Polygalacturonase-inhibiting proteins in plant defence

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Polygalacturonases (PGs) are secreted by pathogens to solubilize the plant cell wall and are required for pathogen virulence. Plant proteins that inhibit PG limit the growth of pathogens and at the same time also elicit defence responses in plants by increasing the lifetime of oligogalacturonoides. These polygalacturonase-inhibiting proteins (PGIPs) are one of the well-elucidated leucine-rich repeat proteins. We review the properties of PGIPs, molecular aspects of their interaction with PG and their possible application to the development of disease-resistant transgenic crop plants.

PECTIN is a major component of the primary walls of dicotyledonous and non-graminaceous monocotyledonous cells. During pathogenesis, cell walls act as the first line of defence that pathogens encounter to colonize the plant tissue and obtain nutritional requirements. A pathogen has to breach the pectin layer for its ramification. A wide range of enzymes, including exo- and endo-polygalacturonase, pectatelyase, pectin methyl esterase and beta-galactosidase are involved in pectin modifications[1]. Pectin-degrading enzymes, including endo-polygalacturonase (EPG), are among the first glycanases to be secreted during fungal infection. Polygalacturonase (PG) is the first cell-wall-degrading enzyme synthesized by phytopathogenic fungi cultured on isolated cell walls[2]. PGs are known to be secreted by phytopathogenic fungi, bacteria and nematodes. PGs that break down the polygalacturonate chain in a random manner into small chains of oligogalacturonate are called EPG and other PGs that cleave the polygalacturonate chain in terminal manner and release monomeric products, i.e. galcturonic acids are called exo-polygalacturonase[3]. EPG (poly-α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15) degrades unesterified regions of homogalacturonan (HGA), the 1,4 linked α-D-galactosyluronic acid polymer found in pectin of plant cell wall. There are also strong correlative evidences supporting the involvement of EPG in causing symptoms in diseases characterized by soft-rotting or tissue maceration[3–5].

EPG fragmentation of HGA results in the transient formation of elicitor-active oligogalacturonoides (OGAs) with degrees of polymerization between 9 and 15. These OGAs are rapidly (within 15 min) converted into smaller, biologically inactive fragments by EPG. Thus, factors that limit fungal EPG action are likely to increase the lifetimes and concentrations of biologically active OGAs and may result in enhanced or prolonged plant defence responses[6]. Plants secrete proteins that specifically bind to PGs and modify their enzyme action. Cervone *et al.*[7] hypothesized that polygalacturonase-inhibiting proteins (PGIP) benefit the plant by retarding the hydrolytic activity of PG-catalysed hydrolysis of polypectate and lead to the formation of oligomers with a degree of polymerization greater than four. Such oligomers elicit active defence mechanism in plants. Therefore, the action of PGIP *in vivo* is to counteract fungal invasion by causing PGs to increase their elicitation of plant defence responses. The PGIP family of proteins, present in the walls of dicots and non-graminaceous monocots, are in some cases capable of inhibiting greater than 92% of the activity of fungal PGs[8] and sometimes[9] even to 100%. A major goal in plant pathology is to understand the molecular basis of pathogen recognition by plants. Though PGIP is one among the few well-elucidated, pathogenesis-related (PR) proteins of known ligand–protein binding model, research on this group of PR proteins has not been reviewed.

OGAs in eliciting plant resistance

Oligosaccharides derived from fungal and plant cell-wall polysaccharides are one class of well-characterized elicitors that, in some cases, can induce defence responses at a very low concentration, e.g. in nanomoles[10]. Cell-wall oligosaccharides elicit numerous defence mechanisms that have evolved in plants to prevent invasion by pathogenic fungi. The following list of observations support this: (i) OGAs of plant cell walls released by EPG elicited the induction of casbene, a phytoalexin in castor bean[11]; (ii) pectin and EPG of *Cladosporium cucumerinum* triggered the lignification of cell wall in cucumber hypocotyls[10]; (iii) fragments of endogenous plant cell walls elicited hydroxyproline-rich glycoproteins and ethylene in soybean and melon[12]; (iv) OGAs increased phenylalanine ammonia lyase in *Citrus limon* seedlings[13] and (v) OGAs inhibited auxin-induced elongation and interfered with auxin-induced ethylene production in pea stems[14]. These examples demonstrate the induction of resistance reactions by OGAs. Cervone *et al.*[7] unambiguously showed that the active

