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Cloning, characterization, and expression of the gene encoding polygalacturonase- inhibiting proteins from strawberry

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Abstract: Polygalacturonase-inhibiting proteins (PGIP) play important roles in the defense against plant pathogens, especially fungi. The sequence of strawberry *PGIP* gene was obtained using reverse transcription PCR (RT-PCR) and its expression in different tissues was studied by semi-quantitative RT-PCR. A cDNA fragment of *FaPGIP* gene was cloned and the sequence analysis shows that the fragment contains a full ORF of 999 bp encoding 332 amino acids. The *FaPGIP* gene from genomic DNA shows a single 168bp intron that is efficiently spliced out of the *FaPGIP* pre-mRNA transcript. The *FaPGIP* has a high degree of identity with previously isolated *PGIP* genes and the encoded polypeptide shows all the characteristic features of PGIP peptides. The 3 dimensional model of the protein contains 12 α-helices and 21 β-sheets, and the center LRR structural domain is composed of 10 tandem LRR motifs. The semi-quantitative RT-PCR revealed *FaPGIP* gene displaying high expression levels in fruit and leaf, middle expression in flower and root, and weak expression in stem. The expression level of *FaPGIP* gene is particular to tissues.

Key words: Strawberry, polygalacturonase-inhibiting proteins (PGIP), cDNA cloning, tissue expression

Introduction

The plant cell wall provides one of the main barriers to almost all phytopathogenic organisms. Pectolytic enzymes produced by many pathogenic fungi impair the natural structure of the cell wall, which helps the pathogen to find its way into the cell. These enzymes include polygalacturonases (PGs), which hydrolyze pectin, one of the cell-wall components, in pathogenesis (Albersheim and Anderson 1971). PGs cleave the glycoside bond in α-1, 4-D-homogalacturans, expediting pathogen penetration into host cells. PGs have been shown to be an important pathogenicity factor for soft-rot fungi (De Lorenzo et al. 2001). Conversely, plant species have evolved polygalacturonase inhibitor proteins (PGIP) that specifically recognize and inhibit fungal PGs (De Lorenzo and Ferrari 2002).

Gene encoding PGIPs have been isolated from raspberry (Ramanathan et al. 1997), apple (Arendse et al. 1999), and peach (Liang et al. 2005). It is clear that PGIP plays an important role in the resistance to infection by fungal pathogens. Plants have evolved different PGIP with specific recognition abilities against the PGs produced by fungi (De Lorenzo et al. 2001). The proposed mode for PG/PGIP interaction is that the inhibiting protein partly covers the active site of PGs and prevents their substrate binding (Frederici et al. 2001). Different PGIPs show variation in

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recognition abilities and specificity. PGIP is an important family of defense protein. In order to gain more information on the specific structural and regulatory characteristics of individual members of the protein family, it is necessary to isolate and analyze a number of PGIP genes from different species.

Strawberry (*Fragaria* × *ananassa*) is one of the most economically and biologically important fruit trees in the world. In strawberry production, the polyphagous grey mould fungus (*Botrytis cinerea*) is undoubtedly the most important pathogen causing fruit rot. In organic or unsprayed fields, the losses due to fruit rot can be up to 55% (Daugaard 1999). To increase a strawberry cultivar resistant to *B. cinerea* is one of the main targets in strawberry breeding. In this paper, the isolation, characterization, and protein structure of a *PGIP* gene from strawberry 'Toyonaka' and the expression of *FaPGIP* gene in different tissues from strawberry were reported as a part of a larger study to enhance the resistance of strawberry to fungal diseases.

Materials and methods

DNA and RNA extraction and gene cloning

Genomic DNA was isolated from young leaves by the method of Pich and Schubert (1993). Total RNA was extracted as described previously (Lu 1999). First-strand cDNA was synthesized by MMLV reverse transcriptase (Toyobo Life Science) according to the product instruction manual. A pair of specific primers Fa-PF:5'-TTCTCATCTGAGTTCTCACTGA-3' and Fa-PR: 5'-AGCATCCCTCCGT CCCATTA-3' were designed based on the conserved region of *PGIP* genes in GenBank from NCBI website and synthesized by Invitrogen Biotechnology Co., Ltd. PCR was performed with a 25 μL reaction mixture containing 20 ng of template DNA, 800 μM dNTPs, 1.5mM $MgCl_2$, 1 × PCR Buffer without Mg^{2+}, 0.2 μM of each primers, and 0.75 unit of *Taq* DNA Polymerase using the following program: 94 °C for 3 min, then 35 cycles each with 94 °C for 45 s, 55 °C for 45 s, 72 °C for 90 s, and finally 72 °C for 10 min. PCR product was analyzed on 1% agarose/EtBr gel and the corresponding DNA bands were recovered and then cloned into the pMD18-T Vector (Takara Life Science) for sequencing.

