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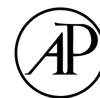

Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors

HENRIK U. STOTZ[1]*, JOHN G. BISHOP[2], CARL W. BERGMANN[3], MARCUS KOCH[4], PETER ALBERSHEIM[3], ALAN G. DARVILL[3] and JOHN M. LABAVITCH[5]

[1] *Max-Planck-Institute of Chemical Ecology, Carl-Zeiss Promenade 10, D-07745 Jena, Germany,* [2] *School of Biological Sciences, Washington State University, Vancouver, WA 98686, U.S.A.,* [3] *Complex Carbohydrate Research Center, University of Georgia, Athens, GA, 30602-4712, U.S.A.,* [4] *Institute of Systematic Botany and Geobotany, Gregor-Mendel-Str. 33, A-1180 Vienna, Austria and* [5] *Department of Pomology, University of California, Davis, CA 95616, U.S.A.*

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Plant polygalacturonase inhibitor proteins (PGIPs) bind fungal polygalacturonases (PGs), but inhibition specificities and kinetics vary within and among species. Purified bean PGIP inhibited all fungal PGs we tested, including *Fusarium moniliforme* PG. Pear PGIP, however, was only effective against *Botrytis cinerea* PG. Moreover, tomato PGIP inhibited *B. cinerea* PG more than *Aspergillus niger* PG. Models of codon evolution for 22 dicot PGIPs and 19 fungal PGs indicated that advantageous substitutions dominate the molecular evolution of these genes and identified 9 amino acid residues, each, that are likely to evolve adaptively in response to natural selection. Many of these residues are within the β-strand/β-turn region of the PGIP LRR, including two sites known to alter inhibition specificities of bean PGIPs, but others lie outside this region. Our results complement existing molecular and biochemical studies of resistance specificity, and suggest new target amino acids for manipulating PG-inhibition.
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Keywords: *Lycopersicon esculentum*; *Phaseolus vulgaris*; *Pyrus communis*; *Fusarium moniliforme*; *Botrytis cinerea*; *Aspergillus niger*; protein interaction; evolution.

INTRODUCTION

Protein–protein interactions play a definitive role in plant disease resistance. For instance, products of plant resistance (*R*) genes determine the outcomes of many interactions between plants and pathogens by recognizing and initiating induced responses to corresponding pathogen (*avr*) avirulence gene products. A key feature of most *R* genes is a leucine-rich repeat (LRR) region that is implicated in protein–protein interactions. These interactions are often highly specific, but direct binding of an *avr* gene product to a resistance protein with LRRs was demonstrated only recently [*7*]. LRRs are also known in plant defense proteins that may not be directly involved in pathogen recognition, such as polygalacturonase inhibiting proteins (PGIPs). However, binding of PGIPs to fungal endo-polygalacturonases (PGs) [*16*] appears to be less specific than the ligand binding of R gene products [*24*].

* To whom all correspondence should be addressed.
Abbreviations used in text: *avr* gene, avirulence gene; $K_a:K_s$, replacement:silent ratio; K_i, inhibition constant; LRR, leucine-rich repeat; pI, isoelectric point; PG, polygalacturonase; PGIP, polygalacturonase-inhibiting protein; *R* gene, resistance gene; RI, ribonuclease inhibitor; TBS, Tris buffered saline.

Microbial PGs contribute to the degradation of pectin polymers in plant cell walls, facilitating penetration of plant tissues by both bacteria [*40*] and fungi [*46*]. By inhibiting fungal PGs, PGIPs directly interfere with host cell wall degradation. In addition, PGIPs may modulate PG activity to prevent complete degradation of pectic oligomeric elicitors of inducible defense systems [*13*]. Individual PGIPs vary greatly in their effectiveness against PGs from different fungal species [*31*] and in pear differential inhibition of fungal PGs correlates with the resistance to fungal pathogens producing these enzymes [*2*]. In addition, PGIPs from different plants vary greatly in their effects on fungal PGs [*43*, *15*]. This variability in target PG specificity likely reflects counter-adaptations between fungal PGs and plant PGIPs that lead to specialization.

As the ligand of PGIP is well known, it may serve as a model for the evolution of specificity in LRR-containing proteins. Plants contain multiple PGIP genes as, for instance, in bean (*Phaseolus vulgaris*) where at least five genes are present. The two *P. vulgaris* PGIP gene products characterized differ in their ability to inhibit PGs from *Fusarium moniliforme* and *Aspergillus niger*. Using directed mutagenesis Leckie *et al.* (1999) [31] identified a small number of amino acid substitutions responsible for most of

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the differences in inhibition. Structural models based on porcine ribonuclease inhibitor (RI) indicate that each of the 10·5 LRRs in PGIP possesses a segment that forms a solvent-exposed β-strand/β-turn structure [*31*] and most amino acid substitutions separating the two bean PGIPs, including those responsible for differences in inhibition, reside in this solvent-exposed region. These structural and experimental analyses of PGIP have greatly advanced the understanding of LRR mediated protein–protein interactions.

Successful pathogens, which decrease plant fitness, might select for amino acid substitutions that increase PGIP specificity or decrease K_i for a particular PG. Generally, most substitutions are not expected to have adaptive consequences, making it difficult to identify which substitutions are responsible for adaptations of enzymes. However, if advantageous substitutions occur frequently enough, it becomes possible to identify individual sites subjected to selection based on the ratio of synonymous (silent) and non-synonymous (amino acid changing) substitutions, providing a novel method of identifying residues important for PG or PGIP function and specificity [*35*].

In this study, we present analyses of the specificity of pear, tomato and bean PGIPs against PGs from three fungal species, allowing us direct comparisons of PGIP effectiveness among different plant species. We also present evolutionary analyses in which we demonstrate that molecular evolution of both PGIPs and PGs is driven by adaptive substitutions. This analysis also identifies individual peptide residues that are likely targets of natural selection and thus likely to strongly affect protein function.

MATERIALS AND METHODS

Materials

Endo-PG II with a pI of 5·2 and a molecular mass of 34 kDa was purified from *A. niger* pectinase (Sigma, St. Louis, MO, U.S.A.), as previously described [*11*]. A PG with a pI of 5·8 was purified from *F. moniliforme* (82093) as described [*8*]. Differential glycosylation and charged residues that are not surface exposed are responsible for minor discrepancies between predicted and actual pIs of the product of the *F. moniliforme pgA* gene (L02239). *Botrytis cinerea* DEL 11 was obtained from Richard M. Bostock (Department of Plant Pathology, University of California, Davis) and grown in modified Czapek medium [*39*] containing 0·2 % (w/v) glucose and 3 % (w/v) pear cell walls [*17*] on a rotary shaker at 125 rpm. After 1 week, the culture medium was collected by filtration through cheesecloth and Whatman 1MM paper, dialysed and lyophilized. *B. cinerea* PG was used without further purification. PGIPs were purified from pods of bean (*P. vulgaris*) [*12*] and fruits of pear (*Pyrus communis*) [*44*] and tomato (*Lycopersicon esculentum*) [*43*] according to published procedures.

