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ORIGINAL PAPER

The bean polygalacturonase-inhibiting protein 2 (PvPGIP2) is highly conserved in common bean (*Phaseolus vulgaris* L.) germplasm and related species

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Abstract Polygalacturonase-inhibiting proteins (PGIPs) are extracellular plant protein inhibitors of endo-polygalacturonases (PGs) that belong to the leucine-rich repeat (LRR) protein family. In bean, PGIP is encoded by a small gene family of four members among which *Pvpgip2* encodes the most wide-spectrum and efficient inhibitor of fungal PGs. In order to evaluate the sequence polymorphism of *Pvpgip2* and its functional significance, we have analyzed a number of wild and cultivated bean (*P. vulgaris*) accessions of Andean and Mesoamerican origin, and some genotypes from the related species *P. coccineus*, *P. acutifolius*, and *P. lunatus*. Our analyses indicate that the protein encoded by *Pvpgip2* is highly conserved in the bean germplasm. The few detected polymorphic sites correspond to synonymous substitutions and only two wild genotypes contain a *Pvpgip2* with a single non-synonymous replacement. Sequence comparison showed a slightly larger variation in the related bean species *P. coccineus*, *P. acutifolius*, and *P. lunatus* and confirmed the known phylogenetic relationships with *P. vulgaris*. The majority of the replacements were within the xxLxLxx region of the leucine rich repeat (LRR) domain and none of them affected residues contributing to structural features. The variant PGIPs were expressed in *Nicotiana benthamiana* using PVX as vector and their inhibitory activity compared to that of PvPPGIP2. All the variants were able to fully inhibit the four fungal PGs tested with minor differences. Taken together these results support the hypothesis that the overall sequence conservation of PGIP2 and minor variation at specific sites is necessary for high-affinity recognition of different fungal PGs.

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Introduction

Pathogens produce a number of molecules to overcome the host barriers. Among these, cell wall degrading enzymes (CWDE) play an important role in several host–pathogen interactions. To counteract these arrays of enzymes and hamper the invasion process, plants produce protein inhibitors among which the polygalacturonase-inhibiting proteins (PGIPs) have been shown to play an important role in limiting host tissue colonization by fungal pathogens (Powell et al. 2000; Ferrari et al. 2003; Manfredini et al. 2006; Aguero et al. 2005; Joubert et al. 2006; Janni et al. 2008).

Polygalacturonase-inhibiting proteins are extracellular plant inhibitors of fungal and insect endo-polygalacturonases (PGs) that belong to the superfamily of leucine-rich repeat proteins (LRRs) of the extracytoplasmic type (Jones and Jones 1997). They contain 9–10 imperfect LRRs of 24 amino acid each that are organized to form two β sheets, one of which—sheet B1—occupies the concave inner side of the molecule and contains residues crucial for PG

recognition (Leckie et al. 1999; Di Matteo et al. 2003; D'Ovidio et al. 2004a).

Pgip genes have been characterized in a number of plants, including both monocot and dicot species. These analyses showed that PGIP is encoded by small gene families whose members undergo a different transcriptional regulation and possess different inhibiting properties against fungal and insect PGs (De Lorenzo et al. 2001; D'Ovidio et al. 2004b).

The pgip family of common bean (*Phaseolus vulgaris* L.) is one of the best studied. The full complement comprises four clustered genes (*Pvpgip1*, *Pvpgip2*, *Pvpgip3*, *Pvpgip4*) spanning 50 Kbp region (D'Ovidio et al. 2004a) on the linkage group B2 (Geffroy et al. 2000). Characterization of the encoded products of these genes revealed both partial redundancy and sub-functionalization against fungal and insect PGs, with *Pvpgip2* encoding the most effective and wide spectrum PG inhibitor of fungal PGs (D'Ovidio et al. 2004a). Structure-function studies of PvPGIP2 identified residues crucial for PG inhibition (Leckie et al. 1999; D'Ovidio et al. 2004a), PG–PGIP interaction (Di Matteo et al. 2003; Federici et al. 2006; Spinelli et al. 2009) and pectin binding (Spadoni et al. 2006). These analyses involved site-directed mutagenesis of residues identified by sequence comparison or computer modelling analyses, whereas searching for natural sequence variation was restricted to the paralogous genes of two genotypes of the commercial class Pinto (hereafter PvPpgip) and the line BAT93 (hereafter PvBpgip). Differences between these two genotypes are limited to a single non-synonymous 1-nt replacement (*PvPpgip1*/*PvBpgip1* and *PvPpgip3*/*PvBpgip3*) or a 9-nt indel (*PvPpgip2.2*/*PvBpgip2*) that includes the triplet encoding the glutamine residue 224, previously identified by Leckie et al. (1999) as crucial for the inhibition of PG of *Fusarium moniliforme* FC-10 strain [now reclassified as *F. phyllophilum* (Mariotti et al. 2008)]. Moreover, four synonymous substitutions distinguish the two Pinto genes *PvPpgip2.1*, described by Leckie et al. (1999), and *PvPpgip2.2* (D'Ovidio et al. 2004a). Functional studies demonstrated that only the three amino acid deletion in PvBPGIP2 modified the inhibition properties of this protein and abolished the inhibition of the PG of *F. phyllophilum* FC-10 (D'Ovidio et al. 2004a).