Sequence analysis of strawberry PGIP gene

The program of Basic Local Alignment Search Tool (BLAST) at the NCBI website was used for similarity search. The software DNAMAN (Version 3.0, Lynnon BioSoft) was used to analyze the ORF to deduce the amino acid sequence of the *PGIP* gene, to predict the protein molecular weight (MW), isoelectric point (pI), hydrophilicity, and hydrophobicity, and to analyze homology. The SignalP 3.0 Server (Bendtsen et al. 2004), an application available at The Center for Biological Sequence Analysis at the Technical University of Denmark DTU, was used to predict the signal peptide. The EsyPred3D Web Server 1.0 (Lambert et al. 2002) was available through the website:(http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/), which was used to predict the protein 3-dimensional structures.

Expression analysis of strawberry PGIP gene

For semi-quantitative RT-PCR analysis, total RNA of roots, stems, leaves, flowers, fruits, and 5 different strawberry tissues were extracted as described previously (Lu 1999). RT-PCR was performed using primer Fa-PF and Fa-PR. The housekeeping gene *FaGAPDH2* (AF421145) was used as a control in the experiment, which was amplified with the primers 5'-CAGACTTGAGAAGAAGGCCACCTA-3' and 5'-GATACCC TTCATCTTTCCCTCAGA-3'. RT-PCR reaction reagents were used as described previously and the initial RNA contents were 1 μg. Amplification was performed using the following profile: 94 °C for 3min, then 35 cycles each with 94 °C for 60 s, 58 °C for 60 s, 72 °C for 60 s, and finally 72 °C for 10 min. All PCR reactions were performed in triplicate.

Results

Isolation of strawberry PGIP gene

In this study, primers prepared based on the conserved regions of *PGIP* genes in raspberry, apple, pear, and other plants were used to amplify DNA fragments from strawberry genomic DNA and cDNA. PCR amplification generated DNA products with the expected size of approximately 1.3 and 1.1 kb, respectively. The DNA fragment amplified from genomic DNA was approximately 170 bp longer than

that amplified from cDNA. Cloning and sequencing of the 2 products revealed that the170bp DNA fragment was an intron of strawberry *PGIP* gene. The comparison of homology of the nucleotide sequence and deduced amino acid sequence at the website of NCBI using the BLAST program revealed that they shared the highest sequence homology with other known *PGIP* genes. This suggested that the cloned fragment from strawberry was a real *PGIP* gene, which was named as *FaPGIP* and the sequence was deposited in the GenBank database (accession numbers EU117213).

Structural analysis of strawberry PGIP gene

The analysis of the sequences revealed that the cDNA fragment of *FaPGIP* gene was 1053 bp in length and contained a full length open reading frame (ORF) of 999 bp encoding 332 amino acids with a predicted molecular mass of 37.1 kDa and an isoelectric point of 7.67. The comparison of the results between the sequences of the *FaPGIP* gene and its DNA sequence indicated only one 168 bp intron was present in the gene, which showed a high AT content (67.9%) characteristic of dicotyledonous plant introns (Figure 1a). The intron had good 5′ and 3′ splice site consensus CAG-GT and ACAG-GT, respectively.

The signal peptide of the deduced amino acid sequence was predicted using the hidden Markov models and neural networks methods, respectively. The results indicated that the protein cleavage site was between amino acid residues 24 and 25 and the 24 amino acids in the N-terminal were considered to be a signal peptide. Protein hydrophilicity and hydrophobicity analyses indicated that there was a hydrophobic region of 24 amino acids in the N-terminal, which was coincided with the results of signal peptide prediction. The sequence analysis indicated that there were 23 codons of 6 types in *FaPGIP* belonging to rare codons of *E. coli*, and there were 5 potential N-glycosylation (N-X-S / T; where X is any amino acid except P) sites at the amino acid positions 108, 146, 156, 240, and 294 in the protein. The N-terminal and the C-terminal region contain both 4 cysteine residues at highly conserved positions compared with PGIP from other fruit plants (Figure 1b).

The deduced amino acid sequence for the ORF of *FaPGIP* gene shows a high degree of similarity with previously isolated PGIPs, and contains characteristics of PGIPs found in other plants. The ORF of *FaPGIP* gene encodes for a polypeptide that shows 73.9%, 47.1%, 65.4%, 76.0%, 73.3%, and 87.5% similarity with PGIPs from apple, bean, citrus, peach, pear, and raspberry, respectively. The PGIP protein from strawberry was more similar to raspberry PGIP protein. Figure 2 shows the phylogenetic relationship of amino acid sequences between strawberry PGIP and other PGIPs. The encoded polypeptide shows the common features of that and other *PGIP* genes.

