A polygalacturonase-inhibiting protein with a role in pea defence against the cyst nematode *Heterodera goettingiana*

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SUMMARY

A cDNA of 312 bp, similar to polygalacturonase-inhibiting proteins (PGIPs), was isolated by cDNA-amplified fragment length polymorphism (cDNA-AFLP) from pea roots infected with the cyst nematode *Heterodera goettingiana*. The deduced amino acid sequence obtained from the complete *Pspgip1* coding sequence was very similar to PGIPs described from several other plant species, and was identical in both MG103738 and Progress 9 genotypes, resistant and susceptible to *H. goettingiana*, respectively. Reverse transcription-polymerase chain reaction (RT-PCR) expression analysis revealed the differential regulation of the *Pspgip1* gene in the two genotypes in response to wounding and nematode challenge. Mechanical wounding induced *Pspgip1* expression in MG103738 within 8 h, but this response was delayed in Progress 9. In contrast, the response to nematode infection was more complex. The transcription of *Pspgip1* was triggered rapidly in both genotypes, but the expression level returned to levels observed in uninfected plants more quickly in susceptible than in resistant roots. In addition, *in situ* hybridization showed that *Pspgip1* was expressed in the cortical cells damaged as a result of nematode invasion in both genotypes. However, it was specifically localized in the cells bordering the nematode-induced syncytia in resistant roots. This suggests a role for this gene in counteracting nematode establishment inside the root.

INTRODUCTION

Sedentary endoparasitic nematodes are obligate biotrophic pathogens that undergo complex interactions with their hosts.

Cyst and root knot nematodes have developed molecular mechanisms that lead to the re-differentiation of root cells into complex feeding structures on which the nematodes depend for the nutrients required for their development. The cyst nematode *Heterodera goettingiana* is a host-specific pathogen of pea (*Pisum sativum* L.) in every area in which this crop is grown. After invading the plant roots, the nematode migrates intracellularly to the vascular cylinder where it selects a cell which becomes its initial feeding site. In response to repeated stimulations by the parasite, this cell develops into a syncytium (Jones, 1981). The syncytium is formed by the breakdown of plant cell walls and subsequent fusion of neighbouring cells. Although the nematode uses its own endogenous cell wall-degrading enzymes to soften host cell walls during migration through the root (Baum *et al.*, 2007), degradation of the cell walls within the syncytium is achieved using the plant's own cell wall-degrading enzymes, which are induced during the development of the syncytium (Goellner *et al.*, 2001).

The plant cell wall is the first barrier to pathogen colonization of plant tissue. Therefore, the degradation of the main cell wall components, including pectin and cellulose, is essential for phytoparasitic organisms. Pectin degradation requires the combined action of several enzymes. Biotrophic and necrotrophic plant-pathogenic fungi and bacteria secrete an array of cell wall-degrading enzymes during infection of their hosts (ten Have *et al.*, 2002; Hugouvieux-Cotte-Pattat *et al.*, 1996; Walton, 1994). Among these enzymes, endopolygalacturonases (endoPGs; EC 3.2.1.15) are considered to be the key pathogenicity determinants for fungal pathogens. Polygalacturonases (PGs) are the first enzymes secreted by phytopathogenic fungi, and their action on the outer component of the cell wall is a prerequisite for further cell wall degradation by other cell wall-degrading enzymes (De Lorenzo *et al.*, 2001). PGs are also produced by bacteria, insects and some nematodes (Girard and Jouanin, 1999; Huang and Allen, 2000; Jaubert *et al.*, 2002). These enzymes act by cleaving the α -1,4 linkages between D-galacturonic acid residues in non-methylated homogalacturonan, the main component of pectin,

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causing cell separation and maceration of host tissues. Plants have developed a mechanism to counteract the action of PGs through the production of proteins, known as polygalacturonase-inhibiting proteins (PGIPs). These proteins are thought to contribute to resistance against pectinase-producing fungi (De Lorenzo *et al.*, 2001). Overexpression of PGIPs in transgenic plants limits fungal colonization (Agüero *et al.*, 2005; Ferrari *et al.*, 2003; Janni *et al.*, 2008; Joubert *et al.*, 2006; Powell *et al.*, 2000), whereas the silencing of PGIPs increases disease symptoms (Ferrari *et al.*, 2006), demonstrating the role of these proteins in defence against pathogens. PGIPs inhibit a broad range of fungal PGs, and their activity leads to the accumulation of elicitor-active oligogalacturonides. These oligosaccharides activate plant defence responses, such as the synthesis of phytoalexins, lignin and ethylene, the expression of pathogenesis-related (PR) proteins and the production of reactive oxygen species (Ridley *et al.*, 2001). PGIPs are typically cell wall bound, tissue specific, developmentally regulated and inducible by various stimuli, including pathogen attack, wounding, salicylic acid, jasmonic acid (JA), oligogalacturonides and cold stress (Joubert *et al.*, 2007). PGIPs form a subclass of extracytoplasmic proteins which contain leucine-rich repeats (LRRs) (Jones and Jones, 1997). The LRR structure is shared by many plant proteins involved in the recognition of pathogens, including the majority of the resistance gene products (Martin *et al.*, 2003). PGIPs typically contain 10 imperfect LRRs of 24 residues, each containing the consensus xxLxLxx motif, which is thought to be responsible for PG recognition (Di Matteo *et al.*, 2003).

Genes encoding PGIPs have been cloned from various plants, and are usually encoded by small gene families. Interestingly, PGIPs from different plants not only differ in their inhibitory activity, but PGIPs from a single plant often exhibit specificities against PGs from different fungi or different PGs from the same fungus (De Lorenzo and Ferrari, 2002).