PGIP activity assay

PGIP activity was determined by assaying the PG-catalysed release of reducing sugars [*21*] from sodium polypectate (Sigma, St. Louis, MO, U.S.A.) in the presence or absence of PGIP, as described [*44*]. PG and PGIP were mixed and incubated at room temperature for 5 min prior to the addition of substrate. The reactions were stopped after different time intervals and initial reaction rates were determined. The standard assay contained 0·05 % (w/v) sodium polypectate and sufficient PG (4×10^3 units) to produce 80 nmol of galacturonic acid reducing equivalents in 20 min at 30°C. PGIP concentrations were determined by quantitative amino acid analysis.

Generation of antisera

Purified pear fruit PGIP was chemically deglycosylated using trifluoromethane sulfonic acid [*25*], gel-purified and electroeluted [*22*]. This PGIP preparation (100 μg) was mixed with Freund's complete adjuvant and injected intrapopliteally into two New Zealand White rabbits. Two boosts of 50 μg deglycosylated PGIP in Freund's incomplete adjuvant were administered at 2 week intervals by the same route. The serum collected after 8 weeks was used for subsequent experiments. Production and purification of polyclonal antibodies against the N-terminal region of bean PGIP were described previously [*4*]. To obtain polyclonal antibodies against glycosylated bean PGIP, 50 μg of the purified bean PGIP glycoprotein in Freund's complete adjuvant was injected intramuscularly into New Zealand White rabbits, followed by boosts of 25 μg PGIP in incomplete Freund's adjuvant at 4 week intervals. Sera collected 10 days after the third and fourth boosts were used for the subsequent experiments.

Gel electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE as described [*29*]. Polyacrylamide gels (10 %) were electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). Blots that were screened with antibodies against bean PGIP glycoprotein were pretreated for 1 h with 1 % (w/v) sodium periodate, washed with TBS and blocked with Tween 20 (Sigma, St. Louis, MO, U.S.A.) according to Birkett *et al.* [*5*]. Antibodies against an N-terminal peptide of bean PGIP were used at a dilution of 1:200 [*4*], whereas antibodies against deglycosylated pear PGIP and bean PGIP glycoprotein, and alkaline phosphatase-conjugated goat anti-rabbit IgG were used at a dilution of 1:2000. Blots were developed with nitroblue

TABLESTABLE 1. *Descriptions and accession numbers of PGIP sequences used for evolutionary analyses*

Sequence	Species	Accession number	Analysis
pgip	*Malus domestica*	U77041	Dicot [1]ML
pgip	*Pyrus communis*	L09264	Dicot ML
pgip	*Prunus armeniaca*	AF020785	Dicot ML
pgip	*Lycopersicon esculentum*	L26529	Dicot ML
pgip	*Actinidia deliciosa*	Z49063	Dicot ML
pgip	*Antirrhinum majus*	X76995	Dicot ML
Moj9.3	*Arabidopsis thaliana*	AB010697	Dicot ML
Moj9.4	*A. thaliana*	AB010697	Dicot ML
pgip 1	*Phaseolus vulgaris*	X64769	Dicot and legume ML
pgip 2	*P. vulgaris*	none; ref. 31	Legume ML
pgip 1	*Glycine max*	X78274	Dicot and legume ML
pgip 2	*G. max*	S60713	Legume ML
pgip	*Citrus sinensis*	Y08618	Dicot ML, [2]$K_a:K_2$
pgip 1	*C. jambhiri*	AB013397	$K_a:K_s$
pgip 2	*C. jambhiri*	AB015198	$K_a:K_s$
pgip	*C. unshiu*	AB016204	$K_a:K_s$
pgip 1	*C. sp. cv. sannumphung*	AB015356	$K_a:K_s$
pgip 2	*C. sp. cv. sannumphung*	AB015643	$K_a:K_s$
pgip 1	*C. iyo*	AB016205	$K_a:K_s$
pgip 2	*C. iyo*	AB016206	$K_a:K_s$
pgip	*Poncirus trifoliata*	AB020528	$K_a:K_s$
pgip	*Fortunella margarita*	AB020529	$K_a:K_s$

[1]ML = Maximum Likelihood analysis of codon evolution.
[2]$K_a:K_2$ = Comparisons of rates of amino acid and synonymous substitutions.

tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as a substrate.

Evolutionary analyses

To detect positive selection, we applied two approaches. First, we simply compared the number of synonymous (silent) substitutions per synonymous site (K_s) with the number of non-synonymous (amino acid replacing) substitutions per non-synonymous site (K_a) according to Li [*32*]. $K_a = K_s$ represents the rate of neutral (i.e. neither advantageous nor deleterious) substitution. Most non-synonymous mutations are deleterious, resulting in purifying selection and $K_a \ll K_s$. A rate of amino acid replacement greater than the rate of silent substitution, $K_a > K_s$, provides extremely stringent evidence of adaptive sequence evolution, but will not detect many cases of weaker positive selection [*32*, *47*]. The analysis was performed on unaltered PGIP sequences, then repeated for cases with $K_a > K_s$ after removing conserved residues of the xxLxxLxxLxxLxLxxNxLxGxIP motif. This procedure is justifiable because we can reasonably identify those residues that are part of the motif and are supposed to fulfill structural roles, as suggested by previous studies [*33*, *34*, *45*]. We used *t*-tests [*47*] to determine whether $K_a:K_s$ ratios of pairwise sequence comparisons were significantly greater than the neutral expectation ($K_a:K_s = 1$).

To detect more subtle positive selection in PGIP and PG proteins, we employed maximum likelihood models of codon substitution. Using the CODEML program in the PAML package [*35*] we compared two scenarios: 1) a null model in which substitutions are assumed to be either neutral or deleterious and 2) a positive selection model that includes a third category of advantageous substitutions. Neutral sites had a ratio of $K_a:K_s = 1$ (replacement rate = silent rate), deleterious sites had a ratio of $0 < K_a:K_s < 1$ and positively selected sites had a ratio of $K_a:K_s > 1$. For each codon site, the probability of observing the data was computed using the proportions of sites belonging to these different categories. The log likelihood is the sum of these probabilities over all codon sites in the sequence. The likelihood ratio of the two models tests whether the positive selection model fits the data better than the null hypothesis. Twice the difference in log likelihood between the two models was compared with a χ^2 distribution with df = 2. We ran each model using several phylogenies estimated by different algorithms to avoid the possibility of an outcome dependent upon a phylogeny in which some branches receive little statistical support. Models were also run under a variety of assumptions regarding codon usage [*35*]. An empirical Bayesian approach implemented in CODEML was used to infer which category (neutral, deleterious or advantageous) each amino acid site most likely belonged to. Sites with posterior probabilities > 0·95 were considered significant. Descriptions and accession numbers of PGIP (Table 1) and PG sequences (Table 2) used for maximum

Table 2. *Descriptions and accession numbers of PG sequences used for maximum likelihood analysis of codon evolution*

Sequence	Species	Accession number
pg	*Sclerotinia sclerotiorum*	L12023
pg	*Aspergillus parasitica*	L23523
pg	*A. aculeatus*	AF054893
pecA	*A. flavus*	U05015
pecB	*A. flavus*	U05020
pga	*A. niger*	X52903
pgaI	*A. niger*	X58892
pgaII	*A. niger*	X58893
pgaC	*A. niger*	X64356
pg	*A. oryzae*	D14282
pg	*Cochliobolus carbonum*	M55979
pg1	*Claviceps purpurea*	Y10165
pg2	*C. purpurea*	Y10165
pg	*Cryphonectria parasitica*	U49710
pg	*Fusarium oxysporum*	AB000124
pg	*Kluyveromyces marxianus*	AJ000076
pg	*Ophiostoma novo-ulmi*	AF052061
pg	*Penicillium expansum*	AF047713
pg	*P. janthinellum*	D79980

likelihood and nucleotide substitution analyses are provided.

