

Plant–pathogen arms races at the molecular level

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Advances in research into the genetics of plant–pathogen interactions include an embracing of evolutionary ideas and methodologies. Recent studies reveal positive selection and selective maintenance of variation in plant resistance and defense-related genes. Coevolution between plants and their enemies involves many interactions at the molecular level.

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Abbreviations

Avr	avirulence
GIP	β -1,3-endoglucanase inhibitor protein
IP	inhibitor protein
K_a	nonsynonymous (amino-acid changing) substitution rate
K_s	synonymous (silent) substitution rate
kb	kilobase
LRR	leucine-rich repeat
PG	polygalacturonase
PGIP	PG inhibitor protein
PR	pathogenesis-related
R	resistance

Introduction

At its simplest, antagonistic coevolution between a plant and its enemy is a three-step process: first, the enemy attacks and exploits the plant; second, enemy exploitation reduces plant fitness, thereby selecting for a novel defense that spreads through the plant population; and third, effectively defended plants decrease pathogen fitness, thus selecting for a genotype that can overcome the defense, which then spreads through the enemy population. When this dance of adaptation and counter-adaptation is ongoing, it is often called an arms race [1]. Beyond the simplest conception, many coevolutionary outcomes are possible [2]. The essential feature of an arms race is escalation [3], which is marked by progressive exaggeration of phenotypic features, serial fixation of new adaptive alleles, or accumulation of molecular interfaces of attack and counter-attack, any of which may impose increasing costs of defense or virulence. Alternatively, antagonistic coevolution can result in ceaseless cycling of allele frequencies at a locus in one or both participants. This outcome involves counter-adaptation but may occur without escalation. If costs are too great or species' responses to selection are genetically constrained, the arms race may be abandoned. These outcomes depend on the genetic bases of coevolutionary traits, the geographic structure of host and pathogen populations, and spatiotemporal variation in the coevolutionary interaction itself, and can result in complex genetic and population dynamics [4*].

Here, we review molecular evolutionary findings and their implications for explaining how plants and their enemies coevolve. Arms races are ultimately determined at the phenotypic level, but examining individual antagonistic genetic systems, such as resistance (R) genes and their corresponding avirulence (Avr) genes, and cell-wall attacking enzymes and their inhibitors, can provide new insights. Because natural selection leaves its signature at the molecular level, evolutionary analysis can suggest how arms races are produced, and to what extent genetic variation is shaped by coevolutionary outcomes.

Recognition and evasion

Plant R genes confer gene-for-gene resistance to a wide array of natural enemies by recognizing pathogen Avr gene products and effectuating downstream response pathways. Altered or eliminated Avr genes allow pathogens to evade recognition and overcome resistance. Thus, plants and their enemies are engaged in an information race.

Evolutionary analyses have been conducted on a number of cloned and sequenced resistance loci (Table 1). Many comprise complex clusters of R genes, and allow evolutionary comparisons among R-gene family members. Ratios of amino-acid changing (nonsynonymous [K_a]) to silent (synonymous [K_s]) substitution rates greater than one ($K_a : K_s > 1$) show that the solvent-exposed residues of R-gene leucine-rich repeat (LRR) regions, which are predicted to function in recognition [5], evolve in response to positive selection. Characterization of multiple alleles or complex locus haplotypes often reveals gross polymorphism for resistance locus structure.

Tomato resistance to *Cladosporium fulvum*, encoded by the *Cf-4/9* and *Cf-2/5* loci, exemplifies complex R-gene locus evolution. Haplotypes conferring different resistance specificities (or no resistance) to *C. fulvum* have been introgressed from wild relatives into cultivated tomato. Their sequences reveal multiple R-gene homologs with intragenic recombination within and between haplotypes [6,7]. Accelerated nonsynonymous substitution rates in 5' LRR residues indicate the action of positive selection [7,8]. Analysis of shared and unique polymorphisms among R genes reveals the evolutionary consequences of *Cf-4/9* locus structure [9**]. The *Arabidopsis* resistance to *Peronospora parasitica* locus *Rpp5* shows an even more dramatic pattern of locus complexity and adaptive evolution [10**]. The *Rpp5* haplotype from the resistant ecotype Landsberg *erecta* contains ten R genes, and was compared across an unprecedented 90 kilobases (kb) with the genome project sequence from the susceptible ecotype Columbia, which contains eight R genes. $K_a : K_s > 1$ suggests positive selection in most pairwise comparisons between the eighteen genes, and recombination produces a mosaic of shared segments among them. The most closely

Table 1

R-gene locus sequences providing evolution or polymorphism information.