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EPG of *Aspergillus niger* formed OGAs from pectin which were capable of eliciting resistance response, while either the heat-killed or antibiotic-inhibited enzyme or HGA failed to produce such a response in *Vigna unguiculata*. Therefore, microbial wall polymerases might be as important to the plant as warning signals as they are to a pathogen as virulence factors. In most cases, it appears that plants do not recognize wall depolymerases directly, but rather respond to the enzyme products[15]. The term 'oligosaccharin' was coined by Albersheim and co-workers[16] to describe biologically active oligosaccharides that are produced as a result of the action of either endogenous or microbial enzymes on larger and inactive polysaccharides. Elicitor activity of OGA was reported in many dicotyledonous plants, but there was no report in monocot plants. This might be due to the much lower content of pectic polysaccharides in monocot cell walls.

Discovery of PGIP and early investigations

Conventionally, the inactivation of pectinolytic enzymes was thought to be due to the action of non-proteinaceous and non-specific inhibitors like phenolics, though only in some cases such as oxidized polyphenols[17] and tannins of chestnut bark[9], was there a good evidence. Contrary to this, few researchers found that phenolics do not inhibit EPG. For example, as high as 200 $\mu g\ ml^{-1}$ *p*-coumaric acid and ferulic acid of tomato did not inhibit the PG of *Botrytis cinerea*[18]. Albersheim and Anderson[16] reported that proteins extracted from the cell walls of red kidney bean hypocotyls, tomato stems, and suspension-cultured sycamore cells can completely inhibit the activity of PGs secreted by the fungal plant pathogens *Colletotrichum lindimuthianum*, *Fusarium oxysporum* and *Sclerotium rolsfii*. They also found that the inhibitors showed varying degrees of inhibition on PGs of different pathogens and their physical properties were similar to those of phytohemmaglutinins and plant glycoproteins capable of agglutinating transformed animal cells. During the 1970s, not much of focused research on PGIP was carried out, except for the contributions of Albersheim and his colleagues at the Department of Chemistry, University of Colorado[16,19]. Figure 1 depicts the model proposed by Albersheim and co-workers on the role of oligosaccharide and PGIPs in the induction of plant defence response[6].

Little or no activity of PG was detected in crude extracts of peach tissue infected with *Monilinia* spp., though its presence was confirmed by isoelectric focusing after fractionation. Further experiments confirmed the presence of proteinaceous inhibitors present in apple and peach tissues infected with *Monilinia* spp.[20]. While assessing susceptibility of pear fruit to three potential pathogenic fungi in relation to its maturity, a non-phenolic inhibitor was identified and was found to be cell-wall-bound proteins[21]. In 1980s, PGIPs from different plants have been characterized biochemically, but little progress was made on the elucidation of structure and regulation of expression of PGIPs, which is crucial for the understanding of the *in vivo* function of this class of proteins.

In India, major research on the inhibition of pectinolytic enzymes has been correlated with phenolic compounds and phenol oxidases. Our studies on the inhibition of pectinolytic enzymes, polymethyl galacturonase, pectin lyase and pectate lyase of *Colletotrichum capsici* and *C. gloeosporioides* (pathogenic fungi of chilli) by fungitoxicants have shown better inhibition *in vivo* than *in vitro*; PG was not inhibited *in vitro*, but was inhibited by the same fungitoxicant *in vivo*[22]. *Capsicum annuum* (chilli) plants treated with fungitoxic chemicals showed less phenolic content and less activity of peroxidases than the untreated and infected plants[23]. PG activity, however, showed a reverse pattern. These results suggested that phenols are not involved in the inhibition of PG and this led us to search for proteinaceous inhibitors in chilli plants. Proteinaceous inhibitors for partially purified PG of *C. capsici* from chilli seedlings have been identified and their purification is in progress.

In general, the proteinaceous inhibitors for PG were isolated only from dicotyledonous plant species. Recently, and for the first time, PGIP from a monocotyledonous species was purified from a non-graminaceous plant, *Allium porrum*[24], and from a graminaceous member, wheat[25]. Its presence was confirmed by biochemical and immunological evidences[26]. But the levels of PGIP were low in wheat seedlings, and this can be explained by the presence of one-tenth the amount of pectin in wheat cell wall compared to dicot plants.