In previous studies, Mahalingam *et al.* (1999) analysed the behaviour of plant PG and PGIP following cyst nematode infection in soybean, and established that PGIP did not behave as a resistance factor in the *Glycine max*–*H. glycines* interaction. In this article, we report a pea *pgip* (*Pspgip1*) gene upregulated during nematode infection. *Pspgip1* expression profiles, examined in both compatible and incompatible interactions between pea and *H. goettingiana*, as well as in mechanically wounded roots, suggest a role in defence against this pathogen.

RESULTS

Isolation and characterization of a PGIP differentially expressed during nematode infection

Roots of pea genotypes MG103738 (resistant) and Progress 9 (susceptible) were analysed for differential gene expression after

Fig. 1 Section of cDNA-amplified fragment length polymorphism (cDNA-AFLP) gel images. Templates were derived from pea roots 48 h after *Heterodera goettingiana* infection (+) compared with control (–). The arrow indicates the fragment corresponding to the *Pspgip1* gene. Molecular size marker (M) is shown on the left.

nematode infection using cDNA-amplified fragment length polymorphisms (cDNA-AFLPs) (Vos *et al.*, 1995). One differentially expressed fragment (Fig. 1), upregulated following nematode infection of both pea genotypes, showed 85% similarity with a *G. max* PGIP (EMBL accession number AAD45503) and 80% similarity with a *Phaseolus vulgaris* protein PGIP2 (EMBL accession number CAI11358).

The full-length sequence of the pea *pgip* cDNA (*Pspgip1*) (EMBL accession number AJ749705) was obtained by 5' and 3' rapid amplification of cDNA ends (RACE), and consisted of a 1020-bp open reading frame (ORF) which could encode a protein of 339 amino acids. The ORF was flanked by a 17-bp 5' untranslated region (UTR) and a 187-bp 3' UTR containing two overlapping polyadenylation signals. Primers encompassing the complete coding region were used to amplify the genomic regions of the *Pspgip1* gene from MG103738 and Progress 9. Sequencing of these sequences revealed the absence of introns in both genotypes, and the presence of intra- and inter-genotype variation in the *Pspgip1* gene. Within MG103738, two forms were identified differing only in eight synonymous substitutions and one nonsynonymous substitution (Val₁₀ instead of Ala₁₀) within the coding region (Table 1). Progress 9 exhibited a varied pattern: three different clones showed the presence of synonymous or nonsynonymous substitutions when compared with each other or with the MG103738 *Pspgip1* gene (Table 1), this latter situation resembling an intraspecific variation.

Southern blot analysis (Fig. 2) suggested that the *Pspgip1* gene belonged to a small gene family consisting of at least three genes in MG103738 and two genes in Progress 9.

Table 1 Nucleotide and amino acid substitutions in MG103738 and Progress 9 pea genotypes.

Accession	Substitutions		
	Coding region (nt)	Mature protein (aa)	Position and type
MG103738 (8)	9	1	Ala ₁₀ –Val ₁₀
Progress 9 (2)	1	0	Asn ₂₀₇ –Asp ₂₀₇
Progress 9 (4)	2	1	Leu ₁₂₁ –Pro ₁₂₁
Progress 9 (5)	3	2	Ser ₁₄₆ –Pro ₁₄₆

Nucleotide (nt) and amino acid (aa) differences from *Pspgip1* (EMBL accession number AJ749705) are indicated. Numbers in parentheses indicate the number of cDNA clones.

Fig. 2 Genomic Southern analysis of the *pgip* gene family in pea MG103738 and Progress 9. DNA (10 µg) of both resistant (MG103738) and susceptible (Progress 9) pea genotypes was digested with *Eco*RI (E), *Hind*III (H) and double digested with *Eco*RI–*Hind*III (E–H), and hybridized with a 779-nucleotide fragment corresponding to the *Pspgip1* open reading frame (ORF) encoding the mature protein. The probe hybridizes to a series of bands indicating the presence of a multigene family. DNA size standards in kb are shown on the left.

Analysis of the putative PGIP protein

The PsPGIP1 predicted protein showed typical PGIP topology. A 25-amino-acid signal peptide for secretion (domain A), was predicted by SignalP (Bendtsen *et al.*, 2004), followed by a 52-amino-acid N-terminal domain (domain B), an LRR region (domain C) and a C-terminal domain (domain D) (Fig. 3). The resulting mature peptide had a molecular mass of 36 814 Da and a *pI* value of 6.79, lower than that observed for other dicots (Li *et al.*, 2003), as predicted by the ProtParam tool (Wilkins *et al.*,

1999). Pea PGIP1 contains three potential N-linked glycosylation sites (N–X–S/T; where X is any amino acid except P) at positions 35, 198 and 274, two of which are conserved in bean PGIP2 and in soybean PGIP3 (Fig. 3). The predicted N-linked glycosylation site at position 35 has been shown to be occupied by a typical complex N-glycan in PvPGIP2 (Mattei *et al.*, 2001).

PsPGIP1 contains 10 LRRs whose consensus sequence matches the extracytoplasmic LRR consensus sequence of resistance genes (Jones and Jones, 1997). The deduced protein also contains eight cysteine (Cys) residues at conserved positions: four within the N-terminus, one in the 10th LRR and three within the C-terminus (Fig. 3). These have been shown to form four disulphide bridges (Cys₃–Cys₅₃, Cys₃₄–Cys₄₃, Cys₂₈₁–Cys₃₀₃, Cys₃₀₅–Cys₃₁₂) which are important for the maintenance of secondary structures in PGIP.