Analysis and prediction of FaPGIP 3-dimensional models

The *FaPGIP* gene shows a high leucine content in the 332 amino acids of putative strawberry PGIP, there were 54 leucines, including 38 highly conserved ones. Furthermore, from amino acid 76 to 314, the strawberry PGIP contains 10 highly conserved tandem leucine-rich repeats (LRR) motifs (LxLxxNxLTGxIPxxLGxLxxLxx) with an average size of 24 amino acids (Table). The predicted 3-dimensional structure of FaPGIP revealed that the 3-dimensional model (Figure 3) contained 12 α-helices and 21 β-sheets and 10 tandem LRR motifs in the center of LRR structural domain, and contained 8 β-sheet/β-turn/β-sheet/α-helix regions. On the opposite side of the protein, 12 α-helices are almost parallel to β-sheets. The first 9 helices have a comparable length and are regularly spaced, whereas the 3 helices in the C-terminal portion are variable in length and position. Like other LRR proteins, the α-helices and β-sheets are connected simply by loops or β-turns, the β-sheet/β-turn occupies the concave inner side of the structure (Figure 3).

The expression of strawberry PGIP gene in different tissues

RT-PCR was used to examine the tissue expression of the *FaPGIP* gene in strawberry. Total RNA was treated with DNase I (GIBCOLBRL) to avoid the genomic DNA contamination before reverse-transcription. The expected 1.1 kb fragment of *FaPGIP* and 188 bp fragment of *FaGAPDH2* gene were obtained (Figure 4). The semi-quantity RT-PCR revealed that *FaPGIP* was expressed in all the tissues