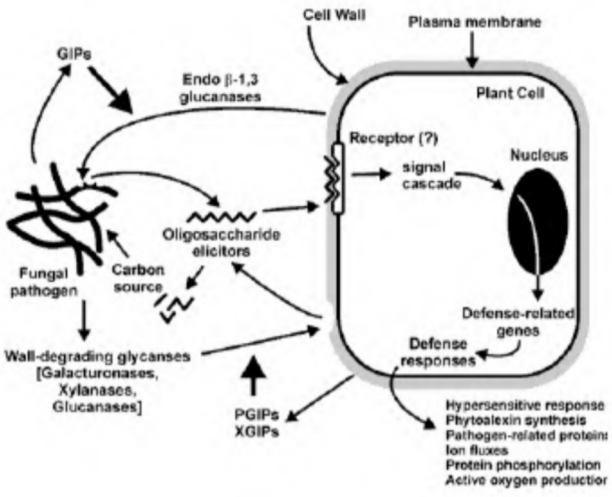

Figure 1. Model depicting the role of oligosaccharide and PGIPs in the induction of plant defence response. (Proposed by Albersheim and co-workers, Complex Carbohydrate Research Center, University of Georgia, USA; www.ccrc.uga.edu/-mao/plapath/Pptext.htm). Reproduced with permission from Dr P. Albersheim.

Isolation and purification methods

For preliminary screening and crude extraction of PGIP, ground plant tissue is extracted in a buffer with or without NaCl: with NaCl to extract the wall-bound and without NaCl to extract the soluble inhibitor proteins of PG. No single or standard method of purification is available at present. Most of the methods have been used only once and they were successful to a limited extent.

The first successful purification was achieved by Albersheim and Anderson[16]. The buffered extract was passed through DEAE cellulose, Sephadex-gradient, Sephadex concentrate, Bio Gel P100 and Bio Gel P150. The yield was low and only 7% of the inhibitors could be recovered. Use of affinity chromatography using covalently attached Sepharose 4B with PG isolated from a pathogenic fungus was found to efficiently purify bean PGIP to 2850-fold and yield[27] was up to 86%. Many workers still follow this method because it is a single step purification method with high yields, even though the preparation of covalently attached Sepharose 4B with PG is time-consuming and cumbersome. Recent attempts of purification using FPLC, HPLC, IEF along with conventional methods of purification are in the experimental stages. Combination of gel filtration (FPLC system of Superdex 75 column), affinity chromatography (Concanavalin A–Sepharose 4B column) and ion exchange (cation exchange column), for purification of PGIPs from *Allium porrum* yielded as many as 18 isoforms[28].

Properties of PGIP

PGIP is a glycoprotein with varying size. The smallest PGIP reported was from peach, with molecular mass 15 kDa[20], and the largest was isolated from pear with a molecular mass of 91 kDa[29]. However, most of the identified PGIPs fall in the range of 34- to –54 kDa. The PGIPs are relatively heat-stable up to 50°C and they slowly lose their activity when heated[16,27,29] above 55°C. Protease inhibitors such as leupeptin (20 µg/ml), polymethyl sulphonyl fluroide (2 mM) and pepstatin (50 µM) did not inhibit PGIP of bean[27], while trypsin destroyed PGIP of peach[20]. Kinetic studies have suggested that the inhibition caused by these proteins is competitive[29]; PG–PGIP complex dissociates at a pH lower than 4.5 and higher than 6 and at a salt concentration above 500 mM Na-acetate[27]. PGIPs from different plant species are likely to differ in their inhibition kinetics and target-PG specificity (Table 1).

Specificity of PGIPs

PGIPs are specific inhibitors; they do not inhibit other cell-wall degrading enzymes, and the per cent inhibition of different fungal PGs differs. PGIP of pear inhibited only PGs secreted by pathogenic fungi of pear and did not inhibit PGs of *A. niger* and *F. oxysporum*[30]. This suggests that PGIPs can discriminate between PGs. The degree of susceptibility of PGs to PGIPs depends on the mode of action of the fungal PG. PGs exhibiting strictly endo mode of cleavage are selectively inhibited only by certain PGIPs, but those cleaving pectin in between the exo and the endo modes and the exo-polygalacturonases are easily inhibited by many PGIPs. PGs of *C. lindemuthianum*, *Cochliobolus sativus*, *Cryphonectria parasitica* and *A. niger* exhibited an intermediate substrate degradation and these exo/endo PGs were inhibited greater than 90% by all the four PGIPs. However, EPGs of *A. niger* and *Fusarium moniliforme* were inhibited by PGIPs extracted from Pinto and Blue Lake cultivars of bean, but not of tomato and pear. EPG of *Postia placenta* was not inhibited by any of these PGIPs[31]. In general, it appears that PGIPs from various plants show inhibition of PGs from fungi to which the plant was not exposed. This observation is strengthened by a report that bean PGIP-2 interacts strongly with a PG of maize pathogen, *Stenocarpella maydis* that causes epidemics in USA[8]. PGIP isolated from *Glycine max* was found to effectively inhibit PGs of soybean cyst nematode[32].