In order to obtain more information on sequence variation of the strong and wide-spectrum PvPGIP2 inhibitor we have analyzed the common bean (*P. vulgaris*) germplasm and several genotypes of the related bean species *P. coccineus*, *P. lunatus* and *P. acutifolius*. Moreover, to verify the functional impact of the polymorphism, the PGIP2 variants identified have been heterologously expressed and their inhibiting activity tested against four different fungal PGs.

Materials and methods

Plant material

Common bean (*P. vulgaris*) genotypes were kindly provided by Centro Internacional de Agricultura Tropical (CIAT), P12S and P12R were kindly provided by Dr. Valerie Geffroy (Universitè Paris-Sud, France) and additional ones were from our collection (Table S1). Genotypes PHA8067 and G25362 of *P. lunatus* were gently provided by Dr. Lioi (Istituto di Germoplasma, Bari, Italy). Genotypes PA-T8a-1, PA-T15-1 and PA-Pi 321638-1 of *P. acutifolius* and the line SL770 and the cultivars Corona and Venere of *P. coccineus* were kindly provided by Prof. Soressi (Università della Tuscia, Viterbo, Italy). Seeds were surface sterilized by immersion in sodium hypochlorite (0.5% v/v) for 30 min, and then rinsed thoroughly in sterile water. Plants were lightly grown in sterilized moist vermiculite for 7 days at 24°C.

DNA extraction, nucleic acid manipulation, sequence analysis and primer development

Genomic DNA was extracted from 0.5 g of green material following the procedure reported by Tai and Tanksley (1991). DNA manipulation, PCR and cloning were performed according to standard procedures. Oligonucleotide primer pairs specific for the *Pgip2* gene were as follows: −154F, 5′-ATCCAATGGTGCGTACATGC; −117F, 5′-GAACACTTGCACTGGCTTT; −14F, 5′-ATATCCCCAGCAACCATGTCC; 1022R, 5′-GGAAATGATTAAGTGCAGGC. Amplicons were subjected to sequencing reactions using the ABI PRISM dye terminator cycle sequencing ready reaction kit and DNA sequences were determined with the semiautomatic ABI PRISM 310 sequencer (Applied Biosystem, Monza, Italy). Nucleotide sequences were also determined through the MWG-BIOTECH AG (Ebersberg, Germany). Sequence analyses were performed using the DNAMAN software (Lynnon Biosoft, Quebec, Canada) and sequence alignment was performed with a gap open penalty of 10 and a gap extension penalty of 5. Trees were generated by DNAMAN software by using the Neighbor-Joining method (Saitou and Nei 1987). Bootstrap values were based on 1,000 replications.

The nucleotide sequences of all *Pgip2* genes analyzed are deposited at the EMBL database under the accession numbers FM246845–FM246878 and FM253097–FM253113.

PVX-mediated expression of PGIPs

The coding region of the novel *Pgip* genes was amplified by PCR from each specific amplicon using sequence–

specific oligonucleotides including restriction sites for *Cla*I and *Sal*I at the 5′ and 3′ ends, respectively. The amplified fragments were double digested with *Cla*I and *Sal*I and cloned into the pPVX201 expression vector. The plasmids obtained were used to inoculate *Nicotiana benthamiana* plants using 30 μg of DNA/plant as described by Baulcombe et al. (1995).

Since the genotypes of the related bean species possessed identical sequences, we expressed only the *Pgip2* of the genotypes PHA8067, PA-T8a-1, and Corona for *P. lunatus, P. acutifolius* and *P. coccineus*, respectively.

Fungal growth, PG preparation and enzymatic assays

Fungal cultures and induction of PG were performed as previously described: using the following fungi *Colletotrichum acutatum* isolate SHK788, reclassified as *C. lupini* (Lotter and Berger 2005) and *Botrytis cinerea* strain B05-10 (D'Ovidio et al. 2004a); *Aspergillus niger* (Cervone et al. 1987); *F. moniliforme* isolate FC-10 (Caprari et al. 1996), now reclassified as *F. phyllophilum* (Mariotti et al. 2008).