The predicted mature protein sequence of the pea PGIP1 showed a high degree of similarity (86% and 83%, respectively) with the sequences of *G. max* PGIP (GmPGIP3) and *P. vulgaris* PGIP2 (PvPGIP2). Comparison of the pea PGIP1 and GmPGIP3/PvPGIP2 sequences showed that the Gln residue at position 224, responsible for the recognition of PG of *Fusarium moniliforme* strain FC10 (Leckie *et al.*, 1999), is conserved, and that the amino acids forming the negatively charged pocket are present at conserved positions (Asp₁₃₁, Ser₁₃₃, Thr₁₅₅, Asp₁₅₇, Thr₁₈₀ and Asp₂₀₃).

A maximum likelihood phylogenetic tree of PGIP nucleotide sequences showed, as expected, two main groupings representing leguminous and brassicales species (Fig. 4). Although there was a reduced phylogenetic signal in the leguminous group involving *Glycine* and *Phaseolus* sequences (node bootstrap support less than 50), the position of the PGIP nucleotide sequence of *P. sativum* was highly supported at the base of the leguminous clade (Fig. 4). Moreover, the phylogram showed a different rate of substitution per site between brassicales and leguminous species, with *P. sativum* close to the latter grouping. A similar situation was found when the phylogenetic tree was inferred by PGIP protein sequences. The *P. sativum* protein sequence again clustered at the base of the leguminous grouping supported by a high bootstrap value (100). However, this tree (available as Fig. S1, see Supporting Information) was not completely solved, and relationships within leguminous species

A	
PsPGIP1	MQNFPSCMLLLLILTANLLVPSLS
PvPGIP2	---MS.SLSII...VS--.STAH.
GmPGIP3	----M.KLSI.FLLVLS--FSSV..
B	
PsPGIP1	ERCNPDDKKALLQIKKEFGNPTKLSSWDPTTDC C <u>NGT</u> WSGVSCD T DTQTFRV ⁵²
PvPGIP2	.L...Q...Q.....DL...T...L..... <u>R</u> .L..L.....Y..
GmPGIP3	.L...Q...Q.....DL...T...LL..... <u>R</u> .L.....Y..
C	
	xxLxLxx
PsPGIP1	NDL D LDLDPQPVSI P PSITNLPFL ⁷⁸
PvPGIP2	.N....G.N..K.YP..S.LA...Y.
GmPGIP3	N..K.Y.....A...Y.
PsPGIP1	FYLSLSRIPNLVGSIPPSISSLT K L ¹⁰³
PvPGIP2	NF.YIGG.N...P...A.AK..Q.
GmPGIP3	NF..I..T.T.I.Q..SA.AK..Q.
PsPGIP1	RYLYLSHSSLTGVIPNTLSQIKGL ¹²⁷
PvPGIP2	H...IT. <u>TNVS</u> .A..DF.....T.
GmPGIP3	IT. <u>TNVS</u> .P..DF.....T.
PsPGIP1	LTLD F SYNKL S GPLPDTLSSLPNL ¹⁵¹
PvPGIP2	V.....A...T..PSI.....
GmPGIP3	V.....T...K..AS.....
PsPGIP1	IGITFDGNQLSGSIPESYGSPD S F ¹⁷⁶
PvPGIP2	V.....RI..A..D.....SKL.
GmPGIP3	V.....I..A..D.....SKL.
PsPGIP1	TVLTLSRNSLSGTIPASLAKNL ¹⁹⁹
PvPGIP2	.SM.I...R.T.K..PTF.N...
GmPGIP3	.SM.I...R.T.K...TF.N...
PsPGIP1	<u>S</u> FVDLSRNSLVGDASVLF G SNKNT ²²³
PvPGIP2	A.....M.E.....D...
GmPGIP3	A.....M.E.....TE...
PsPGIP1	QKIVLATNSLAFDIGVGLSANL ²⁴⁶
PvPGIP2	...H..K.....L.....K..
GmPGIP3	...L..K.M....L.....K..
PsPGIP1	NYL D LRNNKIYGTLP E GLTGLKFL ²⁷⁰
PvPGIP2	.G.....R.....Q...Q....
GmPGIP3	.G.....R.....Q...A....
PsPGIP1	HNFNVSYNSLCGQIPQGDN ²⁸⁹
PvPGIP2	.SL...F.N...E....G.
GmPGIP3	.S...F.D...E....G.
D	
PsPGIP1	LRRFDEYCYAHNKCLCGSPLPACKT ³¹⁴
PvPGIP2	.Q...VSA..N.....T-
GmPGIP3	MQ...VSS..N.....S.T-

Fig. 3 Sequence alignment of PsPGIP1, PvPGIP2 and GmPGIP3. Numbering refers to the PsPGIP1 sequence and starts from the first residue of the mature protein. Typical polygalacturonase-inhibiting protein (PGIP) domains are indicated (A, signal peptide; B, presumed N-terminus of the mature protein; C, 10 leucine-rich repeat (LRR) modules; D, C-terminus). The xxLxLxx region is boxed. Dashes indicate gaps. Dots represent invariant amino acids. Cysteine residues are in bold; putative glycosylation sites are underlined and in italics.

Fig. 4 Maximum likelihood phylogenetic tree of polygalacturonase-inhibiting protein (PGIP) and related leguminous nucleotide sequences inferred by the PhyML program (Guindon *et al.*, 2009). Node labels represents bootstrap support values. Branch lengths are in substitutions per site.

could not be assessed satisfactorily, even though two main clades involving leguminous and brassicales species were clearly identified. Inconsistencies in the phylogenetic tree deduced by protein PGIP sequences were mainly a result of a higher conservation degree at the protein than at the nucleotide level.