PGIPs do not inhibit EPGs of plant origin. For example, PGIP of *Phaseolus vulgaris* did not inhibit EPG of tomato and exo-PG of pollens of water oak. However, it inhibited the exo-PG of corn pollen and sorghum[33]. Likewise, pear PGIP inhibited *Pencillium expansum*, *Botrytis cinerea* and *Dothiorella gregaria*, while it did not inhibit PG of pear fruit[30].

Many plants possess more than one PGIP with differential abilities to inhibit different PGs of pathogens[31]. Specificity of PG : PGIP is based on the existence of multiple molecular forms of PGIP in a single plant. In *A. porrum*, more than 20 isoforms[28] and in bean five isoforms have been detected[34]. Expression of PGIPs in tissues of plants is differential: PGIPs in tomato, apple, and raspberry were predominately expressed in fruit tissues[35–37], whereas in others such as bean it was expressed in vegetative parts[38,39]. PGIP is a constitutive protein; the amount of PGIP gene transcripts varies at different stages of maturity and at different distances from the diseased region. In apple fruits, high level of PGIP gene was expressed in the decayed area and in adjacent areas of the fruit inoculated with *Penicillium expansum* and *B. cinerea*. However, there was no apparent increase in PGIP in tissues away from the decayed region, suggesting that the PGIP gene is readily activated by fungal infection[40].

Structural and molecular characteristics

PGIP belongs to the super family of leucine-rich repeat (LRR) proteins. The motif is assigned for the specific protein–protein recognition and interaction. PGIP is closely related to the products of resistance genes (*R*-genes), viz. *Cf-9* of tomato (contributing resistance to *Cladosporium fulvum*)[41] and *Xa21* of rice (for bacterial blight resis-

Table 1. Properties of polygalacturonase-inhibiting proteins

Plant source	Pathogen	Percentage inhibition	Isoforms	Mr (kDa)	pI	pH	Temperature (°C)	Reference
Lycopersicon esculentum (tomato)	*Fusarium oxysporum*	100	2	34–41	9.0			36, 52
	Aspergillus niger	100						
	Botrytis cinerea	>75						
Glycine max (soybean)	*S. sclerotiorum*		3	37–40	8.21			32, 53
	A. niger							
	Heterodera glycines (soybean cyst nematode)							
Phaseolus vulgaris (French bean)	*A. niger*	93	5	45	9.5	4.5–6	60	8, 27
	F. moniliforme	74						
	Colletotrichum lindemuthianum	68						
	Stenocarpella maydis	66–83						
Pisum sativum (pea)	*Assochyta pisi*	>50		42				54
Gossypium hirsutum (cotton)	*Verticillium dahliae*	>25		34				55
	A. niger	>50						
Castanea mollissima (Chinese chestnut)	*Cryphonectria parasitica*	100		–	–		60	9
	C. lindemuthianum	100						
	Rhizopus sp.	0						
Pyrus communis ('Barlett' pear fuit)	*Penecillium expansum*	>50	2	91–44	6.6			21, 29
	B. cinerea	>50			7.7			56
	Dothiorella gregaria	>40						
Pyrus domestica (peach)	*Molnilinia fructigena*	98					80	20
	M. laxa	99						
Malus domestica (apple)	*M. fructigena*	96		34	7.0			20, 40
	M. laxa f.sp. *mali*	90						
	Nectria galligena	90						
	B. cinerea	98						
Citrus sinensis (orange)	*Diplodia natalensis*	>80		54		3.6–8	60	57
Allium porrum (leek)	*F. moniliforme*	0	20	39, 42	5–7	–	–	24, 28
	Sclerotium cepivorum	0						
	S. sclerotiorum	>95						
	B. aclada	>60						
Triticum aestivum (wheat)	*Cochliobolus sativus*	>80		40.3				26
	A. niger	>5						
	F. moniliforme	>5						
	C. lindimuthianum	0						
	Postia placenta	0						
	Ralstonia solanacearum	0						
Citrus jambhiri (rough lemon)	*Alternaria citri*							58