Endo-polygalacturonases of *F. phyllophilum* and *A. niger* were purified at homogeneity as previously described (Cervone et al. 1987; Caprari et al. 1996), while those of *B. cinerea* and *C. lupini* were from culture filtrates.

Enzymatic activity of PGs and inhibitory activity of PGIPs were evaluated using an agarose diffusion assay (Taylor and Secor 1988) as modified by Ferrari et al. (2003) in the presence of 20 mM Na acetate pH 4.7. PG activity was expressed as agarose diffusion units, with one agarose diffusion unit defined as the amount of enzyme that produced a halo of 0.5 cm radius (external to the inoculation well of 0.5 cm radius) after 18 h at 30°C. Inhibitory activity was expressed as inhibitory units, with one inhibitory unit defined as the amount of PGIP that inhibited one agarose diffusion unit of PG by 50%.

Preparation of PGIPs and western blotting

Transiently expressed PGIPs were extracted from leaves of *N. benthamiana* plants infected with single PVX-Pgip constructs or with the empty vector. Leaves were homogenized in 1 M NaCl and 20 mM sodium acetate, pH 4.7 (2 mL/g tissue), incubated with gentle shaking for 1 h at 4°C, and centrifuged for 20 min at 10,000*g*. Supernatants were filtered through Miracloth (Calbiochem), centrifuged to remove debris and stored at –20°C. Purification of PGIPs was performed by ammonium sulfate precipitation and fast protein liquid chromatography (FPLC) fractionation. Sample were brought to 25% $(NH_4)_2SO_4$ incubated at 4°C for 12 h and centrifuged. Proteins were precipitated from supernatant with 85% $(NH_4)_2SO_4$, recovered by centrifugation and redissolved in 0.5 M NaCl, 20 mM Na acetate pH 4.7. Samples were dialysed for 24 h against 20 mM Na acetate (pH 4.7) and loaded on a cation exchange (SP-Sepharose) column. Bound proteins were eluted with a 40-min linear gradient of 0–1 M NaCl in 100 mM sodium acetate, pH 4.7 at a flow rate of 1 ml min^{-1}. Fractions (1 mL) were collected and assayed for inhibitory activity against *A. niger* PG.

SDS-PAGE and western blot analyses were used to estimate corresponding amount of each PGIP2 variant and Pinto PvPPGIP2. The absolute amount of these proteins was also estimated by including in the analysis a quantified Pinto PvPPGIP2.

Protein samples were fractionated on 15% SDS-PAGE ($T = 15$, $C = 0.5$) and stained by silver nitrate. For western blotting analysis, gels were blotted using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) in Tris–glycine buffer (25 mM Tris, 192 mM glycine, 0.04% SDS) for 1 h at 100 V on a PVDF membrane. Polyclonal antibodies raised against PGIP purified from *P. vulgaris* pods were used for immunoblotting experiments. The secondary antibody (anti-rabbit, Santa Cruz Biotechnology, Inc.) was used in 1:5,000 ratio. Detection was performed by using luminol reagent sc-2048 (Santa Cruz Biotechnology, Inc.). Densitometric analysis was performed by using the DC 120 camera and the computer software "Kodak Digital Science 1D" (Kodak).

Results

Design of primers specific for the complete coding region of *Pvpgip2* and assessment of sequence variability in common bean germplasm and related bean species

The nucleotide sequences of the four *Pgip* genes of the Pinto genotype of *P. vulgaris* (*PvPpgip1*, *PvPpgip2.2*, *PvPpgip3*, and *PvPpgip4*; accession numbers AJ864506, AJ864507, AJ864508, and AJ864507, respectively) were aligned and the sequences immediately flanking or minimally overlapping the coding region were chosen as sites for primers design.

The primer combination −14F/1022R was selected and tested for specificity in PCR assays containing, in separate samples, each of the four bean *Pgip* genes as a template. Only samples containing the *PvPpgip2.2* gene as a template produced the expected amplicon of about 1,000 bp, whereas the other samples (*PvPpgip1*, *PvPpgip3*, and *PvPpgip4*) did not produced any amplification product (Fig. S2).