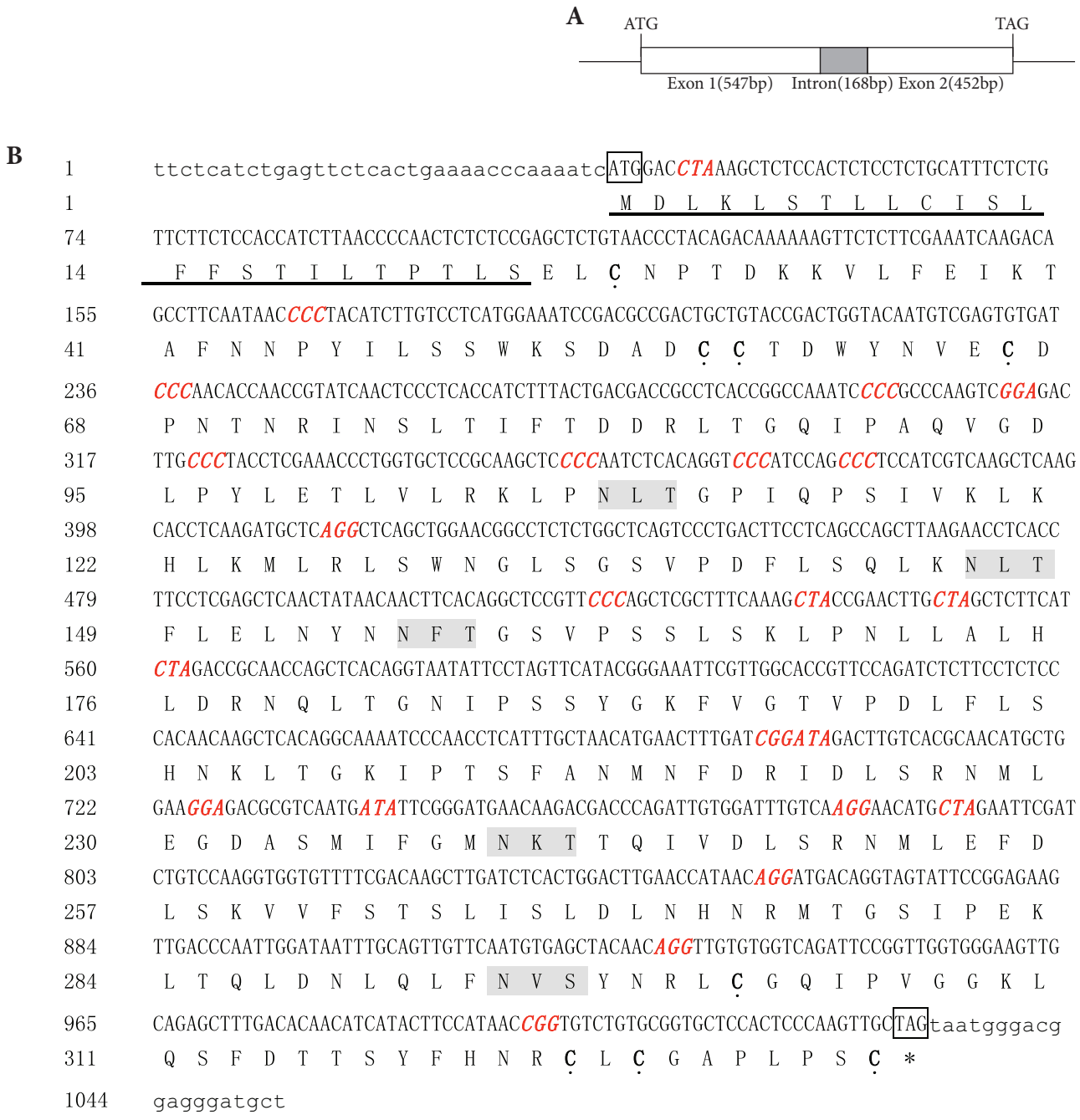

Figure 1. Gene structure (a) and deduced amino acid sequence (b) of *FaPGIP* gene
Note: The lowercase characters indicate noncoding regions; double underline sequence indicates the signal peptide; ATG indicates the start codon; TAG indicates the stop codon; the bold-italic letters indicate the rare codons of *E.coli*; the bold letters indicate the cysteine residues sites; dashed area indicates the potential N-glycosylation sites.

and organs tested (root, stem, leaf, flower, and fruit). The results suggested that under control condition, *FaPGIP* displayed high expression levels in fruit and leaf, middle expression in flower and root, and weak expression in stem.

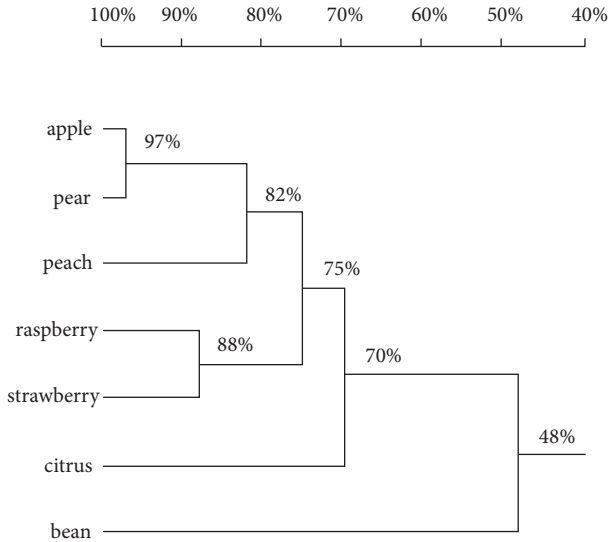

Figure 2. Cluster of deduced strawberry PGIP and other PGIPs.
Note: Numbers indicated similarity.

Table. Comparison of 10 LRR sequences in deduced strawberry PGIP.

LLR No.	Location	Sequence	Length
1	76-99	LTIFTDDRLTGQIPAQVGDLPYLET	24
2	100-124	LVLRKLPNLTGPIQPSIVKLKHLKM	25
3	125-148	LRLSWNGLSGSVPDFLSQLKNLTF	24
4	149-172	LELNYNNFTGSVPSSLSKLPNLLA	24
5	173-197	LHLDRNQLTGNIPSSYGKFVGTVPD	25
6	198-220	LFLSHNKLTGKIPTSFANMNFDR	23
7	221-244	IDLSRNMLEGDASMIFGMNKTTQI	24
8	245-267	VDLSRNMLEFDLSKVVFSTSLIS	23
9	268-290	LDLNHNRMTGSIPEKLTQLDNLQ	23
10	291-314	LFNVSYNRLCGQIPVGGKLQSFD	23
Cons.		LxLxxNxLTGxIPxxLGxLxxLxx	24

Note: Cons. indicated the LRR consensus sequence of FaPGIP; x represents any amino acid.

