

**CHARACTERIZATION OF A *BETA VULGARIS* POLYGALACTURONASE-
INHIBITING PROTEIN: A DEFENSE RESPONSE GENE**

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Abstract

Polygalacturonase-inhibiting proteins (PGIPs) are plant cell wall proteins that inhibit pathogen and pest polygalacturonases (PGs). PGIPs are members of the leucine-rich repeat (LRR) protein family that play crucial roles in development, pathogen defense and recognition of beneficial microbes in plants. Two sugar beet *PGIP* genes, *Bv(FC607)PGIP1* and *Bv(FC607)PGIP2*, were cloned from a breeding line FC607. Sequence analysis showed that both genes encoded about 380 amino acids and shared 74.8% sequence similarity. They were most closely matched to PGIPs of a sugar beet line KWS2320 and a subgroup of *M. truncatula* PGIP (GenBank No: XP_003621816). FCPGIPs exhibited characteristics of other plant PGIPs, including the presence of an N-terminal signal peptide and LRR repeats that were 50-60 amino acid longer than has been reported for most other PGIPs. In 2-month old plants, RT-PCR

analysis demonstrated that each *PGIP* gene was expressed constitutively, with maximum expression being observed in roots, followed by leaves then petioles and hypocotyls, suggesting that the gene is developmentally regulated. A study of PGIP inhibitory effect on pathogens and pests is ongoing.

Introduction

Polygalacturonase-inhibiting proteins (PGIPs), produced in most plant cell walls, effectively and specifically combine with pathogen and pest polygalacturonases (PGs) and inhibit their activity used for overcoming plant defense responses (D'Ovidio et al., 2004). Phytopathogenic fungi, bacteria, nematodes and insects are known to secrete PG enzymes for breaking down the polygalacturonate chain in plant cell walls. The interaction between PGs and plant PGIPs favors the accumulation of oligogalacturonides which elicit a wide range of plant defense responses (Gomathi and Gnanamanickam 2004; Schacht et al., 2011). Many plants possess more than one PGIP with differential abilities to inhibit different PGs of pathogens. Like the products of many resistance genes, PGIPs belong to the subclass of proteins containing leucine-rich repeats (LRRs) of the extracytoplasmic type (Jones and Jones 1997). In our laboratory, one of the sugar beet genes that codes for PGIP has been found to be induced by the sugar beet root maggot in roots (Puthoff and Smigocki, 2007). In order to characterize the structure and functional features of sugar beet PGIPs, several PGIP genes were cloned and characterized from several sugar beet breeding lines. Here we report on the cloning of two PGIP genes from sugar beet line FC607 and on their tissue-specific expression in 2-month old plants. These findings will facilitate further studies on PGIP inhibition of pathogens and pests and will

advance the development of novel approaches for more effective disease and pest control in sugar beet.

Materials and Methods

Plant materials

A sugar beet breeding line FC607 was used in this study. Seeds were germinated at room temperature in Pro-Mix (Professional Horticulture) soil. Seedlings were grown in the growth chamber at 24°C with a 16 h photoperiod.

Full-length cDNA cloning and sequence analysis

The full-length sequence of the sugar beet *PGIP* cDNA was obtained through rapid amplification of cDNA ends polymerase chain reaction (RACE PCR) using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA). Gene specific primers were designed and the RACE fragment was amplified as previously reported (Padmanaban et al., 2011). PCR amplified fragments were sequenced and nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches were performed using default parameters. Amino acid sequences and opening reading frames were determined by ExpASy translation tool (<http://web.expasy.org/translate/>). Using genomic DNA and gene specific primers, *PGIP* genomic DNA was cloned by PCR and the resulting DNA fragment was sequenced.

The SignalP 4.1 server (Petersen et al., 2011) was used to predict the signal peptide. The LRR domains were predicted according to sequence alignment of BvPGIPs and the bean PvPGIP2 whose secondary crystallographic structure has been determined (Di Matteo et al., 2003). Amino acid sequences of BvPGIPs and other PGIPs were aligned by ClustalW through

MEGA6 program (www.megasoftware.net). Phylogenetic tree was constructed with the neighbor-joining method (Tamura et al., 2013).