The primer combination −14F/1022R was then used in PCR assays involving 25 cultivated and 18 wild common bean accessions, both of Andean or Mesoamerican origin. To maximize the genotypic variation, samples growing

from the sea level up to 3,500 m s.l. were included in the analysis (Table S1). All genotypes analyzed produced an amplicon with the expected size of about 1,000 bp. Direct nucleotide sequence of these amplicons showed a limited sequence variation of *Pvpgip2* in both cultivated and wild type genotypes, with a maximum of four single nucleotide polymorphisms (SNPs), as for example in the accessions G1441, G785, G50968, and G19407 (Table S1). The variation included previously identified *PvPpgip2.1* and *PvPpgip2.2*, but not *PvBpgip2*, as well as novel *Pvpgip2* alleles. As expected, allelic diversity was higher in wild germplasm compared to the cultivated, although *PvPpgip2.1* was not among wild alleles observed (Table S1). The distribution of the different alleles was related neither to the geographical origin of the genotype nor to their altitude.

The majority of SNPs identified corresponded to synonymous substitutions and only the Mesoamerican accessions G13505 and the Andean G23581, G23583, G23585 showed a single SNP corresponding to a non-synonymous substitution (Table 1). Moreover, the G23581, G23583, G23585 have an identical SNP.

We also analyzed *Pgip2* sequence variability in the related bean species *P. coccineus* (3 genotypes), *P. acutifolius* (3 genotypes) *P. lunatus* (2 genotypes) using the same primer combination used to amplify *Pvpgip2* (−14F/1022R). Since these primers produced an amplicon of the expected size only in *P. acutifolius*, two additional primer combinations (−117/1022R and −154F/1022R) were designed, tested for specificity as specified above and then used to amplify *Pgip2* of *P. coccineus* and *P. lunatus*, respectively. Direct nucleotide sequence of all the amplicons obtained showed the absence of intra-specific variation and a high sequence identity to *PvPpgip2*. Differences were mainly due to SNPs, with the majority of them corresponding to synonymous substitutions. However, 13, 11 and 7 of these were non-synonymous substitutions in *P. lunatus*, *P. acutifolius*, and *P. coccineus*, respectively (Table 1).

Table 1 Nucleotide (*nt*) and amino acid (*aa*) substitutions detected in the PGIP2 variants of common bean and related bean species

Species	Accession	Substitutions		
		Coding region (nt)	Precursor protein (aa)	Mature protein (aa)
P. vulgaris	G13505	2	1	1
	G23581	3	1	1
	G23583	3	1	1
	G23585	3	1	1
P. coccineus		17	7	5
P. acutifolius		25	11	10
P. lunatus		31	13	8

Nucleotide (*nt*) and amino acid (*aa*) differences to *PvPpgp2.2* (EMBL accession number AJ864507) are indicated

Nucleotide sequence alignment between all the *Pgip2* genes, here, characterized and *PvBpgip2*, *PvPpgip2.1*, and *PvPpgip2.2* confirms the reported phylogenetic relationships between the four *Phaseolus* species analyzed (Delgado-Salinas et al. 1999), where *P. lunatus* and *P. coccineous* are the most distantly and closely related species to *P. vulgaris*, respectively (Fig. 1).

In summary, our search identified two novel PGIP2 variants in the common bean germplasm [Pv(13505)PGIP2 and Pv(23581)PGIP2], and the PGIP2 of *P. coccineus* (PcPGIP2), *P. acutifolius* (PaPGIP2) and *P. lunatus* (PlPGIP2). Moreover, the analysis of sequence diversity of *Pgip2* genes confirms the previously reported phylogenetic relationships between the analyzed *Phaseolus* species.

Sequence comparison of PGIP2 variants in common bean and related species

The deduced amino acid sequences of the five PGIP2 variants identified [Pv(13505)PGIP2, Pv(23581)PGIP2, PcPGIP2, PaPGIP2, and PlPGIP2] and the variant from the

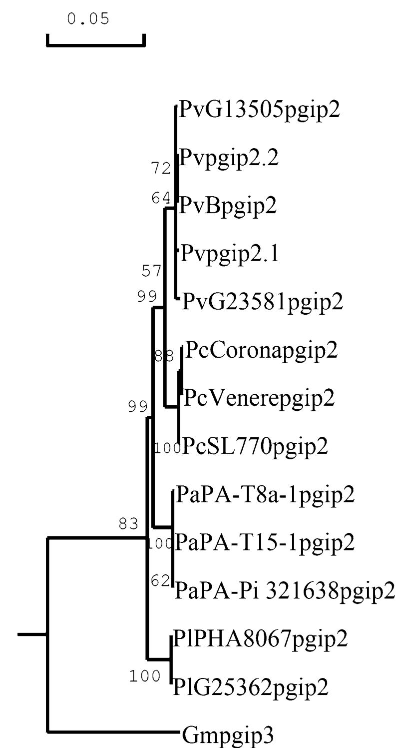

Fig. 1 Phylogenetic tree of *Pgip2* sequences from bean and related species. The nucleotide sequences for the complete coding region of *Pgip2* were aligned using the DNAMAN software with a gap open penalty of 10 and a gap extension penalty of 5. The tree was set up with the distance matrix using the Neighbor-Joining method. *Numbers* at branches indicate bootstrap values. *Gmpgip3* was used as outgroup sequence