Phylogenies were inferred by estimating the number of nucleotide substitutions per site [*26*] and construction of bootstrap probability estimates over 100 replicates [*18*] of neighbor-joining trees [*41*]. Characters were run unordered, equivalent to equal weighting at all nucleotide sites [*20*]. Additional phylogenies used in codon substitution models were estimated using parsimony and maximum likelihood. All phylogenetic estimates were performed with PHYLIP, version 3·57 [*19*].

Protein structure modelling

The threading program of the SwissModel server was used in the First Approach Mode [*36*] to partially align the structures of fungal PGs to the known structure of PehA from *Erwinia carotovora* ssp. *carotovora* [*37*]. The alignment was optimized with the Swiss-PdbViewer software. The sequence alignment between 19 fungal sequences and the bacterial sequence was consulted to correctly interpret the model. Graphical representations were created using the SYBYL 6·5 software package.

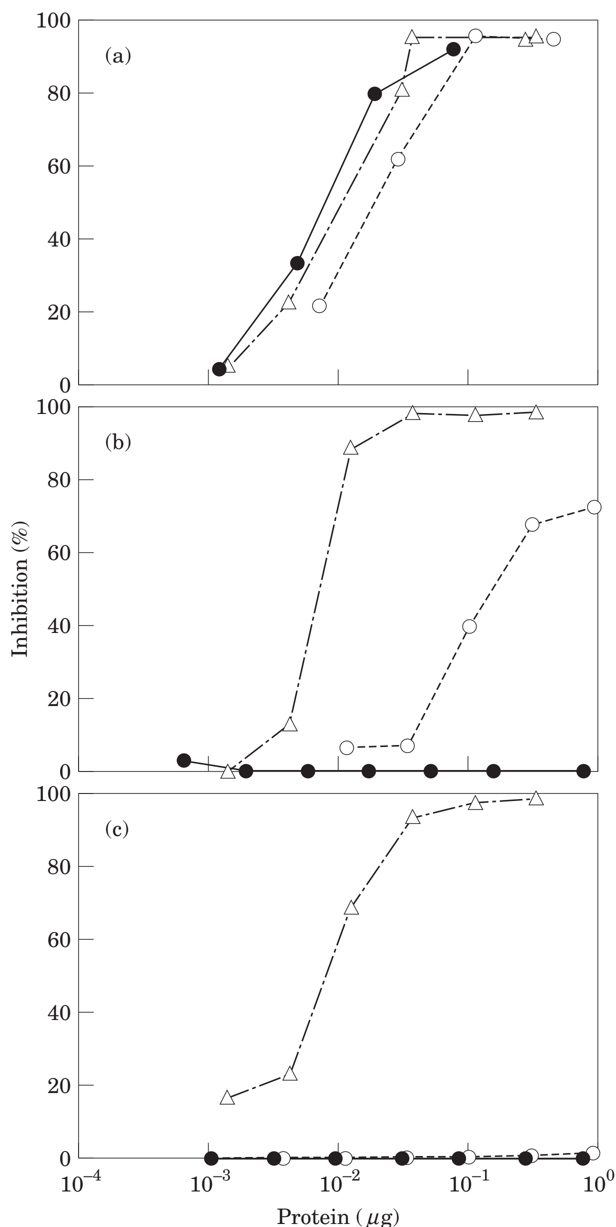

Fig. 1. Inhibition of fungal PGs by plant PGIPs. Increasing amounts of pear (●) bean (△) and tomato (○) PGIPs were tested for inhibition of (a) *B. cinerea* (4·3 nmol min^{-1}), (b) *A. niger* (2 nmol min^{-1}) and (c) *F. moniliforme* (2·1 nmol min^{-1}) PGs by analysing enzyme catalysed release of reducing sugars. Inhibition was expressed relative to PG-activity in the absence of PGIP. Reported protein concentrations are based on amino acid analysis of the purified PGIPs.

RESULTS

Inhibition specificities and kinetics of plant PGIPs

PGIPs from bean, pear and tomato were tested for inhibition of the PGs from *A. niger*, *F. moniliforme* and *B. cinerea* to determine differences in target PG selectivity (Fig. 1). The purified PGs from *A. niger* and *F. moniliforme* are each encoded by single genes [*11*, *9*]. In contrast, the culture filtrate from *B. cinerea* contained several PG isozymes as well as various unidentified proteins. PGIPs from bean, pear and tomato were pure as determined by SDS-PAGE and silver staining (data not shown). However, bean pods contain at least two PGIP forms that are biochemically distinguishable by their inhibition specificities [*16*].

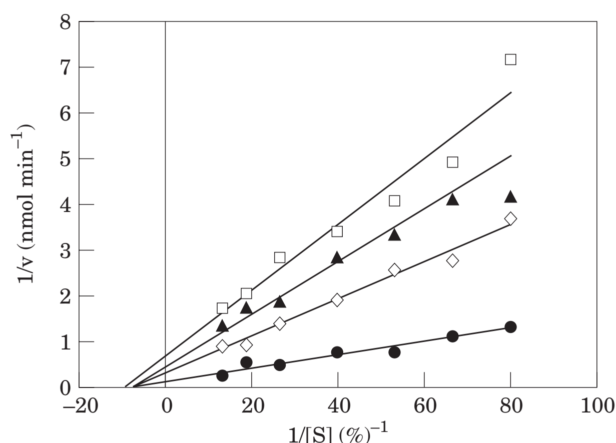

FIG. 2. Kinetics of inhibition of *A. niger* PG by tomato PGIP. Lineweaver-Burk plot analysis of the reaction rates at different substrate concentrations in the absence (●) or presence of 0·08 mg ml^{-1} (◇), 0·24 mg ml^{-1} (▲) and 0·72 mg ml^{-1} (□) tomato PGIP.

