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Molecules of Interest

Polygalacturonase-inhibiting protein (PGIP) in plant defence: a structural view

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Abstract

Polygalacturonase-inhibiting proteins are plant extracellular leucine-rich repeat proteins that specifically bind and inhibit fungal polygalacturonases. The interaction with PGIP limits the destructive potential of polygalacturonases and might trigger the plant defence responses induced by oligogalacturonides. A high degree of polymorphism is found both in PGs and PGIPs, accounting for the specificity of different plant inhibitors for PGs from different fungi. Here, we review the structural features and our current understanding of the PG–PGIP interaction.

Keywords: Plant innate immunity; Polygalacturonase; Polygalacturonase inhibiting protein; Crystal structure; Protein–protein interaction

1. Introduction

Plant innate immunity is based on an ancient system of molecules that defend the host against infection. Defence relies on the capability of each cell to recognize the presence of pathogens and activate downstream responses. The first defence line is the plant cell wall, a physiological barrier that has a critical role in controlling pathogen invasion. Microorganisms have a number of enzymes that degrade the polysaccharides of the cell wall. *Endo*-polygalacturonase (PG), one of the enzymes secreted at the early stages of infection, depolymerizes the homogalacturonan, the main component of pectin, by cleaving the α-1,4 glycosidic bonds between the galacturonic acid units (De Lorenzo et al., 2001).

PGs are produced by bacteria, fungi, nematodes and insects (De Lorenzo and Ferrari, 2002; Jaubert et al., 2002; Girard and Jouanin, 1999) and their involvement in pathogenesis has been demonstrated for several fungi such as *Botrytis cinerea* (ten Have et al., 1998; Kars et al., 2005), *Aspergillus flavus* (Shieh et al., 1997), *Alternaria citri* (Isshiki et al., 2001), *Claviceps purpurea* (Oeser et al., 2002) and *Sclerotinia sclerotiorum* (Li et al., 2004) and bacteria such as *Ralstonia solanacearum* (Tans-Kersten et al., 2001) and *Agrobacterium tumefaciens* (Rodriguez-Palenzuela et al., 1991). PGs from salivary glands of phytophagous insects are considered a main cause of plant damage (Girard and Jouanin, 1999; Boyd et al., 2002; Frati et al., in press). Many plants produce extracellular polygalacturonase-inhibiting proteins (PGIPs) that specifically recognize and inhibit fungal and insect PGs (De Lorenzo and Ferrari, 2002; D'Ovidio et al., 2004). The PG–PGIP interaction limits the destructive potential of polygalacturonases and leads to the accumulation of elicitor active oligogalacturo-

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nides as demonstrated in vitro. These oligosaccharides may activate plant defence responses such as synthesis of phytoalexins, lignin and ethylene, expression of proteinase inhibitor I and β-1,3-glucanase and production of reactive oxygen species (Ridley et al., 2001).

The importance of PGIPs in plant defence has been corroborated by in vivo studies. The overexpression of the genes *Atpgip1* and *Atpgip2* in Arabidopsis limits the colonization by *B. cinerea* and reduces disease symptoms (Ferrari et al., 2003). A significant increase of PG-inhibitory activity and a decrease in susceptibility to *B. cinerea* has been found in transgenic tomato and grapevine plants overexpressing a pear *pgip* (Powell et al., 2000; Agüero et al., 2005), and in tobacco and Arabidopsis plants overexpressing a bean *pgip* (*Pvpgip2*) (Manfredini et al., in press). Arabidopsis plants with antisense expression of *pgip* genes have a reduced inhibitory activity in response to biotic and abiotic stress and are more susceptible to *B. cinerea* (our unpublished data). These findings suggest that PGIPs are important players in plant innate immunity.