P. vulgaris BAT93 genotype (PvBPGIP2) were aligned with PvPPGIP2.

Amino acid substitutions occur along the entire sequence except in the IV, VI and IX LRRs. The IInd and the VII LRR motifs contain the higher number of amino acid replacements (Fig. 2). Some of the replacements are species-specific, while other are present in more than one species. For instance, substitutions L60H and A213T are present only in the *P. vulgaris* Pv(13505)PGIP2 and Pv(23581)PGIP2, respectively, whereas substitutions N79S and A297S distinguish PGIP2 of *P. vulgaris* and that of *P. coccineus*, *P. acutifolius*, and *P. lunatus*. Notably, L60H and A297S are two of the eight substitutions differentiating PvPPGIP2 and PvPPGIP1 and produced very little variation or a reduction 3.5-fold in the affinity for the PG of *F. phyllophilum* FC-10, respectively (Leckie et al. 1999).

The majority of the substitutions occur within or close to the xxLxLxx region and none of them affect residues forming the negative pocket (residues D131, D157, D203, S133, T155, and T180) or those interacting with pectin (residues R183, R206, K230, and R252).

Variation on residues forming part of secondary structure (such as the sheet B1 and B2, and 3_{10}-helix) occurs only in the following two cases: on residues forming the sheet B1 of the II LRR in *P. coccineus*, *P. acutifolius*, and *P. lunatus* and on residues involved in the formation of sheet B1 of the III (H110S) and VIII (H227N) LRRs in *P. lunatus* (Fig. 2).

No variation was observed in the consensus sequence Lt/sGxIP that characterize the plant-specific LRR subfamily to which PGIP2 belongs (Kajava 1998), in the eight cysteine residues that form the disulfide bridges Cys-3–Cys33, Cys-34–Cys43, Cys-281–Cys-303 and Cys-305–Cys312, and in the three putative N-linked glycosylation sites (Fig. 2).

Finally, among the polymorphic sites only those at positions 39 and 224 correspond to positively selected sites identified using the maximum likelihood codon-evolution models (Stotz et al. 2000; Bishop 2005). However, while site 224 was confirmed as a site of functional importance, the contribution of site 39 on the inhibition activity of PvPGIP2 has not been determined.

Heterologous expression of PGIP2 variants and analysis of inhibitory activities

In order to determine the impact of the amino acid variations on the activity of the different PGIP2 variants, these were individually expressed in *N. benthamiana* using PVX as a vector. In this analysis PvBPGIP2 was included as a control, because its inhibitory activity is known (D'Ovidio et al. 2004a), whereas Pv(13505)PGIP2 was not included because the effect of the only substitution present (LH60) had been previously analyzed (Leckie et al. 1999). The expressed proteins were purified and subjected to SDS-PAGE and western blot analyses to estimate corresponding amount of each PGIP2 variant and PvPPGIP2. An example of these experiments is reported in Fig. S3.

The inhibitory activity of variant against the PG of *Botrytis cinerea* (BcPG), *F. phyllophilum* (FpPG), *C. lupini* (ClPG) and *A. niger* (AnPG) was compared to that of PvPPGIP2. The inhibitory activity of PvBPGIP2 was as already reported (D'Ovidio et al. 2004a), i.e. it failed to inhibit FpPG. The other PGIP2 variants were all able to completely inhibit all the four PGs tested and showed inhibition efficiencies comparable to that of PvPPGIP2. Only Pv(23581)PGIP2 showed inhibitory activities slightly lower against all four PGs (Table 2).

Discussion

The interaction between PG and PGIP differs in terms of strength and inhibition kinetics, and should reflect the counter-adaptation occurring in both enzymes and inhibitors (Stahl and Bishop 2000). During this evolutionary arms race, pathogens have evolved PGs with different activities to successfully colonize the host tissue and, conversely, plants have evolved PGIPs with different specificities to counteract the different PGs. For instance, all members of the bean PGIP family inhibit, although with different efficiencies, PGs from *B. cinerea* and *C. acutatum*, but only PvPPGIP2 inhibits the PG from *F. phyllophilum* FC-10 strain and only PvPGIP3 and PvPGIP4 inhibit PGs of insects (D'Ovidio et al. 2004a; Leckie et al. 1999).