Pea *pgip1* is induced in response to *H. goettingiana* infection

Pspgip1 expression was investigated by real-time reverse transcription-polymerase chain reaction (RT-PCR) in uninfected and nematode-challenged roots of MG103738 and Progress 9 genotypes. The analyses were performed at 24, 48 and 72 h

post-infection (hpi) with *H. goettingiana* using a primer set targeting the *Pspgip1* 3' UTR in order to detect this gene specifically. *Pspgip1* behaviour in response to nematode infection showed a different trend in the two genotypes (Fig. 5). *Pspgip1* levels were high at 24 hpi and peaked at 48 hpi in the resistant genotype, and were decreased at 72 hpi. Surprisingly, in the uninfected tissues, an induction of the gene was observed at 48 and 72 hpi, possibly in response to physiological changes occurring during root development. In susceptible pea roots, *Pspgip1* expression increased strongly at 24 hpi but, by around 48 hpi, the transcript levels started to decrease and returned to the levels observed in uninfected plants at 72 hpi. This result is consistent with those obtained by cDNA-AFLP analysis (Fig. 1),

Fig. 5 Gene expression in pea roots after nematode infection. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analyses were performed on total RNA from root apices and elongation zones of resistant (MG103738) and susceptible (Progress 9) pea genotypes at the time points indicated (h) after nematode infection using primers targeting the *Pspgip1* 3' untranslated region (UTR). Control samples were uninfected roots from seedlings kept under the same conditions as infected ones. Expression levels in each sample were normalized to the expression of the 26S rRNA gene and were calculated relative to uninfected control plants at 24 h. All genes were analysed in triplicate from three independently isolated RNA samples. Values are expressed as the mean $\pm$ standard error. All mean comparisons were carried out according to Duncan's multiple range test. Differences were significant at $P < 0.05$ (*) and $P < 0.01$ (**).

where differential expression of the *Pspgip1* gene was observed between resistant and susceptible pea at 48 hpi. This suggests that this gene could be important in the early response to nematode challenge; whereas, in resistant pea roots, a longer expression might be important to limit the spread of the infection, in susceptible pea roots, *Pspgip1* expression is suppressed as a result of a successful nematode establishment in the root.

It has been suggested that soybean PG and PGIP are under mutual transcriptional control during the *G. max*–*H. glycines* compatible interaction (Mahalingam *et al.*, 1999). Therefore, we investigated whether levels of the *Pspgip1* transcript were correlated with the expression of the only pea PG gene (EMBL accession number AF361321) available in data banks. RT-PCR experiments on total RNA extracted at 24, 48 and 72 hpi did not show any pea PG transcript induction, even with a large number of amplification cycles (data not shown). Therefore, as far as it is possible to tell with currently available data, *Pspgip1* does not seem to be induced by the expression of endogenous PG in pea. It is feasible that *Pspgip1* could be induced by nematode PG, as a PG has been identified in the root knot nematode *Meloidogyne incognita* (Jaubert *et al.*, 2002). However, no similar sequences have been reported from cyst nematodes, and no sequences similar to PG are present in expressed sequence tag (EST) datasets or partially completed genome sequences for these nematodes.

Expression analysis following wounding and methyl jasmonate (MeJa) treatment

The response of *Pspgip1* to wounding and MeJa treatment was analysed using the primer sets described above. RT-PCR analyses were performed on total RNA extracted from either root

tissues needle-punctured and sampled 2, 4, 8, 24 and 48 h after treatment or roots exposed overnight to MeJa. RT-PCR experiments demonstrated a different behaviour between the two genotypes (Fig. 6). In the susceptible roots, little or no induction of the gene was observed in response to wounding. By contrast, transcripts of *Pspgip1* accumulated 8 h after wounding in the resistant germplasm (Fig. 6). *Pspgip1* gene expression was induced by MeJa in both susceptible and resistant pea roots (Fig. 6).

Localization of *Pspgip1* transcript during nematode infection

To determine the site of expression of *Pspgip1*, *in situ* hybridization experiments were carried out on serial cross-sections of uninfected and 24 hpi/48 hpi roots. Probes specific for *Pspgip1* were used in these experiments. In both resistant MG103738 and susceptible Progress 9 infected roots, a purple staining, more intense at 24 hpi (Fig. 7a,a') than at 48 hpi (Fig. 7e,e'), was detected in the cytoplasm of cortical cells affected by nematode migration, indicating a prompt induction of the *Pspgip1* gene in these cells in response to nematode-induced injury. In addition, in resistant roots, a specific strong signal was found in the cytoplasm of the cells very close to or inside the vascular cylinder and surrounding the nematode feeding site (Fig. 7b–d,f–h). These cells appeared to be part of the response to nematode infection, in which cells surrounding the feeding site prevent the further development of the feeding site. Syncytia at 24 hpi (Fig. 7d) and 48 hpi (Fig. 7h) did not show any staining. In susceptible roots, the signal was very evident at 24 hpi in the cortical cells along the nematode's migration path (Fig. 7b'–d') and in the cells bordering the nematode. Syncytia appeared to be

Fig. 6 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *Pspgip1* expression in response to mechanical wounding and methyl jasmonate (MeJA) treatment. Time points in hours (h) are indicated. C₀, untreated root; C₂, plantlets kept for 2 h on filter paper, with the rootlets in the dark, soaking in Hoagland solution (HS) in wounding experiments; C, roots watered with a solution of 0.1% methanol, 0.02% Tween-20, in MeJA treatments. The 26S rRNA gene was used as a control for amplification.

unreactive except when they were adjoining the nematode (Fig. 7d'). At 48 hpi, the signal became less intense and was specifically localized in cells directly injured by the nematode. Syncytia appeared to be unstained (Fig. 7f'–h') and were surrounded by reacting endodermal cells (Fig. 7h').