To accommodate pathogenesis to different environmental conditions and on various hosts, fungi produce PG isoenzymes variable in terms of sequence, specific activity, pH optimum and substrate preference (De Lorenzo et al., 2001; Poinssot et al., 2003). Conversely, plants have evolved PGIPs with different recognition specificities encoded by differentially regulated *pgip* genes (De Lorenzo and Ferrari, 2002; Ferrari et al., 2003). Also plants produce PGs that play a role in the cell wall development (Torki et al., 2000) but these PGs do not interact with PGIPs, suggesting that the inhibitors are specialized for plant defence (Federici et al., 2001). The finding that transgenic tobacco and Arabidopsis plants overexpressing PGIPs do not show morphological alterations is consistent with this vision (Ferrari et al., 2003; Capodicasa et al., 2004; Manfredini et al., in press). PGIPs belong to the leucine-rich repeat (LRR) superfamily of proteins (Kobe and Kajava, 2001) and are characterized by the tandem repetition of a consensus motif rich of leucines (Mattei et al., 2001). LRR proteins are ubiquitous in the life kingdoms and provide a versatile recognition surface for protein–protein interactions. The LRR domain of plant proteins is often fused with other domains and acts in recognition of hormonal signals and pathogen-derived elicitors. Plant LRR proteins involved in plant defence include: (i) those containing an extracellular LRR domain and a single membrane-spanning helix, such as the Cf proteins of tomato (van der Hoorn et al., 2005); (ii) proteins containing an extracellular LRR domain and a cytoplasmic serine-threonine kinase, connected by a transmembrane helix, such as the rice resistance gene products Xa21 (Song et al., 1995) and Xa26 (Sun et al., 2004); (iii) cytoplasmic proteins containing a LRR domain, a putative nucleotide binding sites (NBS) and a N-terminal putative leucine-zippers (LZ) or Toll interleukine receptor (TIR) domain (Staskawicz et al., 2001).

LRRs of the extracellular type homologous to PGIP are found in pattern-recognition receptors involved in non-host specific defence such as the Arabidopsis flagellin receptor FLS2 (Gomez-Gomez et al., 2001), in carrot proteins with antifreeze activity (Worrall et al., 1998), in the Arabidopsis protein SHY with a role in pollen tube growth (Guyon et al., 2004), in several receptors involved in development, perception of hormones (Becraft, 2002) and in bacterial and fungal symbiosis (Kistner and Parniske, 2002). Plant proteins with extracytoplasmic LRRs (eLRRs) are specifically characterized by the tandem repetition of the same 24-residues motif: xx**L**x**L**xx**N**x**L**t/s**G**x**IP**xx**L**xx**L**xx**L**. This repeat differs from those found in plant cytoplasmic LRRs as well as other LRR subfamilies thus suggesting that eLRR proteins might share the same three-dimensional arrangement (Kobe and Kajava, 2001; Di Matteo et al., 2003).

2. The structure of PGIP is the prototype of the plant eLRR proteins

The crystal structure of the isoform 2 of PGIP from *Phaseolus vulgaris* (PvPGIP2) is the only available structure of plant LRR proteins (Di Matteo et al., 2003) (Fig. 1). PvPGIP2 displays the right-handed superhelical

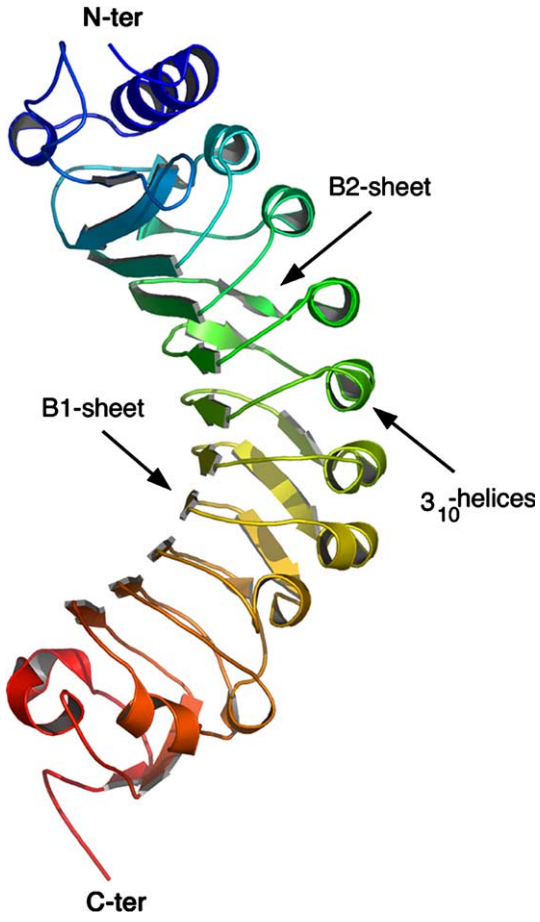

Fig. 1. Ribbon representation of the crystal structure of PGIP2 from *P. vulgaris*.