All three plant PGIPs inhibited the *B. cinerea* PG mixture with similar efficiency [Fig. 1(a)]. In separate experiments tomato PGIP was slightly less active than pear and bean PGIPs but the latter two were not significantly different. Pear PGIP did not inhibit *A. niger* PG [Fig. 1(b)]. Tomato PGIP was approximately 25 times less inhibitory than bean PGIP against this fungal PG. *F. moniliforme* PG was inhibited only by bean PGIP [Fig. 1(c)]. These data demonstrated differences in PG-inhibition specificities among PGIPs from different plant species, an observation that prompted us to examine whether these differences are due to an evolutionary arms race between fungal PGs and plant PGIPs.

The interaction between specific plant PGIPs and fungal PGs also differ in their inhibition kinetics, suggesting different molecular associations. Lineweaver-Burk double reciprocal plot analysis indicated non-competitive inhibition of *A. niger* PG by tomato PGIP with a Ki of 0·06 mg ml^{-1} (Fig. 2). PGIPs from bean [*30*] and raspberry [*23*] act by noncompetitive inhibition as well, whereas PGIPs from pear [*1*] and orange fruits [*3*] have been reported to be competitive inhibitors of fungal PGs. As these studies used distinct PGs, differences in inhibition kinetics may depend on target-PG rather than PGIP properties.

Phylogenetic and immunological relationships between plant PGIPs

PGIPs are members of gene families and their divergence within and among species was estimated using phylogenetic comparisons. The phylogenetic relationship among plant taxa inferred by mature PGIP coding sequences [Fig. 3(a)] is congruent with data for the chloroplast rbcL gene [*14*]. The PGIPs from pear and apple (*Malus domestica*) are closely related and may in fact be orthologous genes, i.e., direct evolutionary descendants from a common ancestral gene. In contrast, based on the long branch lengths separating them, PGIPs from bean and soybean (*Glycine max*) may be paralogous to one another, i.e., descendants of duplication events occurring prior to speciation. An example of gene duplication is the pair of PGIP genes in *Arabidopsis* which are present in tandem with the same orientation on chromosome V. Pairs of bean and soybean PGIPs analysed here also represent duplications after divergence of these species, indicating recent diversification of these genes.

The immunological relationships between bean, pear and tomato PGIPs supported the phylogenetic distance data. Polyclonal antibodies against denatured, deglycosylated pear PGIP [Fig. 3(b)] or native bean PGIP [Fig. 3(c)] were highly selective, cross-reacting with PGIPs from the other species only when an order of magnitude more protein was analysed. Polyclonal antibodies against a synthetic peptide corresponding to amino acid residues 10–21 of mature bean PGIP [Fig. 3(d)] cross-reacted fairly well with tomato PGIP, but not with pear PGIP. This result is due to the lower degree of homology of the pear PGIP at the N-terminus relative to the other two PGIPs [*43*]. The mature pear polypeptide has Ala-Phe as amino acids 17 and 18, whereas both bean and tomato PGIPs have Asp-Leu at the same positions. Thus these antibodies should allow discrimination between endogenous and exogenous PGIPs in transgenic plants expressing PGIPs from a different species.

Analysis of nucleotide substitution patterns of PGIPs and PGs

We applied a maximum likelihood model of codon evolution [*35*] to determine whether positive selection may have played a role in the evolution of plant PGIPs and their fungal PG targets. A set of mature PGIP coding sequences from 10 dicot species [Fig. 3(a)] and a set of four legume sequences both provide strong evidence that evolution of these proteins is driven largely by advantageous substitutions (Table 3). Likelihood ratios between neutral and positive selection models indicated that the positive-selection model fits the data significantly better ($P < 0{\cdot}00001$ for dicots, $P = 0{\cdot}003$ for legumes) than the neutral model.

Bayesian analysis of the model allowed identification of five and four positively selected amino acids in the dicot and legume data sets (Fig. 4), respectively. Most of these sites (six of nine) were located in LRRs 5–7, whereas additional sites were found in β-sheet regions of LRR3 and LRR9 and at the C-terminus. Positively selected sites were as frequent in xxLxLxx β-strand regions as in other parts of the polypeptide. However, the dicot data indicated