In agreement with the results obtained by RT-PCR, uninfected roots of both MG103738 and Progress 9 did not show any reaction when hybridized with the *Pspgip1* probe (Fig. 7i,i'). No *Pspgip1* transcript signal was detected in resistant or susceptible infected root sections when hybridized with a sense probe (Fig. 7j,j').

DISCUSSION

We have identified and functionally characterized a *pgip* gene from *P. sativum*, which is upregulated in response to cyst nematode infection. Genomic analysis revealed the presence of a small gene family in *P. sativum* and showed that, as in the closely related Leguminosae and in the majority of other species analysed to date, the coding sequence is not interrupted by introns in the genomic DNA.

Sequence comparison performed between pea PGIP1 and the available sequences present in databanks showed the highest degree of similarity, 86% and 83%, respectively, with the sequences of soybean PGIP (GmPGIP3) and bean PGIP2 (PvPGIP2). The deduced protein sequence contained typical PGIP sequence features, including 10 LRRs and eight conserved Cys residues, which are thought to be important in the formation of disulphide bridges required for the biological function of these LRR proteins (Mattei *et al.*, 2001). Additional Cys residues have been found in some PGIP sequences, and the PGIP1 sequence has an additional Cys residue (Cys₂₉₇) in the C-terminus as observed in PvPGIP4 (D'Ovidio *et al.*, 2004a).

PGIPs are glycosylated proteins (Mattei *et al.*, 2001), and pea PGIP1 has three putative glycosylation sites, two of which were

conserved in PvPGIP2 and GmPGIP3. In particular, the potential glycosylation site at position 35, which has been shown to be occupied by N-glycan in bean PvPGIP2, and the site within the 10th LRR, which can be occupied by any polysaccharide in PvPGIP2 (Mattei *et al.*, 2001), are conserved in pea. A comparison of the pea PGIP and GmPGIP3/PvPGIP2 sequences showed that the Gln residue at position 224, responsible for the recognition of PG of *F. moniliforme* strain FC10 (Leckie *et al.*, 1999), is conserved, together with the amino acids forming the negative pocket (Asp₁₃₁, Ser₁₃₃, Thr₁₅₅, Asp₁₅₇, Thr₁₈₀ and Asp₂₀₃). These features provide further support for the concept that a small number of residues within or close to the concave surface of PGIP are required to form the PG–PGIP complex (Di Matteo *et al.*, 2003).

It is well established (D'Ovidio *et al.*, 2004b) that, in the presence of PGIP, the enzymatic activity of PGs is reduced in such a way that the balance between the release of elicitor-active oligogalacturonides and depolymerization of active oligogalacturonides into inactive molecules is altered in favour of the accumulation of elicitor-active molecules. This leads to the transcriptional activation of defence-related genes, such as those encoding enzymes of the phenylpropanoid pathway and pathways related to the production of phytoalexins, as well as genes involved in the metabolism and/or synthesis of JA. Jasmonates are hormones that regulate plant growth and development, but they also have an important role in defence responses against various biotic and abiotic stresses (Wasternack, 2007). JA and its derivatives, such as MeJA, are synthesized from linolenic or linoleic acid by the consecutive action of 13-lipoxygenase (13-LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), 12-oxophytodienoic acid reductase (OPR3) and β -oxidative enzymes (Wasternack, 2007). There is strong evidence to suggest that JA and its conjugates are essential wounding signals in a wide range of plants. Wounding activates the expression of defence genes by triggering the LOX pathway described above (Wasternack, 2007). Previous studies have shown an increase in LOX gene expression and activity in pea roots infected with *H. goettingiana* (Leone *et al.*, 2001; Veronico *et al.*, 2006). The importance of this response in the context of the plant response to nematode infection is demonstrated by the presence of a secreted lipid-binding protein on the surface of cyst nematodes, which has been shown to inhibit the LOX-mediated breakdown of linoleic and linolenic acid (Prior *et al.*, 2001).

Pspgip1 gene expression was induced by exogenous application of MeJA, suggesting that the regulation of this gene could be dependent on the jasmonate pathway. However, the behaviour of resistant and susceptible pea roots in response to mechanical wounding was different, with the resistant genotype reacting more strongly than the susceptible genotype in the induction of *Pspgip1* expression. A marked response 8 h after wounding was observed in resistant roots, which was not found

Fig. 7 *In situ* localization of *Pspgip1* transcript in uninfected, 24-h and 48-h nematode-infected resistant MG103738 (a–j) and susceptible Progress 9 (a'–j') pea roots. Cross-sections of roots were hybridized to digoxigenin (DIG)-labelled *Pspgip1* antisense (a–i and a'–i') and sense (j, j') riboprobes. Resistant (i) and susceptible (i') uninfected roots show no hybridization with antisense probe. (a–d) Cross-sections of 24-h resistant infected roots. (a) Specific signals in the cytoplasm of cortical cells challenged by nematode migration. (b) Detail of reacting pericycle. (c) Parenchyma vessel cells react strongly to nematode challenge. (d) Detail of a 24-h syncytium showing pale purple staining. (e–h) Cross-sections of 48 h resistant infected roots. (e) Cortical and endodermal cells react strongly to nematode penetration and its feeding action. (f, g) Strong signals are detectable in the cytoplasm of the cells surrounding the nematode. (h) The developing syncytium adjacent to the vascular cylinder does not show any staining. (a'–d') Cross-sections of 24-h susceptible infected roots. (a') Hybridization signal in the cytoplasm of several cortical cells injured by nematode invasion. (b', c') Details of stained cortical cells promptly reacting to nematode challenge. (d') A developing syncytium close to the head of the nematode shows heavy staining of its cytoplasm. (e'–h') Cross-sections of 48-h susceptible infected roots. (e') Pale purple signal is visible in the cortex. (f', g') Details of cells injured by the migrating nematode and of a small syncytium completely unstained. (h') A large unstained syncytium is surrounded by reacting endodermal cells. (j, j') Unreactive negative controls of resistant and susceptible pea, respectively. a, e, i, j and a', e', i', j': bar, 25 µm; other panels: bar, 16 µm. Symbols: arrow head, nematode; arrow, syncytium; asterisk, root primordium.

in the susceptible plants, where a later and weaker reaction was detected 48 h after wounding. The different induction level in response to wounding suggests that *Pspgip1* expression may either be governed by different systems in the two genotypes or may reflect differences in the sequence of the regulatory regions.