fold typical of LRR proteins such as the porcine and human ribonuclease inhibitors (Kobe and Deisenhofer, 1993; Papageorgiou et al., 1997), U2A′ (Price et al., 1998), RanGAP rna1p (Hillig et al., 1999), Internalin B (Marino et al., 1999), YopM (Evdokimov et al., 2001), Skp2 (Schulman et al., 2000) and decorin (Scott et al., 2004). The fold of PvPGIP2 consists of a central LRR domain (residues 53–289) flanked by the N- and C-terminal cysteine-rich regions (residues 1–52 and residues 290–313, respectively). The LRR domain is characterized by the tandem repetition of 10 coils matching the consensus sequence xx**L**x**L**xx**N**x**L**t/s**G**x**IP**xx**L**xx**L**xx**L**. An extended parallel β-sheet (B1), conserved in all known LRR protein structures (Kobe and Kajava, 2001), occupies the concave inner side of the protein solenoid. B1 is the β-sheet where the residues determining the affinity and the specificity of PGIP2 reside (Leckie et al., 1999; Sicilia et al., 2005). Nine 3_{10}-helices are located on the convex side of the protein. While the majority of LRR proteins have only one β-sheet connected with the helices on the convex side by loops or β-turns, PGIP2, instead, has an additional parallel β-sheet (B2). B2 is distorted because of the twisted shape of the molecule and the variable length of the β-strands.

Specific positions (3, 5, 10, 18, 21 and 24) of the LRR repeats are occupied by hydrophobic amino acids, mostly leucines, that point into the interior of the protein scaffold and stabilize the overall fold topology thorough van der Waals interactions (Fig. 2). Position 8 is usually occupied by asparagine residues that are oriented towards the core of the protein and form hydrogen bonds with the main-chain carbonyls or amide groups originating the typical "asparagine ladder". An additional cooperative stabilization pattern across the LRR domain is generated by the conserved serine or threonine residues at position 17. The glycine at position 12, the isoleucine at position 14 and the proline at position 15 are conserved in plant-derived eLRR proteins. In PGIP2 the characteristic sequence **L**t/s**G**x**IP** is partially involved in the formation of the sheet B2. Interestingly, glycines show a stereochemistry that is forbidden to any other residue and determine the peculiar bending of sheet B2. Sequence alignments suggest that, like PGIP, other plant eLRR proteins display a second β-sheet that may form an additional surface for interaction (Di Matteo et al., 2003). The convex face of the LRR region of PGIP2 is mostly occupied by unstructured segments that are stabilized through water molecules; these are organized in spines along the structure and form H-bond interactions with the protein backbone (Marino et al., 1999; Evdokimov et al., 2001). While contributing to stabilize the protein scaffold, the water network provides a structural flexibility to the molecule and might facilitate the adaptation of the PGIP scaffold to the surface of its interacting partners (Di Matteo et al., 2003).

The N-terminal region contains two disulphide bridges (Cys3–Cys33, Cys34–Cys43) and consists of a 15 residue-long α-helix and a short β-strand that forms H-bonds with residues of the sheet B1. The C-terminal region contains

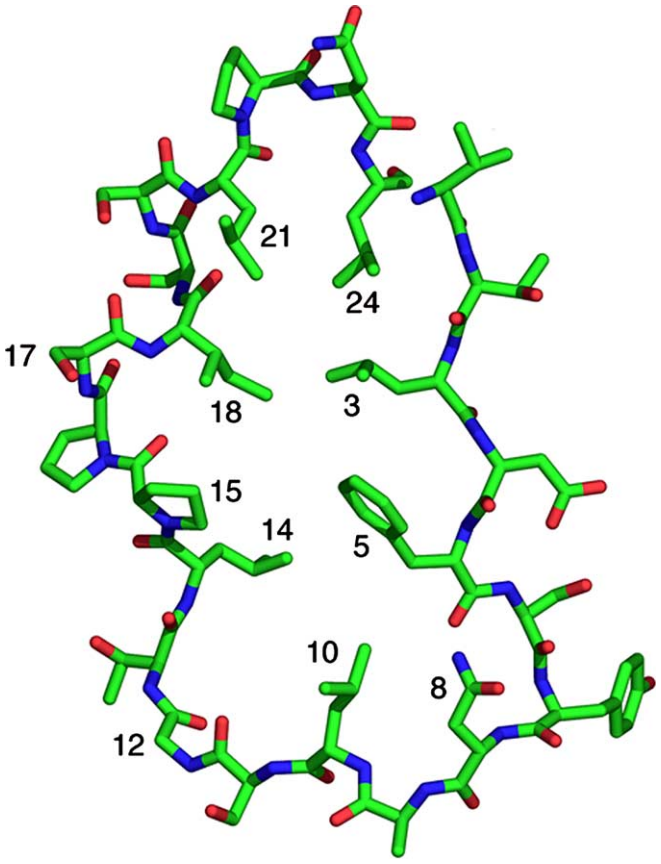

Fig. 2. A typical LRR of PvPGIP2 matching the consensus motif: ^{1}xx**L**x**L**xx**N**x**L**t/s**G**x**IP**xx**L**xx**L**xx**L**^{24}.

two disulphide bonds (Cys281–Cys303, Cys305–Cys312) and consists of the last two 3_{10} helices, the last strand of the sheet B2 and a short loop. The LRR-flanking regions play a structural role in capping the hydrophobic core of the protein (see Fig. 1).