The capability of the same PGIP to interact with different PGs is facilitated by the LRR motif that provides a versatile structural framework for protein–protein interactions (Kobe and Kajava 2001). In particular, computational analysis of PvPGIP2 predicts that the xxLxLxx region within each LRR has a strong propensity to be involved in protein–protein interactions (Sicilia et al. 2005) and biochemical analysis have shown that this region contains residues influencing the specificity of PvPGIP2 (Leckie et al. 1999; D'Ovidio et al. 2004a; Sicilia et al. 2005).

PvPPGIP2 is the most effective inhibitor of many fungal PGs and shows competitive, non-competitive or a mixed mode of inhibition kinetics against the PGs of *F. phyllophilum*, *A. niger* and *B. cinerea*, respectively (Federici et al. 2001; King et al. 2002; Sicilia et al. 2005). Computational analyses have shown that this versatility could involve the formation of complexes with different interacting surfaces (Federici et al. 2006). It has been also suggested that the formation of the PG–PGIP complex could require a network of multiple and relatively weak contacts and only one or very few strong contact that lock the complex, possibly

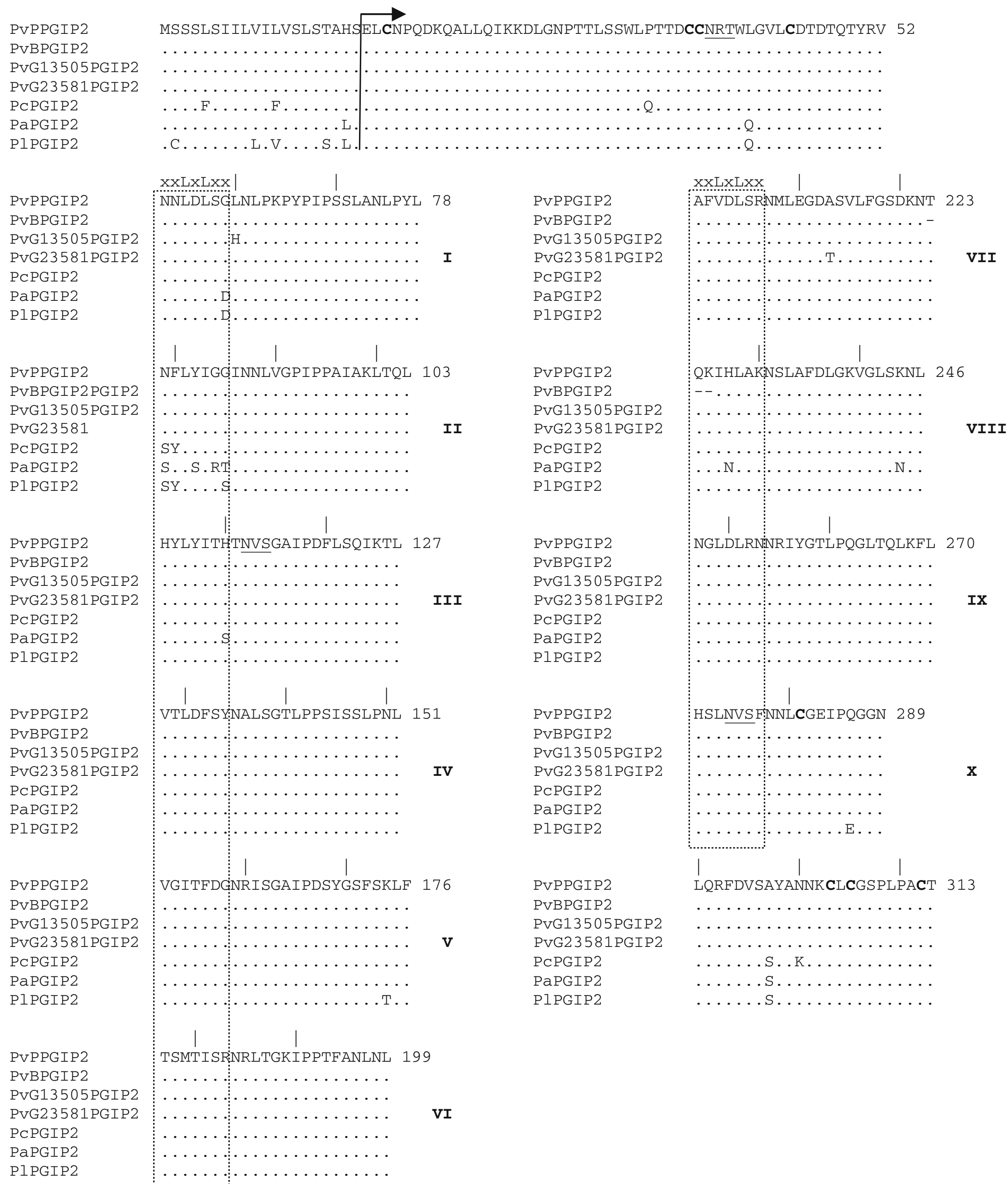

Fig. 2 Deduced amino acid sequence comparison between PvPPGIP2 from Pinto and the PGIP2 variants identified in common bean and related species. Alignment includes also PvBPGIP2, a PGIP2 variant previously identified in the Mesoamerican bean line BAT93 and containing a three amino acids deletion (D'Ovidio et al. 2004a). Numbering is referred to the PvPPGIP2 sequence and starts from the first residue of the mature protein (indicated by *arrow*). Regions, including the modular organization of the LRR motif (residues 53–289), were defined on the basis of the crystal structure of PvPGIP2 (Di Matteo et al. 2003). The xxLxLxx region is *boxed*. Cysteine residues are in *bold*; putative glycosylation sites are *underlined*. Amino acid substitutions in the different genotypes are indicated. *Dash* indicates amino acid deletion. PvPPGIP2, *P. vulgaris* cv Pinto; PvBPGIP2, *P. vulgaris* genotype BAT93; Pv(13505)PGIP2, *P. vulgaris* accession G13505; Pv(23581)PGIP2, *P. vulgaris* accession G23581; PcPPGIP2, *P. coccineus* cv Corona; PaPGIP2, *P. acutifolius* genotype PA-T8a-1; *P. lunatus* geneotype PHA8067

involving residues located in or close to the xxLxLxx region forming the concave surface of PGIP (Di Matteo et al. 2003; D'Ovidio et al. 2004a).

The possibility that PG–PGIP specificity can be controlled by one or a few amino acids is particularly supported by the findings that variation at site 224 of

Table 2 Inhibitory activities of PvPPGIP2 and different PGIP2 variants against *endo*-polygalacturonases of fungal pathogens (top lines)