tance)[42]; not only structurally but also functionally. *R*-gene products are thought to function as receptors for pathogen-encoded avirulence (Avr) proteins and it has been hypothesized that sequence variation within LRRs influences recognition specificity. Comparison of members of the *Cf* family has identified the β-sheet/β-turn region as a 'hypervariable' region, probably reasonable for the ligand specificity in this class of proteins[43]. This hypothesis is confirmed by PG : PGIP ligand binding studies by Leckie *et al.*[44]. They studied PGIP-1 and PGIP-2 of *P. vulgaris* with differential affinities towards PGs of *F. moniliforme* and *A. niger*. Only eight amino acid variations were distinguished between the two PGIPs and five of them were confined within the β-sheet/β-turn (Figure 2). Change in each single amino acid by site-directed mutagenesis decreased the binding nature and a change in Q253 of PGIP-2 (in β-sheet/β-turn) made a major reduction in binding capacity. Conversely, change in amino acid K253 of PGIP-1 to Q253 increased its binding capacity dramatically.

The PGIP was crystallized and subjected to X-ray crystallographic analysis in order to understand the role of LRR motif in ligand recognition. It contains ten repeats of a short (24 amino acid) LRR motif[45]. PGIP of *P. vulgaris* has three domains, the central LRR region and two cysteine-rich flanking domains[46]. PG–PGIP is the most widely studied example of ligand and protein binding.

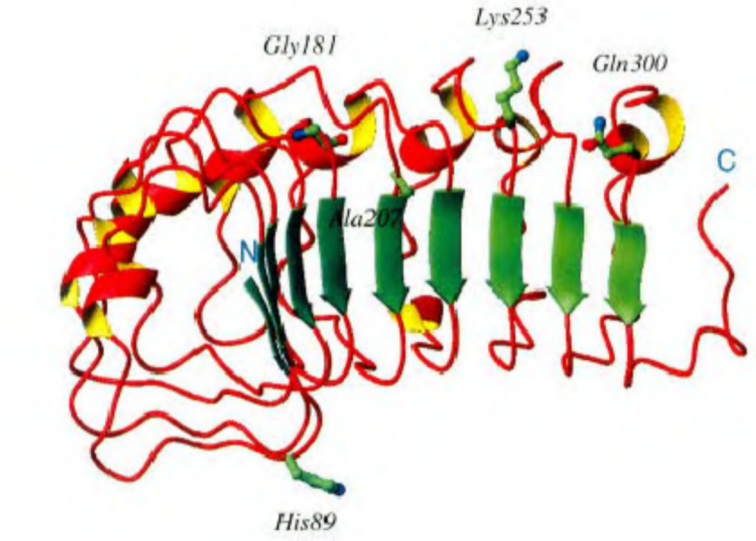

Figure 2. The fold of modelled PGIP-1. β-strands are shown in green and -helices in red/yellow. Positions of the five amino acid distinguished between PGIP-1 and PGIP-2 that lie in the LRR domain are shown. Positions of Gly181 and Lys253 map to the heel of their respective LRR motif preceding the β-strands[44]. Reproduced with permission from Nature Publishing House, London.

Site-directed mutagenesis of PG of *F. moniliforme* at the histidine residue 234 to lysine abolished enzyme activity, but its binding with PGIP of *P. vulgaris* was not affected[47]. This indicates that the critical site for enzyme activity and binding site for PGIP are different.

Transgenic plants

DNA sequences for more than 120 PGIPs have been deposited in Gene Bank (International DNA Sequence Database). Monocots have little pectin composition in their cell walls and therefore, many monocots do not possess protein inhibitors against pathogen PGs. Since cereal crops lack PGIP, insertion and expression of *pgip* gene will make them disease-resistant. Studies on incorporation of *pgip* gene from dicots into monocots have been initiated. Maize Hi-II variety was transformed with *pgip* gene of bean to confer resistance to *Stenocarpella maydis*[48]. Most of the *pgip* genes were isolated in connection with fungal PG inhibition. Transgenic plants carrying *pgip* can be exploited to combat PGs of other pests like nematodes, insects and soft-rot bacteria. The possibility of developing transgenic citrus with *pgip* gene of grapefruit to confer resistance to citrus root weevil is being studied[49]. Transgenic *Vitis vinifera* with inserted *pgip* gene of pear to confer resistance to the bacterium *Xylella fastidiosa* is under trial[50].

Transgenic tobacco and tomato plants expressing PGIP from *P. vulgaris* grew much larger and more vigorously than the wild type. Pectin from transgenic tomato exhibited a higher degree of methylation and acetylation than those isolated from non-transformed plants[51]. It is a well-established fact that highly methylated pectins are not affected by PGs.

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