PGIP is ionically anchored to the cell wall and interacts in vitro with unesterified homogalacturonan and with blockwise de-esterified homogalacturonan by means of positive residues, located between the sheets B1 and B2 and protruding into the solvent to create a regular distribution of charges (Fig. 3). The interaction of PGIP with pectin is competed in vitro by PGs, suggesting that PGIP use overlapping although not identical regions to interact with pectin and PGs. The interaction of PGIP with the cell wall may be instrumental to ensure the presence of the inhibitor where infection initiates and microbial PGs degrade homogalacturonan (our unpublished data).

3. The *pgip* gene families

Gene families code for PGIP isoforms with homologous structures but different specificities (De Lorenzo et al., 2001; Li et al., 2003; Ferrari et al., 2003; D'Ovidio et al., 2004). The arrangement and the similarity among the genes of *Phaseolus vulgaris* suggest that they derive from a com-

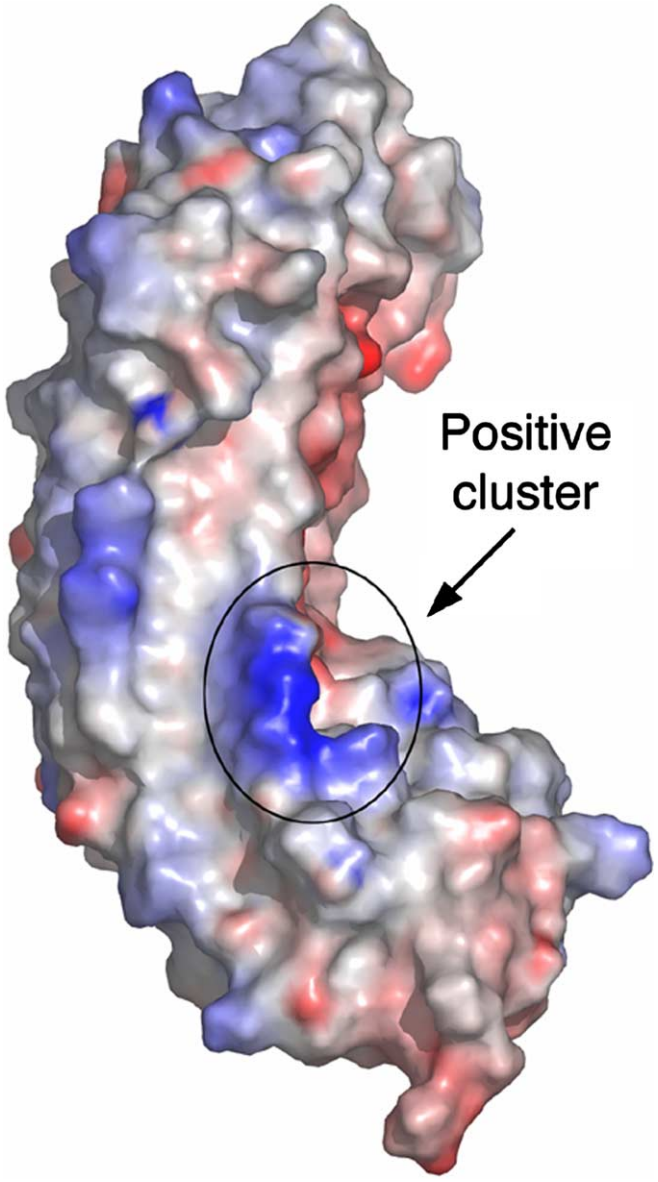

Fig. 3. Electrostatic potential surface of PvPGIP2. Residues are coloured according to their electrostatic potential: blue indicates positive charge while red indicate negative charge. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mon ancestor as a result of a sequence of duplication–divergence–duplication events. The four paralogues *Pvpgip1–4* form two pairs *Pvpgip1*/*Pvpgip2* and *Pvpgip3*/*Pvpgip4* that code for functionally distinct classes of PGIPs, each of which differentially devoted to the recognition of PGs from fungi or insects (D'Ovidio et al., 2004). In *Arabidopsis* two closely related genes code for two distinct products (AtPGIP1 and AtPGIP2) (Ferrari et al., 2003). In *B. napus* the *pgip* family is composed of four different members; two products (BnPGIP1 and BnPGIP2) have been characterized (Li et al., 2003).