The putative role of *Pspgip1* as a defence gene in response to nematode attack is supported by the increase in transcript levels during infection in both genotypes. The expression of the *Pspgip1* gene responded rapidly to the nematode, with the gene induced at 24 hpi in both genotypes. The increase in *Pspgip1* expression was followed by a more rapid return to uninfected expression levels in susceptible than in resistant roots, where the gene was still highly expressed at 48 hpi. It is possible that this reflects the ability of the nematode to successfully suppress host defence responses in the susceptible interaction. The establishment and maintenance of a syncytium require stimuli associated with the injection of secretions from the nematode into the host cells and a co-evolution between the pathogen and its host. Syncytial development on susceptible and resistant pea roots has been studied previously (Bleve-Zacheo *et al.*, 1990), confirming the existence of a striking difference in the reaction to *H. goettingiana*. Indeed, although syncytia occurred in the same regions of the root tissues in both resistant and susceptible peas, they degenerated within a few days after infection in resistant roots, as cells appeared necrotic and showed features of a hypersensitive response. *In situ* localization of *Pspgip1* transcripts revealed staining of cortical cells affected by nematode migration, and this suggests a prompt reaction of the cells to nematode-induced injury. It is possible that, as the *Pspgip1* gene is induced by mechanical wounding, its expression during infection by nematodes might be induced as a result of the breaking and dissolution of cell walls during intracellular migration of the nematodes. Moreover, as the *Pspgip1* gene is induced slowly by mechanical wounding in the susceptible genotype, the increased localized *Pspgip1* expression observed along the nematode's migratory track could be associated with biotic signals from nematodes. In addition, jasmonate levels increase in roots infected by nematodes (Gao *et al.*, 2008; Veronico *et al.*, 2006),

and this could regulate *Pspgip1* expression on nematode infection. However, a different expression pattern was observed in the two genotypes at nematode feeding site level. Although a strong hybridization was detectable in the cytoplasm of the cells surrounding the feeding site at 24 hpi and 48 hpi in the resistant roots, susceptible roots seemed to react promptly at 24 hpi, but were less reactive at this site at 48 hpi. *Pspgip1* expression in the root cells surrounding the nematode in resistant roots could be associated with the defence response of the plant against the pathogen, possibly making a contribution to the development of a physical barrier to feeding site development by the accumulation of lignin, favoured by the release of elicitor-active oligogalacturonides. In the susceptible response, this stimulus is overcome: no such barrier is constructed, either because the syncytium is not detected or because the response of the plant is suppressed. Important interactions occur between the plant and the nematode within 48 hpi, and *Pspgip1* gene induction soon after inoculation seems to be important for blocking pathogen establishment in the root.

In conclusion, the findings of the present study suggest that *Pspgip1* behaves as a defence factor in the pea–cyst nematode interaction. Further investigations are needed to understand the molecular basis of the differences between the responses in susceptible and resistant hosts.

EXPERIMENTAL PROCEDURES

Plant material, nematode culture and treatments

All experiments were performed using germplasm accession MG103738 (*P. sativum* ssp. *transcaucasicum* Govorov, from the Gatersleben collection) and a commercial cultivar Progress 9, resistant (R) and susceptible (S) to the cyst nematode *H. goettingiana*, respectively. Seeds of both genotypes were surface sterilized, germinated on filter paper, transferred to clay pots containing 10 mL of sterilized sand and maintained in a growth chamber with a light intensity of 200 µmol/m²/s at 19 °C and a 16 h : 8 h light/dark cycle. Ten-day-old seedlings were inoculated

with batches of 150 freshly hatched sterile second-stage juveniles of *H. goettingiana*, obtained from cysts collected from a culture maintained on pea. Pools of 0.5 cm of infected roots were sampled at 24, 48 and 72 hpi, immediately frozen in liquid nitrogen and stored at -70°C until use. Uninfected roots were used as controls.

For wounding experiments, seeds of both pea genotypes were surface sterilized, allowed to germinate on filter paper, soaked in tap water, transferred to Hoagland solution (HS) and grown in growth chambers under the same conditions as reported above. One-week-old plantlets were injured with a needle at three different points between the root apex and the elongation zone. Wounded plantlets were kept on filter paper, with the rootlets in the dark, soaked in HS and, 2, 4, 8, 24 and 48 h later, 1-cm portions of roots at the wounded areas were collected. Roots from unwounded seedlings (time 0) and roots kept for 2 h in the same conditions as wounded ones were used as controls.