Analysis of *PGIP* gene expression

Total RNA was extracted from leaves, petioles, hypocotyls, and roots of 2-month old plants using RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RT-PCR was carried out on 100 ng of total RNA using Titanium One-Step RT-PCR Kit (Clontech) under the following conditions: 50°C for 1 h, 94°C for 2 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by 72°C for 5 min. RT-PCR results were normalized to transcripts of the constitutively expressed plant actin gene (Smigocki et al., 2013). Gene expression was quantified by densitometry with an AlphaImager HP (Alpha Innotech, San Leandro, CA). RT-PCR analyses were repeated three times.

Results and Discussion

Cloning and analysis of *Beta vulgaris* *PGIPs*

To investigate intergenotype variation in sugar beet *PGIP* genes, the full complements of *PGIP* genes of the *B. vulgaris* breeding line FC607 were cloned and characterized. To date, two cDNA fragments were cloned from FC607 using primers based on the *PGIP* EST sequence (GenBank No: DV501910) isolated from sugar beet root after insect feeding (Puthoff and Smigocki, 2007). Comparison of the nucleotide and deduced amino acid sequences using BLAST revealed that the cloned cDNAs shared the highest sequence homology with other

known *PGIP* genes. Comparison of *Bv(FC607)PGIP1* and *Bv(FC607)PGIP2* cDNAs and PCR derived genomic DNA sequences revealed that neither gene had introns. The full-length cDNA sequences of 1,152 and 1,146 bp, encoded 384 and 382 amino acids, respectively, with a calculated molecular weight of about 49.0 kDa. These two peptides showed 74.8% similarity, and shared the typical PGIP topology, which included a signal peptide for secretion, a 75-amino acid N-terminal domain, a domain comprising leucine rich repeats (LRRs), and a 21-amino acid C-terminal domain (Fig. 1). A total of eleven imperfect LRR regions of about 24 amino acids each was predicted according to sequence alignment of *Bv(FC607)PGIPs* with the bean *PvPGIP2* whose secondary crystallographic structure has been determined (Di Matteo et al., 2003). However, according to the LRR search program (<http://lrrsearch.com/>), only 10 LRR domains (excludes the first one) were predicted for *Bv(FC607)PGIP1* and only 9 LRRs (excludes the second and the ninth LRRs) were predicted for *Bv(FC607)PGIP2*. Nevertheless, the LRR region of these two genes exhibited the identical consensus sequence (LxxLxLxxNxLxGxIPxxLGxLxx) and matched precisely the extracytoplasmic LRR consensus sequence of other resistance genes (Jones and Jones 1997). There were 4 potential *N*-glycosylation sites (NxS/T; where x can be any amino acid), three of which occurred in the LRR domain, where the third NxS/T site was different between these two genes. Both genes contained 5 cysteine residues at the N-terminal and C-terminal regions.

Sequence alignment by ClustalW revealed that both *Bv(FC607)PGIP1* and *Bv(FC607)PGIP2* showed the closest homology with PGIP or PGIP-like proteins from a *B. vulgaris* line KWS2320, a recently released genome sequence (Dohm et al., 2014). A 97.4% similarity between *Bv(FC607)PGIP1* and *Bv(KWS2320)PGIP* (accession No: XP_010675711) and an 80.0% similarity between *Bv(FC607)PGIP2* and *Bv(KWS2320)PGIP* (XP_010676004)

was observed (Fig. 2A, 2B). The second closest homology of our sugarbeet PGIPs was to *Medicago truncatula* PGIP (MtPGIP2, XP_003621816), followed by *Nicotiana tabacum* PGIP (NtPGIP, AIA22327), and *Arabidopsis thaliana* PGIP (AtPGIP2, AAF69828). Like most of the PGIPs from *B. vulgaris* KWS2320, Bv(FC607)PGIP1 and Bv(FC607)PGIP2 were approximately 50 amino acids longer than the PGIP structure model PvPGIP2 that represents most of other known plant PGIPs (Fig. 2A). The additional amino acids were found in the N-terminal and the LRR regions of Bv(FC607)PGIP1 and Bv(FC607)PGIP2 LRR but not found in most other plant PGIPs, thus potentially being unique to the sugar beet PGIPs .