	FpPG	ClPG	AnPG	BcPG
PvPPGIP2	27	38	4.5	9
PvBPGIP2	∞	39	54	58
Pv(23581)PGIP2	45	70	9	30
PcPGIP2	33	27	3.5	9
PaPGIP2	45	30	4.5	12
PlPGIP2	30	42	4.5	12

Values indicate the amount (in ng) of PGIP that determines 50% inhibition of 1 agarose diffusion unit of PG. The symbol ∞ indicates >2 μg

The Pv(13505)PGIP2, carrying the substitution L60H, was not included in the inhibition assays because this variation produced a very little change in the affinity for the PG of *F. moniliforme* (reclassified as *F. phyllophilum*) and *A. niger* (Leckie et al. 1999)

PvPPGIP2, *P. vulgaris* cv Pinto; PvBPGIP2, *P. vulgaris* genotype BAT93; Pv(23581)PGIP2, *P. vulgaris* accession G23581; PcPPGIP2, *P. coccineus* cv Corona; PaPGIP2, *P. acutifolius* genotype PA-T8a-1; *P. lunatus* geneotype PHA8067; Fp, *F. Phyllophilum*; Cl, *Colletotrichum lupini*; An, *Aspergillus niger*; Bc, *Botrytis cinerea*

PvPPGIP2 can abolish its inhibiting properties against the PG of *F. phyllophilum* FC-10 strain (Leckie et al. 1999; D'Ovidio et al. 2004a) and that variation at site 97 of the PG of *F. verticillioides* PD strain allowed this PG to escape PGIP recognition (Raiola et al. 2008).

Despite the analysis of wild and cultivated genotypes of *P. vulgaris* distributed from the sea level up to 3,500 m s.l., only a very limited sequence polymorphism of *Pvpgip2* has been detected. Variation does not distinguish between genotypes of Andean or Mesoamerican origin but, as previously reported (Gepts et al. 1986), was higher in the wild accessions compared to the cultivated genotypes. Sequence diversity increased, but was still very limited, in the *Pgip2* of the related species *P. coccineous*, *P: acutifolius*, and *P. lunatus,* with *Pcpgip2* being the closest and *Plpgip2* the most divergent with respect to *Pvpgip2*. This is in agreement with the reported phylogenetic relationships among these species (Delgado-Salinas et al. 1999), which all grow in the same area and are predominantly autogamous, except for *P. coccineous* that is predominantly allogamous (Escalante et al. 1994). The strong sequence conservation of *Pgip2* of *P. lunatus* is noteworthy, because this species belongs to a separate clade from that including *P. vulgaris*, *P. coccineous*, and *P. acutifolius* (Delgado-Salinas et al. 1999). Similarly, a strong conservation was observed with the member *Gmpgip3* of the soybean (*Glycine max*) *pgip* family. This gene is closer to *Pvpgip2* than to its *Gmpgip* paralogs. Its encoded product differs from PvPGIP2 by 35 amino and shares similar functional characteristics (D'Ovidio et al. 2006), indicating that it retains all the structural features required for PGIP2 function.