Pgip genes are different not only in terms of recognition specificity of their products but also because they are differentially regulated (Ferrari et al., 2003). For instance *Pvpgip*3 responds to OGs but not to fungal glucan, salicylic acid or wounding. *Pvpgip*1 responds only to wounding while *Pvpgip2*, that encodes the most efficient inhibitor of fungal PGs, is upregulated by all these stress stimuli (D'Ovidio et al., 2004). Both Arabidopsis inhibitors are induced in response to wounding and *B. cinerea* infection. *Atpgip1* expression responds to oligogalacturonides and is independent of salicylic acid, ethylene or jasmonic acid; *Atpgip2* is instead induced by jasmonic acid (Ferrari et al., 2003). Moreover *Atpgip2* is induced by the fungus *Alternaria brassicicola* but not by insect attack (Reymond et al., 2000). In *Brassica napus* PGIP-encoding genes respond differentially to biotic and abiotic stimuli (Li et al., 2003). The ability of *pgip* genes to switch on in response to different stress-related signals ensures the expression of at least one inhibitor if a pathogen evolves the capacity of blocking or avoiding the activation of a particular transduction pathway.

4. The PG–PGIP interaction

PGIPs specifically interact with PGs by forming a bimolecular complex. The PG–PGIP interaction varies in terms of inhibition kinetics and strength, and reflects the counter-adaptation occurring in both enzymes and inhibitors (Federici et al., in press). Pathogens have evolved different PGs to maximize their offensive potential and, conversely, plants have evolved various PGIPs with different specificities to counteract the many forms of PG existing in nature. For instance PGs from *B. cinerea* (BcPGs) and *Colletotrichum acutatum* are inhibited with different efficiencies by all members of the bean PGIP family, while PG from *Aspergillus niger* (AnPGII) is inhibited by PvPGIP1 and PvPGIP2 but not by PvPGIP3 and PvPGIP4 (D'Ovidio et al., 2004). PG from *Fusarium moniliforme* (FmPG) is inhibited only by PvPGIP2 (Leckie et al., 1999). PGs from the phytophagous insects *Lygus rugulipennis* and *Adelphocoris lineolatus* are inhibited by PvPGIP3 and PvPGIP4 (D'Ovidio et al., 2004). PGIPs from Arabidopsis inhibit PGs from *Colletotrichum gloesporioides*, *Stenocarpella maydis* and *B. cinerea* (Ferrari et al., 2003; Manfredini et al., in press) but are uneffective against FmPG and AnPGII (D'Ovidio et al., 2004).

Computational analysis predicts that the hypervariable **L**xx**L**x**L**xx region spanning the sheet B1 in the concave surface of PvPGIP2 has a strong propensity to be engaged in protein–protein interactions (Sicilia et al., 2005; Federici et al., in press). This is the same area where the determinants of PGIP's affinity and specificity are located (Leckie et al., 1999; Sicilia et al., 2005). Homologous regions of other LRR proteins such as the ribonuclease inhibitor (Papageorgiou et al., 1997), internalin A (Schubert et al., 2002) and RanGAP (Seewald et al., 2002) are also involved in recognition. Phylogenetic codon-substitution analysis performed on PvPGIPs showed that only nine out of 313 residues are positively selected and seven of them are

located within the LRR domain (Bishop, 2005). The small number of positively selected residues suggests that PGIPs require the maintenance of features necessary for the basic recognition of PGs through a network of multiple and weak contacts. A limited number of "hot spots" may be responsible for specificity and their mutations lead to the recognition of new molecules.

Variability of recognition and function of PGIPs is not only reflected by their specificity but also by the variable inhibition kinetics played against different fungal PGs: tomato PGIP inhibits AnPGII in a non-competitive manner (Stotz et al., 2000); PGIPs from bean (Lafitte et al., 1984) and raspberry (Johnston et al., 1993) are non-competitive inhibitors of PGs from *Colletotrichum lindemuthianum* and *B. cinerea*, respectively; PGIPs from pear (Abu-Goukh and Labavitch, 1983) and orange fruits (Barmore and Nguyen, 1985) are competitive inhibitors of PG from *Diplodia natalensis*. PvPGIP2 acts competitively against FmPG (Federici et al., 2001), non-competitively against AnPGII (King et al., 2002) and has a mixed mode of inhibition against BcPG1 (Sicilia et al., 2005). This suggests that the orientation of PGIP in the PG–PGIP complexes differs depending on different PG partners. Consistently, molecular docking analysis of the interaction of PvPGIP2 with FmPG, AnPG and BcPG1 predicts that different, although partially overlapping, surfaces of these enzymes are recognized by PGIPs (Sicilia et al., 2005; Federici et al., in press).

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