For MeJA treatments, seeds of both genotypes were surface sterilized, germinated on filter paper, transferred to clay pots containing sterilized sand, watered with HS and maintained in a glasshouse at 20°C . One-week-old seedlings were treated by sand infiltration with 6–8 mL solution containing $100\text{ }\mu\text{M}$ MeJA. Control plants were watered with a solution of 0.1% methanol and 0.02% Tween-20. At 12 h after treatment, root material was harvested and stored in liquid nitrogen for later isolation of total RNA. Three independent biological replicates were carried out for each treatment.

cDNA-AFLP analysis

Total RNA ($2.5\text{ }\mu\text{g}$) was extracted from uninfected and infected (48 hpi) roots of MG103738 and Progress 9, and used for cDNA synthesis. First-strand and second-strand cDNA syntheses were carried out with the SuperscriptTM III First-strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and Universal RiboClone cDNA Synthesis System (Promega Corporation, Madison, WI, USA), respectively, following the manufacturer's instructions. Double-stranded cDNA was digested with *EcoRI* and *MseI*, and the digestion products ($20\text{ }\mu\text{L}$) were incubated with 25 pmol of an *EcoRI*-adapter and 2.5 pmol of an *MseI*-adapter, which were annealed to the cDNA as described by Vos *et al.* (1995). The sequences of the adapters were as follows: 5'-CTCGTAGACTGCGTACC-3' and 3'-CTGACGCATGGTTAA-5' (*EcoRI*); 5'-GACGATGAGTCTGAG-3' and 3'-TACTCAGGAC TCAT-5' (*MseI*).

Following digestion of the cDNA and ligation of *EcoRI* and *MseI* adapters, one-fifth of the ligation mixture was used as a template for primary PCR amplification with the nonselective *MseI* (M00) (5'-GATGAGTCTGAGTAA-3') and *EcoRI* (E00) (5'-GACTGCGTACCAATTC-3') primers. The amplification products were diluted 25-fold and $5\text{ }\mu\text{L}$ were used as a template in a

second PCR with selective primers with 2-bp extensions. Previously, the *EcoRI* primer had been radiolabelled at 37°C for 1 h. AFLP products were electrophoresed through a 6% polyacrylamide denaturing gel; which was then dried onto Whatman paper and exposed to autoradiographic film Kodak (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK).

Following development, autoradiographic films were repositioned on polyacrylamide gels and the segments corresponding to differentially amplified cDNAs were excised. The gel fragments were eluted in $20\text{ }\mu\text{L}$ of sterile distilled water at -20°C overnight and a PCR was performed with a 1 : 5 dilution of the eluted DNA as template and the same primers as those used to generate the cDNA-AFLP profile. The purified PCR products were cloned into a pGEM T Easy vector (Promega Corporation) and sequenced.

Semi-quantitative RT-PCR and real-time PCR analyses

Total RNA was extracted using an RNeasy Plant Mini Kit following the manufacturer's instructions (Qiagen GmbH, Hilden, Germany) and used in RT-PCR analyses.

Semi-quantitative RT-PCR was performed on single-strand cDNAs derived from DNase-treated RNA ($2\text{ }\mu\text{g}$), and reverse transcribed (mMULV) with oligo dT primers, following the manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA). To define the optimal number of PCR cycles for the linear amplification of each gene, a range of PCR amplifications was performed. The conditions were chosen so that none of the RNAs analysed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification. The PCR was conducted in a total volume of $50\text{ }\mu\text{L}$ containing cDNA (200 ng), 1.5 mM MgCl_2 , $200\text{ }\mu\text{M}$ of each deoxynucleoside triphosphate (dNTP), 20 pmol of each specific primer and 1 U of GoTaq DNA Polymerase (Promega Corporation), starting with an initial denaturation at 94°C for 3 min and followed by 26 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 30 s. The reaction was completed by a final extension step at 72°C for 7 min. The *Pspgip1* message was amplified using the primers PGIPfor3 (5'-AGCTTGCAAGACTTGAATTG-3') and PGIPrev2 (5'-AGATAGCT TACTAAGCTTAC-3'). The expected product containing the 3' UTR was 167 bp in size. The 26S rRNA fragment (c. 500 bp) was amplified using primers 26Sfor (5'-AGCATTGCGATGGT CCCTGCGG-3') and 26Srev (5'-GCCCGTCGATTACGCC AAAGTCC-3'), and the signal was used to check for equal amounts of cDNA template. A mock reaction was also performed to check for DNA contamination in RNA samples.

Real-time PCR assays were performed on an Mx3000P Stratagene system (Agilent Technologies Inc., Santa Clara, CA, USA) in $20\text{-}\mu\text{L}$ reaction mixtures containing 250 nM of each primer described above, $10\text{ }\mu\text{L}$ of 2X Power SYBR Green PCR Master mix (Applied Biosystems) and $1\text{ }\mu\text{L}$ (20 ng) of five-fold-diluted

template cDNA. After an initial denaturation step at 95 °C for 10 min, amplifications were carried out for 40 cycles at 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s. The specificity of the PCR product was verified by melting curve analysis after 40 cycles, and agarose gel electrophoresis. Expression levels in each sample were normalized to the expression of the 26S rRNA gene, and the relative gene expression was obtained with Pfaffl's method (2001). In the latter case, raw fluorescence values, as a function of cycles, were exported into the real-time PCR Miner program (Zhao and Fernald, 2005) to calculate the reaction efficiency.

Southern blot analysis

DNA was extracted following a previously described method (Dellaporta *et al.*, 1983), starting from 1 g of pea root tissue. Ten micrograms of genomic DNA from each genotype were digested with *EcoRI*, *HindIII* and *EcoRI* and *HindIII* together, fractionated in a 0.8% agarose gel and transferred onto a nylon membrane (Hybond N+, Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) following standard procedures (Sambrook *et al.*, 1989). A gene-specific probe for *Pgip*, 779 bp in length and completely localized between the restriction sites for *EcoRI* and *HindIII*, was labelled by PCR using a PCR-digoxigenin (DIG) probe synthesis system (Roche Diagnostics GmbH, Mannheim, Germany). This DNA fragment was amplified using the PCR primers PGIPfor1 (5'-CTACCTCAGCCACAGCAGCC-3') and PGIPrev2 (5'-AGATAGCTTACTAAGCTTAC-3'). High-stringency hybridization was performed at 42 °C in DIG Easy Hyb solution (Roche Diagnostics GmbH) overnight, and the filtrate was washed twice at room temperature in 2 × standard saline citrate (SSC)/0.1% sodium dodecylsulphate (SDS) for 5 min, followed by two 15-min washes at 65 °C in 0.5 × SSC/0.1% SDS. Signals on the Southern blot were detected using alkaline phosphatase-conjugated anti-DIG antibody and CDP Star (Roche Diagnostics GmbH) chemiluminescent substrate reaction. The membrane was exposed to high-performance chemiluminescence films (Amersham Biosciences) at room temperature for 30 min.