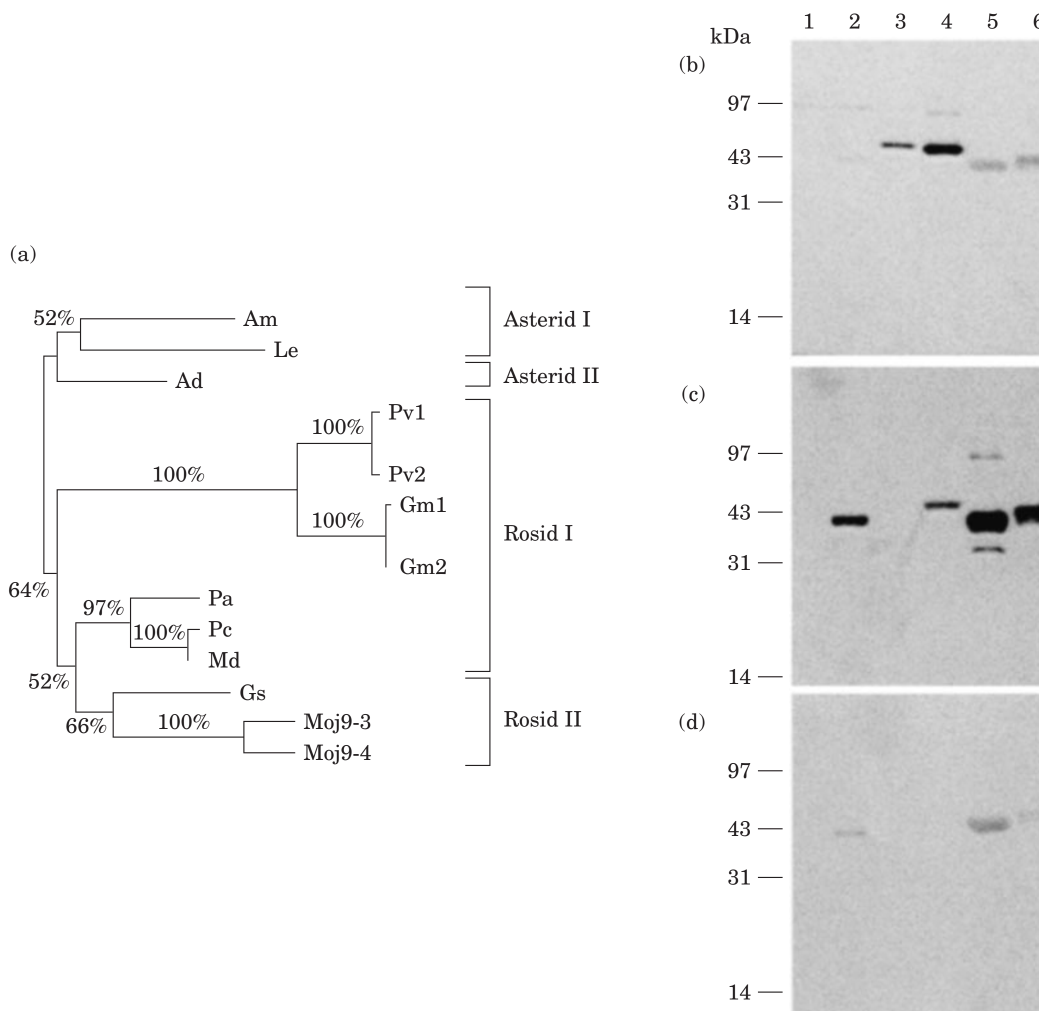

FIG. 3. Phylogenetic and immunological analyses of different plant PGIPs. (a) Phylogenetic relationships of selected mature PGIP coding sequences from *Antirrhinum majus* (Am), *L. esculentum* (Le), *Actinidia deliciosa* (Ad), *P. vulgaris* (Pv), *G. max* (Gm), *Prunus armeniaca* (Pa), *P. communis* (Pc), *M. domestica* (Md), *Citrus sinensis* (Cs) and *Arabidopsis thaliana* (MOJ9·3 and MOJ9·4). Bootstrap supports are indicated above or below branches. Branch lengths are proportional to the number of nucleotide substitutions. The accession numbers of the sequences are listed in Table 1. (b) Purified PGIPs from tomato fruits (lane 1, 50 ng; lane 6, 0·5 μg), bean pods (lane 2, 50 ng; lane 5, 0·5 μg) and pear fruits (lane 3, 50 ng; lane 4, 0·5 μg) were separated by SDS-PAGE and probed with antibodies against the pear PGIP polypeptide, (c) the native bean PGIP and (d) an N-terminal peptide from bean PGIP. Molecular mass standards are indicated.

that amino acid sites located in β-sheet structures were more likely to be positively selected ($P < 0{\cdot}001$) than variable sites located elsewhere (Table 4). One of the adaptively evolving residues in the legume data set (Leu280) is predicted to serve a structural role. The amino acid replacements at this position are conservative, suggesting that this residue is part of the hydrophobic core of the protein. Thus, positively selected residues that are identified by maximum likelihood analysis of relatively few sequences, as in the case of the legume data set, need to be critically examined to assess their functional significance.

Comparisons of replacement (K_a) and silent (K_s) substitution rates among PGIP sequences of the Rutaceae family also supplied evidence that positive selection is the predominant force shaping sequence variation at this locus (Fig. 5). This analysis excluded conserved residues in the LRR motif that are presumed to fulfill structural roles [*33, 34, 43*]. Many pairwise comparisons show $K_a > K_s$, providing evidence for the presence of adaptive sequence evolution [*28*]. However, because of the small number of nucleotide substitutions only three comparisons were statistically significant ($t = 2{\cdot}42$, $P < 0{\cdot}05$) using conservative estimates [*47*]. Thus, these results should be viewed as strongly suggesting, but not proving, positive selection in Rutaceae PGIPs. Interestingly, the replacement rate (K_a) rapidly saturates with increasing silent rate (K_s) in both Rutaceae (Fig. 5) and other dicots (not

TABLE 3. *Positive selection explains the codon substitutions observed in PGIP and PG sequences better than the neutral model*

Gene	Sequences	Model	[1]Neutral	[1]Deleterious	[1]Advantageous	[2]$K_a : K_s$	[3]ln L	[4]χ^2	Probability
PGIP	11 dicots	Neutral	0.25	0.75	0.00	NA	−7807.3		
		Selection	0.25	0.73	0.02	9.00	−7793.6	27.38	< 0.00001
PGIP	4 Legume	Neutral	0.45	0.55	0.00	NA	−2215.6		
		Selection	0.44	0.53	0.03	9.00	−2211.1	9.00	0.003
PG	19 fungi	Neutral	0.17	0.83	0.00	NA	−12800		
		Selection	0.17	0.76	0.07	2.67	−12789	42.84	< 0.00001

[1]Proportion of sites that are neutral, deleterious, advantageous. [2]$K_a : K_s = 9$ is the upper bound set by CODEML. [3]ln L: log likelihood (see "Materials and Methods"). [4]Likelihood ratio test: $\chi^2 = 2 \times (\ln L_{Selection} - \ln L_{Neutral})$. NA: not applicable.

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Orange              MSNTSLLSLFFFLCLCISPSLS 22
Pear              MELKFSTF...TLLFSSVLN.A.. 24
Cherry            MDVKFPT..C.TLLFSTILN.A.. 24
Kiwi                 .KS.TAI..-LLFLSLL..... 21
A. thaliana          .DK.AT.C.L.LFTFLTTCLSK 22
Tomato                M.L...LVVI...-FA..... 20
Bean 1        MTQFNIPVTMS.S..IILVILVSLRTA.. 29

Orange        DLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCD--WYCVTC 63
Pear          .....D......QI..AFGD.......KSD.....-....... 65
Cherry        E....E......QI..AF.D....T..K.E.....-....... 65
Kiwi          .R..........RI.QA.....L......DN....--..N.D. 62
A. thaliana   ....Q...NT...I........H....D.Q....S--..LE. 63
Tomato        VR...K......QI..D.G...H....D.N....Y--..VIK. 61
Bean 1        E....Q..QA..QI..D.G..TT.S..L.T....NRT.LG.L. 72

Orange        DLTT--NRINSLTIFAGDLPGQ--IP 85
Pear          .S..--.........QVS..--.. 87
Cherry        .S..--.........QVS..--.. 87
Kiwi          ....--...IA....S.NIS..--.. 84
A. thaliana   GDA.VNH.VTA....S.QIS..--.. 87
Tomato        .RK.--....A..V.QANIS..--.. 83
Bean 1        .TD.QTY.V.N.DLSGHN..KPYP.. 98

Orange        PEVGDLPYLETLMFHKLPSLTGPIQ 110
Pear          AL..........E...Q.N...... 112
Cherry        TQ..........E...Q.N...... 112
Kiwi          AA.......Q..I.R..SN...Q.P 109
A. thaliana   A...........V.R..SN...T.. 112
Tomato        AA..........E..HVTN...T.P 108
Bean 1        SSLAN....NF.YIGGINN.V...P 123

Orange        PAIAKLKNLKTLRISWTNISGPVP 134
Pear          .......G..S..L....L..S.. 136
Cherry        .S.....L..E..L......S.. 136
Kiwi          S..S..S..MV.L....L..... 133
A. thaliana   .T.......RM..L....LT..I. 136
Tomato        ......T...M..L.F..LT..I. 132
Bean 1        ......TQ.HY.Y.TH..V..AI. 147

Orange        DFIRQLTNLTFLELSFNNLSGTIP 158
Pear          ..LS..K.....D.......T.A.. 160
Cherry        ..LS..K.....D....S..T.S.. 160
Kiwi          S.FS..K.....D.....D.T.S.. 157
A. thaliana   ...S..K..E..........D...S.. 160
Tomato        E.LS..K...L...NY.QFT.... 156
Bean 1        ..LS.IKT.VT.DF.Y.A....L. 171