Signal peptide		
Bv(FC607) PGIP1	MKMTNNVIVSLILLSLFDISHS	
Bv(FC607) PGIP2	--...H...L.F.P..VH...	
N-terminus		
Bv(FC607) PGIP1	QPLPDLCLPHDRDVLRLKKNHFGNPSLSSWDPTDC	37
Bv(FC607) PGIP2	..I...H...KN...H...H... ..	
Bv(FC607) PGIP1	AGWNGIRQNIQGYVEVVQITEAQDIHGPIPPFLDQLSS	75
Bv(FC607) PGIP2VQ...H.TTL...D.E...S.....QF	
LRR domain		
Bv(FC607) PGIP1	LTVLYFKTLPNLGPIPSYIGKLTK	100
Bv(FC607) PGIP2	..T.N.YK.....N	
Bv(FC607) PGIP1	LIELSISETNV ASPIPEFLGQLTN	124
Bv(FC607) PGIP2	.T.MFF.T....G.....	
Bv(FC607) PGIP1	LNRLYLTSNNF TGPVFNFLGKLN	148
Bv(FC607) PGIP2	.DQ...N..K. ...I...AQ...	
Bv(FC607) PGIP1	LIHLDLSSNLL TGPVPMGLGKLTN	172
Bv(FC607) PGIP2	.MY.....V..A..N.	
Bv(FC607) PGIP1	VEILALSSNKL SGPLPEFIAQKLKK	197
Bv(FC607) PGIP2	LKV.S.....QI.D..G..Q	
Bv(FC607) PGIP1	LWVLQLDFNLSL SGQIPTSLGLLPS	221
Bv(FC607) PGIP2	.A...N....P..A....SK	
Bv(FC607) PGIP1	LQTVALTGNQL SGVPVPTSLGRGK	244
Bv(FC607) PGIP2	...ID...K. ...I.KW...N	
Bv(FC607) PGIP1	LAFLYLGQNKL IGDVSEFLFQKSNNN	269
Bv(FC607) PGIP2	.Y.ISV....S..A...E.T.T.	
Bv(FC607) PGIP1	ILILEIQDNLF KFDFTNVDLAPS	292
Bv(FC607) PGIP2	..SVRVEY..L....K.....	
Bv(FC607) PGIP1	LSMLNISHNMI YGSLPLRFGLLS	315
Bv(FC607) PGIP2	.VGM... ..K....R	
Bv(FC607) PGIP1	PDSVDVSYNQL CGPIPNRRFRKR	338
Bv(FC607) PGIP2	..DI.....	
Consensus	LxxLxLxxNxL xGxIPxxLGxLxx	
C-terminus		
Bv(FC607) PGIP1	VDPIIFAHNKQLGGFFPGCK	359
Bv(FC607) PGIP2	.NSN..... ..	

Fig. 1. Sequence characteristics of Bv(FC607)PGIPs. Deduced amino acid sequences of Bv(FC607)PGIP1 and Bv(FC607)PGIP2 are aligned in four regions: signal peptide, N-terminus, LRR domains, and C-terminus. Numbering refers to the Bv(FC607)PGIP1 sequence and starts from the first residue of the mature protein. The LRR consensus sequence (LxxLxLxxNxLxGxIPxxLGxLxx) is shown. Dots indicate identical amino acids. Dashes