Cloning of PGIP cDNA fragment by PCR and genomic fragments

The full-length cDNA of *Pspgip1* (EMBL accession number AJ749705) from pea roots was obtained by 5' and 3' RACE. In 3' RACE, total RNA (2 µg) of R and S genotypes was reverse transcribed using an oligo dT primer, and the resulting single-stranded cDNA was used in PCRs. The combination of primer PGIPfor1 (5'-CTACCTCAGCCACAGCAGCC-3') and an oligo dT adapter was used to clone the 3' end of 815 bp. For 5' RACE, a 5' RACE System kit (Invitrogen) was used, following the manufacturer's instructions. The RNA (1 µg) was reverse transcribed using primer PGIPrev (5'-AGTCTGTGAAAGTGTGTGG-3'); the 5'

modified single-stranded cDNA was amplified using the *pgip* nested reverse primer PGIPrev1 (5'-CAGCAGTCGGTGG TTGGATC-3') and the adapter oligo provided by the kit as forward primer. A product of 193 bp was amplified which harboured the 5' UTR. All PCR products of interest were cloned into pGEM T Easy Vector (Promega) and propagated in *Escherichia coli* XL1 blue cells. Sequencing was performed by the CRIBI service (Università di Padova, Padova, Italy).

The full-length cDNA was reconstituted from its extremities by high-fidelity PCR amplifications and sequenced on both strands. Upstream and downstream primers were designed from the cDNA sequence and used to isolate the *pgip* gene by PCR on genomic pea root DNA. The sequences of products from both genomic DNA and cDNAs were aligned and no introns were found.

In situ hybridization

Pea roots were collected 24 and 48 h after nematode infection and fixation [4% (w/v) formaldehyde in phosphate-buffered saline (PBS)]; embedding in paraffin and *in situ* hybridization were carried out as described by Jackson (1991). The *Pspgip1*-specific probe spanned the 3' UTR region from 1022 to 1188 bp (referring to AJ749705). Sense and antisense DIG-labelled riboprobes were produced by *in vitro* transcription of linearized plasmid DNA (cut with either *NcoI* or *SpeI*), using T7 and SP6 RNA polymerase, respectively (Roche Diagnostics GmbH). Hybridization was performed at 54 °C overnight using 50 ng of riboprobe. The slides were soaked twice in prewarmed (54 °C) 0.2 × SSC for 30 min, rinsed twice for 5 min with NTE solution [500 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8, 1 mM ethylenediaminetetraacetic acid (EDTA)] at 37 °C and incubated at the same temperature for 30 min in a prewarmed NTE solution containing RNase A at 20 µg/mL. The slides were then rinsed twice for 5 min in NTE solution at 37 °C, washed for 1 h in 0.2 × SSC at 54 °C and for 5 min in PBS at room temperature. The signals were detected using an alkaline phosphatase-conjugated antibody (1:1000). The colour reaction, obtained using nitroblue tetrazolium-5-bromo-4-chloroindol-3-yl phosphate (NBT-BCIP) (Roche Diagnostic GmbH) as substrate, was stopped by adding Tris-EDTA. Sections were mounted in Aquatext (Merck, Darmstadt, Germany) on glass slides and viewed under a bright-field microscope. Controls to check for signal background were samples hybridized with *pgip* probe in sense orientation and untreated samples reacting with alkaline phosphatase.

Sequence analyses, alignment and phylogenetics

PGIP nucleotide sequences from leguminous species, as well as sequences from *Arabidopsis thaliana* and *Brassica napus*, were retrieved from the GENBANK database according to their

accession numbers [PvPGIP1, PvPGIP2, PvPGIP3, PvPGIP4 (*Phaseolus vulgaris*, AJ786408 to AJ786411); GmPGIP1, GmPGIP2, GmPGIP3, GmPGIP4 (*Glycine max*, AJ972660 to AJ972663); AtPGIP1 and AtPGIP2 (*Arabidopsis thaliana*, AF229249, AF229250)]. Each sequence, including the *P. sativum* PGIP nucleotide sequence, was aligned using the CLUSTALW (Chenna *et al.*, 2003) program implemented in the MEGA package (Kumar *et al.*, 2008). Nucleotides corresponding to signal peptides were removed before the alignment procedure. The final alignment was checked manually to correct potential inconsistencies.

A maximum likelihood phylogenetic tree was reconstructed using PhyML (Guindon *et al.*, 2009) under the GTR + I + G (general time reversible with estimated rate of invariant positions and estimated gamma shape parameter for among-site rate variation) evolutionary model (Lanave *et al.*, 1984). Clade reliability was examined through a nonparametric bootstrap with 100 replicated samples. The same tree reconstruction strategy was used to build a maximum likelihood tree using PGIP protein sequences under the JTT evolutionary model (Jones *et al.*, 1992) and allowing 100 bootstrap replicates.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Maximum likelihood phylogenetic tree of PGIP protein sequences inferred by PhyML program (Guindon *et al.*, 2009). Node labels represents bootstrap support values. Branch lengths are in substitution per site.

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