Orange        GSLSKLQKLGALHLDRNKLTGSIP 182
Pear          S...E.PN....R.......H.. 184
Cherry        SW..Q.PN.N..RV......IFF 184
Kiwi          S.....TN.D.I.........P.. 181
A. thaliana   S...T.P.IL..E.S........ 184
Tomato        S...Q.PN.L.MY........T.. 180
Bean 1        P.I.S.PN.GGITF.G.RIS.A.. 195

Orange        ESFGTFTG-SIPDLYLSHNQLSGKIP 207
Pear          I...Q.I.-NV...........N.. 209
Cherry        LDISLS.K-CPERN..........T.. 209
Kiwi          N...E...-QV..........T.S.. 206
A. thaliana   ....S.P.-TV...R.......P.. 209
Tomato        ....R.K.PN...........S.T.HV. 206
Bean 1        D.Y.S.SK-LFTMTI.R.R.T.... 220

Orange        ASLGSMDFNTIDLSRNKLEGDAS 230
Pear          T.FAQ...TS............. 232
Cherry        T..AKLN.S...F.......... 232
Kiwi          KT..DLN.TV..V...M.S..I. 229
A. thaliana   K...NI...R.........Q.... 232
Tomato        ....DLN.S.L.F.........V. 229
Bean 1        P.ANLNLAFV.....M...... 243

Orange        FLFGLNKTTQRIDVSRNLLEFNLS 254
Pear          VI........IV.L............ 256
Cherry        MI........IV.L.......I... 256
Kiwi          .M..S...I.IV.F...KFQ.D.. 253
A. thaliana   M...S....WS..L...MFQ.DI. 256
Tomato        ....K...S.V..L.......DI. 253
Bean 1        V...SD.N.KK.HLAK.S.A.D.G 267

Orange        KVEFPQSLTNLDLNHNKIFGSIP 277
Pear          .....T...S..I.....Y.... 279
Cherry        N...SK...S.........T.G.. 279
Kiwi          ..V......S.........Y..L. 276
A. thaliana   ..DI.KT.GI........G.T.N.. 279
Tomato        .S..AE..IS........R....L. 276
Bean 1        ..GLSKN.NG...RN.R.Y.TL. 290

Orange        AQITSLENLGFLNVSYNRLCGPIP 301
Pear          VEF.Q.N-FQ..........Q.. 302
Cherry        VGL.QVD-.Q..........Q.. 302
Kiwi          VGL.K.D-.QY.........H.. 299
A. thaliana   V.W.EAP-.Q.F......K...H.. 302
Tomato        PGLKDVP-.Q.F.........Q.. 299
Bean 1        QGL.Q.KF.QS....F.N...E.. 314

Orange        VGGKLQSFGYTEYFHNRCLCGAPLE--R 327
Pear          ........DEYS............PSCK 330
Cherry        ........DSST............PSCK 330
Kiwi          T.....G.DQ.S............PDCK 327
A. thaliana   T.....T.DSYS....K........ICK 330
Tomato        Q..T....DIYS.L..K....S..PKCK 327
Bean 1        Q..N.KR.DVSS.AN.K....S..PSCT 342
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FIG. 4. For legend see facing page.

TABLE 4. *Probabilities that amino acid sites do not belong to the positively selected category*

Data set	[1]Amino acid site	Part of xxLxLxx β-sheet?	Probability
Dicot PGIPs	Tyr134	Yes	< 0.001
	Gly181	Yes	< 0.001
	Ala193	No	0.040
	Ser200	No	0.031
	Val324	No	0.008
Legume PGIPs	Ala207	Yes	0.005
	Thr222	No	0.030
	Phe223	No	0.005
	Leu280	Yes	0.038

[1]Amino acids correspond to positions in the bean PGIP-1 sequence.

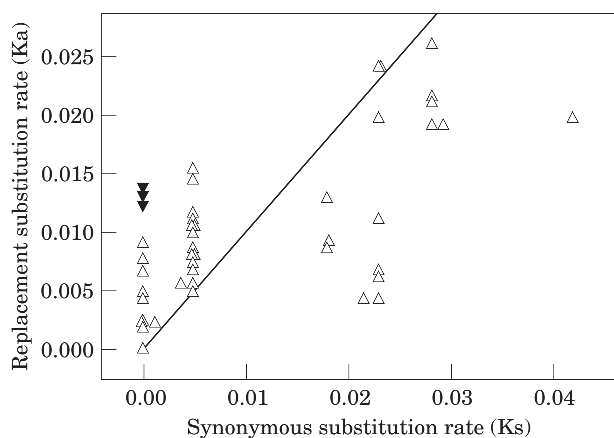

FIG. 5. Analysis of synonymous (K_s) and replacement (K_a) substitution rates among PGIP sequences from the Rutaceae family. The triangles represent K_a/K_s ratios for pairwise comparisons of PGIP sequences. The filled inverted triangles show comparisons that were statistically significant ($P < 0{\cdot}05$). The diagonal corresponds to $K_a = K_s$, defining neutral evolution. Values above the line suggest positive selection; values below the line suggest conservation.

TABLE 5. *Probabilities that amino acid sites do not belong to the positively selected category*

[1]Amino acid site	Probability
Asp120	0.021
Ser208	0.012
Asn218	0.008
Ser241	0.025
Glu258	0.001
Asp268	0.001
Ser297	0.006
Gly345	0.002
Glu346	0.020

[1]Amino acids corresponds to positions in the *E. carotovora* ssp. *carotovora* PehA sequence.

shown), indicating that the number of sites that can change adaptively is highly constrained.

The maximum likelihood model also provided clear evidence for frequent advantageous mutations in PGs ($P < 0{\cdot}00001$) based on the analysis of 19 different PG coding sequences from 14 different fungal species (Table 3). A total of nine positively selected amino acid sites ($P < 0{\cdot}05$) were identified by Bayesian analysis (Table 5). Only two of these amino acid sites are relatively close to the substrate binding cleft, according to structural modelling. These sites are discussed in the context of the crystal structure of PehA from *Erwinia carotovora* ssp. *carotovora* [*37*] that allows some inference on the locations of positively selected sites within the PG molecule.

DISCUSSION

Plant PGIPs and fungal PGs are subject to positive selection

PGIPs differ in PG-target selectivity within and among plant species. Pear, bean and tomato PGIPs are relatively highly diverged, yet all three inhibit PG activity from *B. cinerea*. In contrast, they differ in their ability to inhibit PGs from *A. niger and F. moniliforme*. Moreover, individual plant PGIPs can selectively inhibit particular PG isoforms that a fungus produces. For instance, PG activity assays in substrate overlays after isoelectric focusing demonstrated that pear PGIP preferentially inhibits certain PG isozymes from culture filtrates of *B. cinerea* [*42*]. However, our measurements of the PG-catalysed release of reducing

FIG. 4. Alignment of PGIP amino acid sequences. Positively selected residues are highlighted in black and gray ($P < 0{\cdot}05$) for the dicot and legume data sets, respectively; boxes indicate proposed β-strand regions. Sites shown by Leckie *et al.* (1999) [31] to affect specificity are bold. They propose that it is primarily the × sites in the xxLxLxx β-strand motif that determine specificity. Our results suggest that sites outside this region are also subject to intense natural selection.