The observed nucleotide variation is mainly due to SNPs, mostly of which account for synonymous substitutions. The non-synonymous substitutions occur mainly in the xxLxLxx region, as reported in many resistance (*R*) genes (Hulbert et al. 2001; Meyers et al. 2005) and consistently with the role of this region in ligand recognition (Warren et al. 1998; Leckie et al. 1999; D'Ovidio et al. 2004a; Sicilia et al. 2005). It is noteworthy that variation does not involve residues participating in the formation of secondary structure elements such as the sheet B1 (except in a very few cases), sheet B2 and the 3_{10}-helix, or residues involved in the formation of the negative pocket (Di Matteo et al. 2003) or those interacting with pectin (Spadoni et al. 2006).

Overall, sequence variation in *Pgip2* genes is very limited (with a maximum of ten amino acid replacements in the mature protein) and does not have a strong impact on the inhibiting properties of the variants against the four PGs tested. This variation is much lower than in most LRR-encoding regions in NB-LRR genes, where this region is often hypervariable (Hulbert et al. 2001; Meyers et al. 2005). This may reflect the fact that the majority of the PGIP2 residues, including most of those of the LRR region, play a role in ligand binding and contribute to the specificity and efficiency of recognition. This model supports the hypothesis that the formation of the PG–PGIP complex requires a network of multiple contacts (Di Matteo et al. 2003), where different sets of PGIP solvent-exposed residues are likely involved in binding different pathogen-derived PGs, which share a general similar structure. Although LRR products control gene-for-gene specificity, examples of a single LRR molecule possessing dual ligand specificity have been reported, possibly controlled by different sets of solvent-exposed residues (Ellis et al. 2000). *RPM1* is responsible for resistance to bacterial pathogens containing either of two unrelated avirulence genes (Grant et al. 1995). Similarly, the *Mi* encodes resistance to a nematode and an aphid (Rossi et al. 1998; Vos et al. 1998).

The analysis of the common bean germplasm allowed the identification of only two PvPGIP2 variants, each possessing a single amino acid substitution. Pv(13505)PGIP2 possesses the substitution L60H that has been previously analyzed by Leckie et al. (1999) in a site-directed mutagenesis study aimed at analysing the effect of each of the eight substitutions that distinguish PvPPGIP2 and PvPPGIP1. In that analysis, this mutation produced very little variation in the affinity for the FpPG and AnPG. The other PvPGIP2 variant, Pv(23581)PGIP2, possesses a substitution A213T that determined a minor decrease in the inhibition efficiency against all four PG tested. This residue is outside of the xxLxLxx region but represents a variation of the alanine residue within the GDA sequence of the VII LRR that is conserved in almost all dicot and monocot PGIPs

(De Lorenzo et al. 2001; Janni et al. 2006). Substitutions were more numerous in the related bean species. However, unlike the Q224K replacement in PvPPGIP2 (Leckie et al. 1999) or the naturally occurring deletion of residues 223–225 identified in PvBPGIP2 of the bean genotype BAT93 (D'Ovidio et al. 2004a), the novel substitutions do not significantly influence the inhibiting properties of the variants against the four PGs tested, and therefore did not allow the identification of the contribution of single or specific sets of residues to functionality. However, experiments involving a more diverse set of PGs could better reveal their impact on inhibitory activity.

In this work, attention was focused onto *Pvpgip2*, because it encodes the most effective PGIP so far characterized. However, the bean *Pgip* gene family comprises three additional genes, namely *Pvpgip1*, *Pvpgip3*, and *Pvpgip4* that we suggested, on the basis of their sequence similarities, to derive from a common ancestor by a sequence of duplication–divergence–duplication events (D'Ovidio et al. 2004a). It would be interesting to analyze sequence polymorphism in these additional members to assess whether they evolve at rates different from that of PvPGIP2, like for example the genes composing the lettuce *RGC2* cluster, which contains type I genes that are evolving rapidly and type II genes that are relatively conserved (Kuang et al. 2004).

In conclusion, we have showed that PGIP2 sequence and function are strongly conserved in the bean germplasm and in the related bean species, *P. coccineus*, *P. acutifolius*, and *P. lunatus*. This conservation suggests that during evolution, only a very limited number of amino acid replacements of PGIP2 can be tolerated, likely to maintain wide-spectrum and high-affinity recognition of PGs.

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