B

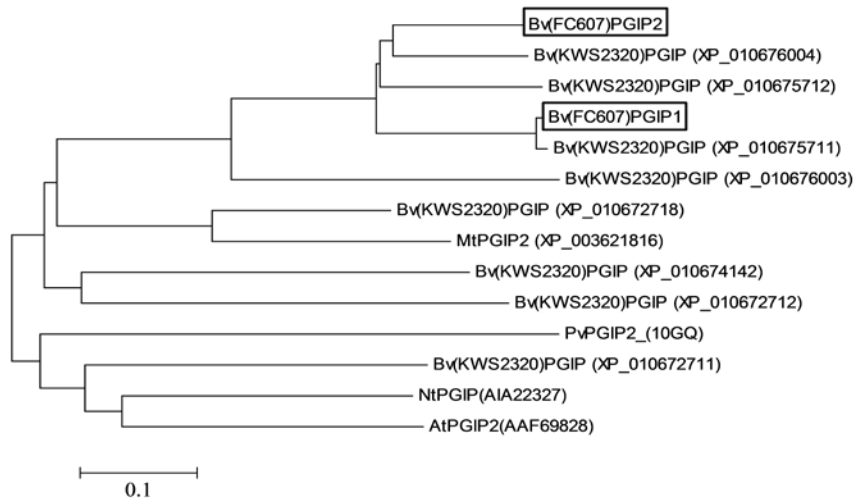


Fig. 2. Sequence alignment (A) and phylogenetic analysis (B) of Bv(FC607)PGIPs with other closely related PGIPs. The sequence alignment was done by ClustalW through MEGA6 program. The phylogenetic tree was constructed by neighbor-joining tree method using MEGA6. Abbreviations are: Bv (*Beta vulgaris*), Mt (*Medicago truncatula*), Pv (*Phaseolus vulgaris*), Nt (*Nicotiana tabacum*), At (*Arabidopsis thaliana*). The scale bar represents the branch length.

Tissue specific expression of *B. vulgaris* PGIPs

The tissue- (or organ-) specific expression of PGIP varies in different plants during different developmental stages (Gomathi et al., 2006). RT-PCR was used to examine the tissue expression of the *PGIP* genes in sugar beet. The expected fragments (about 1.1 kb) were obtained in all the tissues tested, indicating the products encoded by these two genes probably contribute to sugar beet normal growth and development (Fig. 3). The results demonstrated that

in a 2-month old plant, both *Bv(FC607)PGIP1* and *Bv(FC607)PGIP2* genes displayed the highest expression levels in roots, then leaves, petioles and hypocotyls. High expression of *PGIP* in roots has been observed in other plants such as in ginseng (Sathiyaraj et al., 2010), and in rice (Janni et al., 2006). This may be due to the penetration and colonization of roots by soil borne fungi due to the high nutrition content of these tissues and result in the induction of root defense responses, among them the expression of the *PGIP* genes (Sathiyaraj et al., 2010).

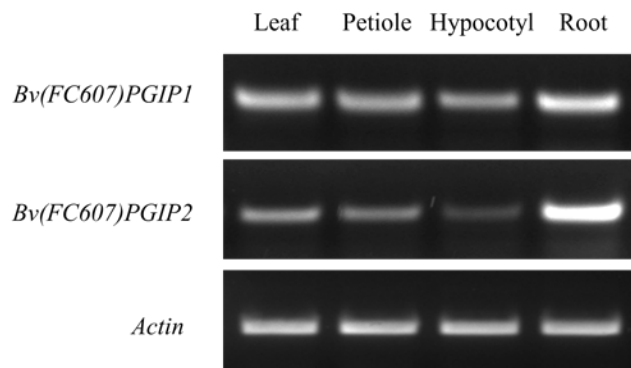


Fig. 3. RT-PCR analysis of *PGIP* gene expression in leaf, petiole, hypocotyl and root tissue of 2-month old FC607 plants.

Conclusion

This study supports the presence of at least two *PGIP* genes in *B. vulgaris* line FC607. Each gene contains 50-60 additional amino acids across the N-terminal and the LRR domain regions that have not been reported in other plant PGIPs. This unique LRR organization could contribute to new recognition capabilities during PGIP-PG interactions. Further studies on

molecular aspects of the PGIP interaction with PG and their effects on plant defense mechanisms would facilitate the development of disease- and pest-resistant sugar beet plants.

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