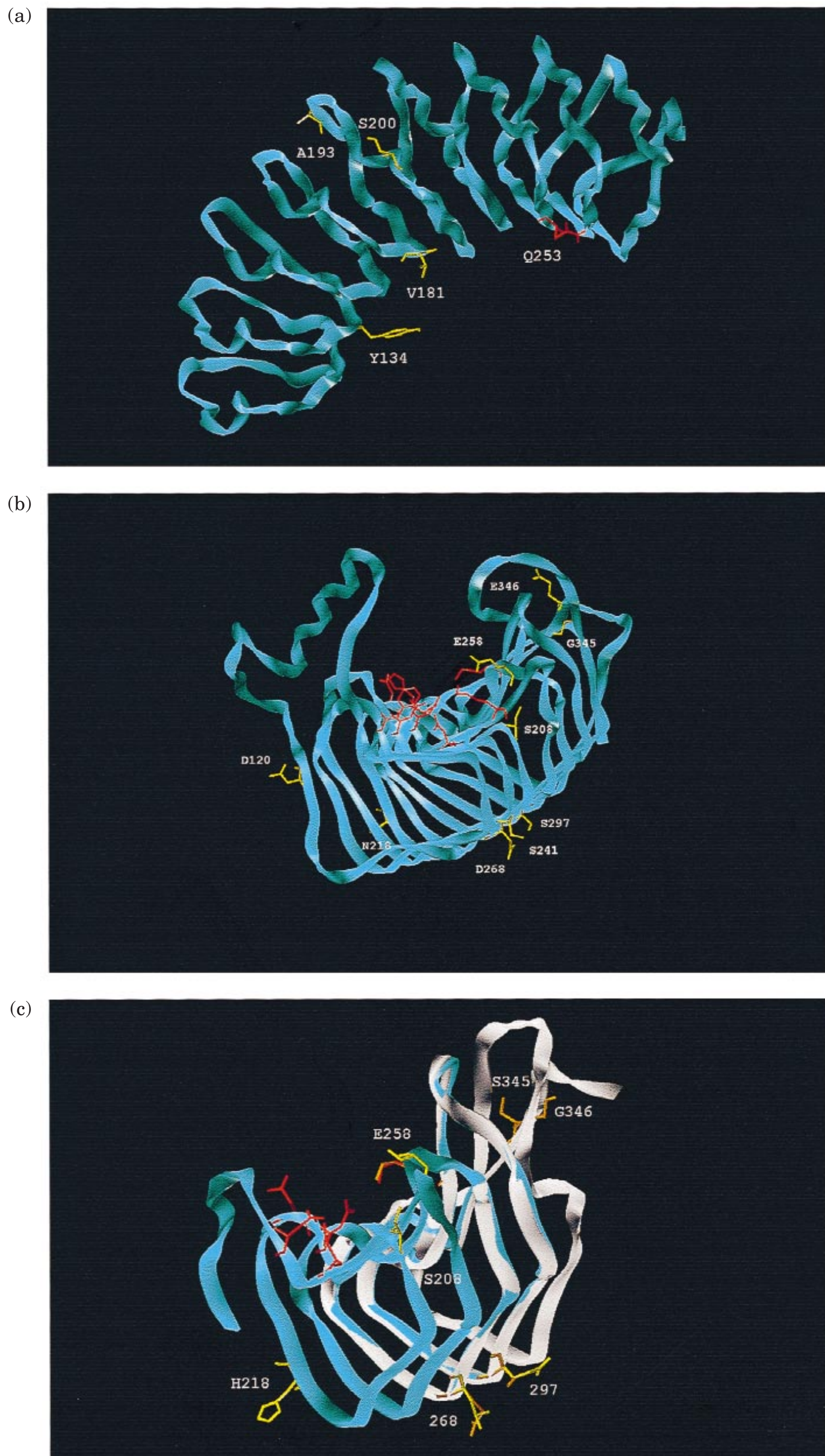

FIG. 6. Modelling of positively selected amino acid sites of plant PGIPs and fungal PGs. (a) The structural model of PGIP-2 according to Leckie *et al*. (1999) [31]. The backbone is colored cyan, positively selected amino acids are yellow and the amino acid that confers specificity to inhibition of *F. moniliforme* PG as determined by Leckie *et al*. (1999) [31] is red. (b) Schematic presentation of the crystal structure of PehA from *Erwinia carotovora* ssp. *carotovora* (Pickersgill *et al*., 1998). The N-terminal 110 amino acids were removed to aid visualization. Yellow amino acids correspond to positively selected sites in fungal PGs. Red amino acids are active

sugars show an almost complete inhibition using such culture filtrates from *B. cinerea* (Fig. 1). This assay-dependent difference is probably due to the higher activity of endo-PGs compared to exo- and endo/exo-PGs in gel assays, whereas the converse is true for the liberation of reducing sugars in solution [*15*]. Besides, plant PGIPs apparently inhibit a range of endo/exo-PGs but discriminate between endo-PGs [*15*]. For instance, pear PGIP inhibits PG I, an enzyme with a mixed endo/exo cleavage mode, but not endo-PG II from *A. niger* [*15*]. This explains the partial inhibition of relatively crude *A. niger* PG mixtures previously compared to the lack of inhibition of purified endo-PG II by pear PGIP observed here [*1*].

Divergence in PG–PGIP interactions may be attributed to evolutionary adaptations of PGIPs to different forms of PG, encountered because of spatial and temporal variation in prevalence of different fungal pathogens, or because of evolutionary response by PG to effective inhibition. This prompted us to examine fungal PGs and plant PGIPs for signatures of positive selection that would indicate continual adaptive substitutions as might occur under rapid plant–pathogen coevolution. The codon evolution models employed here detected positive selection in PG as well as in PGIP dicot and legume data sets, but not in the Rutaceae data set. However, comparisons of replacement (K_a) and silent (K_s) substitution rates among the 10 Rutaceae sequences revealed many pairwise comparisons with $K_a > K_s$, providing evidence for the presence of adaptive sequence evolution [*28*]. The discrepancy between the two analyses of the Rutaceae data set is most likely due to the low number of nucleotide substitutions and the exclusion of conserved LRR residues from the analysis of K_a vs. K_s. To our knowledge this is the first report demonstrating adaptive evolution of both participants in an antagonistic protein interaction between plant and pathogens.

