et al., 2004), and bean (Devoto et al., 1998). Mechanical wounding is generally generated in the infection site where defense-related genes may be active against pathogens (Schenk et al., 2003).

The *BrPGIP2* gene, characterized from Chinese cabbage provides us with the clue on the molecular mechanism on disease resistance. A study of the inoculation of *Pcc*, a bacterial pathogen causing soft rot disease, revealed that the expression of *BrPGIP2* from J3-11M-54, the relatively resistant Chinese cabbage line was strongly and rapidly induced compared to J3-11M-3, a relatively susceptible line. Further analysis of transgenic plants, obtained by the over-expression in tobacco and Chinese cabbage with *BrPGIP2* proved that resistance against soft rot disease of transgenic plants increased by more than 50%, being ascribed to the promotion of *BrPGIP2* activity (Hwang et al., 2010).

It is recognized that PGIPs play an important role in plant defense through studies on improvement of disease resistance involving over-expression of PGIP genes and elucidation of the expression pattern after treatments with several microbial defense elicitors (Di Matteo et al., 2006; Federici et al., 2006; Ferrari et al., 2006). It is known that PGIPs inhibit the action of pathogen PG on cell wall pectic structural components in various plants. This inhibition was investigated through site-directed mutagenesis of particular amino acid residues in the active site and structural portions of PG protein (Federici et al., 2001; Manfredini et al., 2006; Spadoni et al., 2006; van Santen et al., 1999).

Interestingly, *RsPGIP1* was induced by environmental stress such as low temperature and salt (Fig. 6). Low temperature induction of PGIP expression has been reported in *Brassica napus* (Wao et al., 1999), *Arabidopsis* (Ferrari et al., 2003), Chinese cabbage (Ahsan et al., 2005), and apple (Yao et al., 1999). Induction of PGIP by salt stress has been reported in Chinese cabbage (Ahsan et al., 2005). With *Arabidopsis* PGIP genes, expression was not induced by drought or heat stress (Rizhsky et al., 2003). Likewise, in the case of the *RsPGIP1* gene, there was no response to drought stress. In the promoter region of *RsPGIP1*, there exists a PINIIK (*cis*-element) that is related to wounding. Also, LS4 and HSRE exist in relation to pathogen response (Table 2). Therefore, it is assumed that each gene responds selectively to different environmental stress. We observed that *RsPGIP1* gene was induced by JA (Fig. 6). Jasmonic acid is the end product in the octadecanoid pathway involving sequential action of allene oxide synthase (AOS), allene oxide cyclase (AOC) and 12-oxo-PDA-reductase (OPR). This pathway mediates plant response to a wide range of biotic and environmental

stress (Agrawal et al., 2003). A variety of environmental cues can induce the expression of AOS, AOC, and OPR genes in plants and therefore production of relevant enzymes. It has been reported that the rice *OPR1* gene is upregulated by many environmental factors, such as: wounding, heavy metals, osmotic pressure, UV-C irradiation, fungal elicitors, protein phosphatase inhibitors and gaseous pollutants (Agrawal et al., 2003). Since the *RsPGIP1* gene was induced by mechanical wounding, various environmental stress, and JA, we conclude that the JA signal transduction pathway may regulate *RsPGIP1* gene expression. It has also been demonstrated in other systems that induction of defense-related genes by mechanical wounding and JA involves the JA-dependent signal transduction pathway (Pieterse and van Loon, 1999). Based on the above, we postulate that *RsPGIP1* gene expression is also regulated by the JA signal transduction pathway, but more research regarding the relationship between JA and other environmental factors is needed.

The *RsPGIP1* gene isolated from radish ‘Daejinyeorum’ will be useful in research on the molecular mechanism of the resistance to diseases in radish. More investigations are required to extend our knowledge on understanding the mechanism by which *RsPGIP1* plays a decisive role in postharvest diseases like soft rot in radish as well as other *Brassicaceae* crops.

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