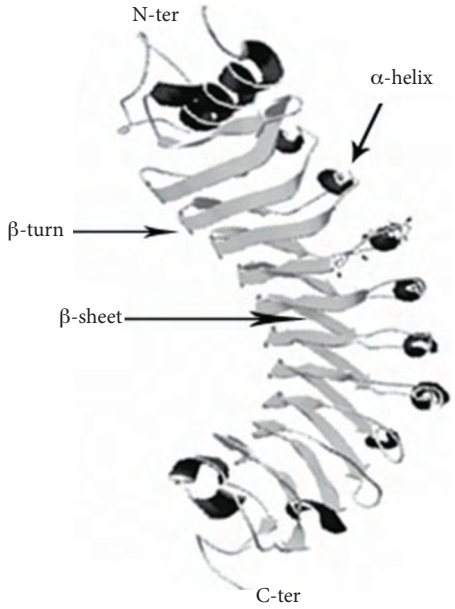

Figure 3. Schematic representations of predicted FaPGIP three 3-dimensional models.
Note: β-sheets are colored grey; α-helices are colored black.

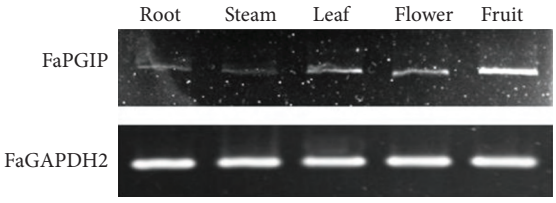

Figure 4. Semi-quantitative RT-PCR of *FaPGIP* gene expression in different tissues of strawberry.

Discussion

PGIP belongs to the plant-specific LRR subfamily characterized by the consensus sequence Lt/sGxIP in the region following the conserved β-sheet/β-turn motif (Stotz et al. 2000) and they are involved in protein–protein interactions (De Lorenzo et al. 2001). The consensus in the strawberry LRR region is similar to plant specific-LRR, which has conserved region, can form β-sheet/β-turn motif, it is assumed to be *Avr* protein recognition site, and is an important part of common cloned plant R gene. The LRR domains have been suggested to be involved in defense signal activation by either interacting with a signaling component or binding to a ligand (Mattei et al. 2001). Strawberry PGIP could therefore interact with their ligands (endo-polygalacturonases) by means of their LRR domains. In strawberry PGIP, there were 5 potential N-glycosylation sites at the amino acid positions 108, 146, 156, 240 and 294 in the protein. The latter 2 seem to be common for all fruit PGIP. The position and number of consensus sites for N-linked glycosylation are not highly conserved among the PGIP rom different plants (Mattei et al. 2001). The N-terminal and the C-terminal region contain both 4 cysteine residues at highly conserved positions when compared with PGIP from other fruit plants. These 4 disulfide bridges flank the LRR domain: 2 bridges are located in the N-terminal region (Cys-27–Cys-57 and Cys-58–Cys-66), and the other 2 are in the C-terminal region (Cys-301–Cys-323 and Cys-325–Cys-332). Both N- and C-terminal regions contribute to cap the hydrophobic core of the protein solenoid and these form 4 disulfide bridges, which were important to maintain local structures and stabilize secondary structures in PGIP (Mattei et al. 2001; Di Matteo et al. 2003).

The three-dimensional structure of a PGIP from bean has very recently been determined (Di Matteo et al. 2003), and its structure reveals a negatively charged surface on the LRR that is likely involved in binding PGs. With respect to the vast majority of previously known LRR proteins, a characteristic feature of this structure is the presence of 2 extended β-sheets that are likely to be conserved in other plant LRR proteins. A peculiarity of the Strawberry PGIP structure is the presence of a cluster of negatively charged residues in the concave surface of the protein likely involved in the interaction with PGs. We expect that this structure will be useful for understanding the mode of interaction of PGIP as well as for designing better inhibitors with tailor-made specificities. This structure may also be useful for modeling other plant LRR proteins, which are known to perform important functions in defense and development and pave the way to elucidate their multiple interactive properties and their mechanism of recognition.

The organ-specific accumulation of strawberry PGIP is different from that reported for PGIP mRNA accumulation in bean (Toubart et al. 1992). Strawberry PGIP mRNA was detected in root, stem, leaf, flower and fruit. The relative expressions of FaPGIP at low levels in stem, root, flower and abundantly in fruit and leaf, which could partially explain the differences in PGIP activity in these different organs. Bean PGIP mRNA was observed in flower, leaf, hypocotyl and more abundantly in cell-suspension cultures (Di Matteo et al. 2003). When the plant pathogenic fungi infection, the fruit and leaf of a higher content of pathogenic fungi with PGIP-specific integration of PGs, thereby reducing the degradation of plant cell wall, contribute to the maintenance of the integrity of plant cell wall, and then slow down on the pathogenic fungi infected plants. The differences in structure and expression of PGIP from strawberry and bean suggest that, although these proteins inhibit fungal PGs in vitro, they may differ in their role in pectin metabolism during pathogen challenge in vivo. The expression of PGIP in these different organs may resolve these important differences and lead to a better understanding of the function of PGIP in plants.

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