In PGIP, the replacement rate (K_a) rapidly saturates with increasing silent rate (K_s), indicating that the number of sites that can change adaptively is highly constrained. This result is similar to Class I chitinase [*6*], but contrasts with that for plant resistance LRRs, where $K_a > K_s$ even among highly diverged genes. This indicates that a far greater proportion of residues in resistance LRRs can interact with ligands to confer new recognition capability than in the PGIP LRR. Several scenarios could explain the constrained evolution of PGIP compared to resistance genes. *R* genes may evolve rapidly to recognize highly divergent elicitors, whereas PGIP may need to respond to much less divergent forms of PG. Constraints may also arise through conflicting patterns of selection. For example, PGIP may be constrained by the need to maintain inhibition to multiple fungal PGs, or by the need not to inhibit endogenous plant PGs. For any of these reasons, there may be only a few residues that will change inhibition specificity advantageously.

Bayesian analysis of the codon evolution model identifies functionally important residues but is restricted to cases where positive selection (i.e., frequent advantageous substitution) is relatively strong. Although more sensitive than the simple comparison of replacement and synonymous substitution rates, the method does require that molecular evolution of the protein is dominated by positive selection and will not be useful in cases where neutral and deleterious mutations far outnumber advantageous mutations. Theoretical analyses suggest that the latter circumstance is most common, though positive selection may occur more frequently than previously realized [*35*, *6*].

In this study, Bayesian analysis identified a low number of amino acid residues that contribute to putative counter-adaptations between PGIPs and PGs. Surprizingly, we detected entirely different sets of positively selected residues in our legume and dicot PGIP data sets indicating that residues that are both under selection and free to respond differ between groups of plants. Associations between plant PGIPs and fungal PGs are sufficiently weak (Fig. 2, $K_i = 1{\cdot}5 \times 10^{-6}$ M) to suggest that only a few amino acid residues contribute to binding. Likewise, just three amino acids of the LRR protein U2A' govern the protein–protein interaction in the splicosomal complex with its ligand U2B' [*38*]. Only about half of all positively selected sites in the dicot and legume data (4 of 9) were found in parallel β-stands that form the inner circumference of PGIP molecules. This suggests that amino acids outside β-sheets contribute to protein binding, either by favoring conformational adjustments or by interacting with parts of the PG molecule that reach into the PGIP structure. Similarly, only 9 out of 28 amino acids that contact ribonuclease A are in β-strands of RI [*27*]. A low proportion of variable sites was also identified in regions outside of β-sheets of LRRs located in the hypervariable C-terminal half of *RGC*2 resistance genes of lettuce [*34*].

Identification of positively selected amino acid sites in PGIP and PG

Functional analysis of bean PGIPs suggested that a single amino acid change in LRR8 (K253Q) conferred the

site residues. (c) Structural model of fungal PGs. The backbone of *F. moniliforme* is cyan, that of *A. niger* PG II is white. Yellow residues from *F. moniliforme* are positively selected sites, red amino acids are active site residues, and orange amino acids are from *A. niger* PG II.

ability to inhibit *F. moniliforme* PG [31]. However, this amino acid does not have a similar effect on tomato or pear PGIPs because they have Gln (Q) at this position but are unable to inhibit *F. moniliforme* PG. Although position 253 has clearly experienced an adaptive substitution in bean PGIP-2, our data suggest that this site has not played a significant role in the adaptive evolution of most plant PGIPs. Furthermore, a mutation in LRR5 (V181G) reduced affinity between bean PGIP-2 and *A. niger* PG but affected the interaction between PGIP-2 and *F. moniliforme* PG much less than the Q253K mutation. Structural modelling [Fig. 6(a)] suggested that G181V, unlike K253Q, causes conformational changes of adjacent amino acids [31]. In fact, position 181 may have general consequences for PG-PGIP interactions because it was the only site where we found evidence for selection favoring amino acid diversification in both the legume ($P = 0{\cdot}059$) and dicot data sets. The third amino acid that had an effect on the interaction between bean PGIP-2 and *F. moniliforme* PG (A326S) is close to the position equivalent to V324 in the bean PGIP sequences which is positively selected in dicots. Seven additional amino acid sites apparently participate in coevolution with fungal PGs, three of them in β-sheet portions. We conclude that amino acids, which have smaller effects on PG-binding, have the freedom to evolve, unlike amino acids that abolish specific interactions altogether or confer novel ones.

We used the known structure of *E. carotovora* ssp. *carotovora* [37] to model positively selected amino acid sites in fungal PGs [Fig. 6(b)]. Only partial structural alignments were possible because fungal and bacterial PGs share less than 20% amino acid identity. In the case of *F. moniliforme* PG 5 of 9 selected residues were predicted [Fig. 6(c)]. The locations of amino acids in the fungal polypeptide backbone matched Ser^{208}, Asn^{218}, Glu^{258}, Asp^{268} and Ser^{297} in PehA. Three additional residues were modelled in *A. niger* PG II, corresponding to Asp^{268}, Gly^{345} and Glu^{346} in PehA. The modelling of these two fungal PGs accounted for 7 of 9 positively selected amino acid sites, including those (corresponding to Glu^{258}, Asp^{268} and Gly^{345} in PehA) with the highest likelihood ($P < 0{\cdot}002$) of being positively selected (Table 3). The remaining two selected residues (equivalent to Asp^{120} and Ser^{241} in PehA) are part of β-sheets and they are preceded by highly conserved aliphatic amino acids that apparently fulfill structural roles. All of the positively selected amino acids of fungal PGs are potentially surface-exposed and able to interact with other proteins, such as plant PGIPs.

We cannot predict the topography of the positively selected amino acid sites with certainty but they appear to be fairly clustered. Taking PehA as a guide, Asp^{268}, Ser^{241} and Ser^{297} are in close proximity on neighboring parallel β-sheets. Ser^{208} and Glu^{258} are relatively close to the active site and the latter one is next to Ser^{257}, an amino acid that may be important in maintaining the geometry of the substrate binding cleft [37]. The residues Gly^{345} and Glu^{346} are close to the C-terminus and separated from the catalytic site by three loops. Two additional sites (Asp^{120} and Asn^{218}) are on yet a different face of the protein on parallel β-stands. It is obvious that a single PGIP cannot contact all of these distinct regions. Rather, different PGIPs target separate domains of fungal PGs, a conclusion that helps explain modes of both competitive and noncompetitive inhibition. The substrate binding cleft appears relatively protected from interference with PGIP. This is consistent with our prediction, based on the observation that neither tomato (data not shown) nor pear PGIP inhibits endogenous fruit PGs [2], that PGIPs would not target the active site of fungal PGs directly. Moreover, mutations in active site residues abolish PG activity without influencing PGIP binding [10].

Plant resistance against pathogens depends on recognition events that activate induced defenses in addition to pre-existing protection mechanisms. Plant PGIPs are defensive proteins that have evolved different specificities and expression profiles [24] to counter the production of multiple PG gene products from different fungi. PGIPs are structurally related to plant *R* genes in as much as both consist of LRRs. The consequences of binding specificities between plant PGIPs and fungal PGs provide a model for the co-evolution of other interacting proteins, including *R* and *avr* gene products. The details of the interaction between PGIPs and PGs remain to be determined but our evolutionary analysis, along with functional analyses, suggest certain regions in both polypeptides that are plausible candidates for physical contacts.

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