Plant locus	Polymorphism*	Complexity [†]	Positive selection [‡]	Reference(s)
Arabidopsis				
<i>Rpp1</i>	1	3	(+)	[43]
<i>Rpp8</i>	2 (2)	2/1	(+)	[44]
<i>Rpp5</i>	2 (2)	10/8	(+)	[10**]
<i>Rps2</i>	2 (1) [§]	1	(+)	[20](a)
<i>Rpm1</i>	2 [§]	1/0	o [#]	[16**]
<i>Rps5</i>	2 (2) [§]	2/1	o	[19*](b)
Tomato				
<i>Cf-4/9</i>	3 (3)	5/5/1**	+	[7,8]
<i>Cf-2/5</i>	3 (3)	2/3/1	nt	[6]
<i>Mi</i>	1	3	(+)	[8,45]
Lettuce				
<i>Dm3</i>	1 ^{††}	24	+	[8,26,27**]
Rice				
<i>Xa21</i>	1	6	+	[46]
Flax				
<i>L</i>	13 (1)	1	+	[13**]

*Number of alleles or haplotypes with distinct resistance specificities (number of different locus structures). [†]Number of R genes for each allele or haplotype sequenced are separated by '/'. [‡]K_a, K_s comparisons between orthologs and paralogs; +, significant; (+), suggestive; o, no evidence; nt, not tested. [§]Within-species variation indicates selective maintenance of polymorphism; [#]Orthologous comparison between species; and **R-gene clusters near *Cf-4/9* provide additional complexity [9**]. ^{††}Within species data available. (a) R Mauricio, T Korves, EA Stahl, J Bergelson, unpublished data. (b) D Tian, EA Stahl, J Bergelson, unpublished data.

related R genes are not in collinear positions in the allelic haplotypes and can actually occur in the same haplotype. Sequence variation and recombination at these complex loci are thought to provide great evolutionary potential for response to positive selection [11]. Genome project studies identifying R-gene clusters in *Arabidopsis* [12*] can test this hypothesis by looking for a correlation between cluster sizes and the extent of adaptive evolution within them.

In the flax–rust interaction, in which gene-for-gene interactions were first described, positive selection acts on allelic resistance specificities [13**]. Thirteen alleles at the flax *L* locus recognize Avr genes of the flax rust fungus *Melampsora lini*. Sequence variation among these alleles and transgenic experiments based on them reveal the mutational and recombinational determinants of their resistance specificity differences, and greater K_a than K_s indicates positive selection in the L-allele histories.

Positive selection in R-gene evolution is likely to reflect 'gene-for-genome' coevolution, in which pathogens overcome resistance by Avr gene loss [14,15] and any R gene in the host may respond to selection for a novel resistance specificity. Thus, a given R gene may show a history of accumulated responses to coevolutionary pressures, and the genome-wide complement of R genes may diversify. In

addition, fine-tuning resistance specificity once it arises by further positive selection [11] may reflect a gene-for-gene arms race, in which an R gene accumulates changes in adapting to a single Avr gene. If Avr genes have innate functions [15], selection should favor mutations in the pathogen that allow evasion of recognition without disrupting function. Avr genes with allelic distinct specificities [14] may reflect this coevolutionary pressure. Evolutionary studies of corresponding gene-for-gene pairs could provide evidence for reciprocal gene-for-gene arms races.

Arabidopsis Rpm1, encoding resistance to *Pseudomonas syringae AvrB* and *AvrRpm1*, has evolved more conservatively [16**]. *Rpm1* segregates for functional and null alleles that have accumulated excess silent differences, indicating that selection maintains the polymorphism. Maintenance of the null allele requires a cost of *Rpm1* resistance in the absence of its Avr elicitor; moreover, maintenance of the resistance allele suggests that *Rpm1* recognizes a natural pathogen of *Arabidopsis* in an interaction that has persisted over evolutionary time. *Rpm1* amino-acid sequence conservation between species implies conservation also in the Avr gene that it recognizes, consistent with a fitness benefit of *AvrRpm1* for *P. syringae* [17]. A cost of R-gene resistance is supported by independent deletions of *Brassica Rpm1* [18] and *Arabidopsis Rps5* [19*]. Within species variation flanking the *Rps5* polymorphism reveals that resistance and susceptibility alleles are again maintained by natural selection because of the cost of resistance, and *Rps5* amino-acid sequence conservation between species, suggests persistence of the *Rps5* gene-for-gene interaction (D Tian, EA Stahl, J Bergelson, unpublished data).

At the *Arabidopsis* resistance to *P. syringae* locus *Rps2*, susceptible ecotypes possess an intact coding sequence that could encode an alternative resistance specificity. Like *Rpm1* and *Rps5*, divergence between *Rps2* resistance and susceptibility alleles is substantial [20]. *Rps2* sequences from 28 ecotypes reveal that silent differences between resistance and susceptibility alleles are clustered because of the selective maintenance of variation in the 5' LRR region. Amino-acid polymorphisms in this region are sufficient to distinguish the alleles of phenotypically distinct ecotypes (R Mauricio, T Korves, EA Stahl, J Bergelson, unpublished data). Thus, disease resistance variation is also maintained at *Rps2*, owing either to the cost of resistance or to the persistence of allelic resistance specificities.

Long-term persistence of a conserved gene-for-gene interaction, as suggested by molecular evolutionary data, implies that a cost must be paid by the pathogen to overcome resistance. Therefore, R genes that show ancient origins and amino-acid sequence conservation might confer durable resistance in applied settings. Evolutionary studies of R genes expected to be durable because of pathogen costs of overcoming resistance, such as pepper *Bs2* [21*], tomato *Cf-ECP2* [22,23] and *Arabidopsis Fls1* [24*,25*], can test the utility of this prediction.

Table 2

Some hydrolytic enzymes involved in attack and defense of cell walls produced by plants and pathogens, and their respective inhibitors.

Pathogen enzyme	Plant enzyme	Enzyme substrate	Reference(s)
Polygalacturonase	Polygalacturonase IP	Plant β -1,4-polygalacturonan	[41**,42**]
Pectin lyases	Pectin lyase IP	Plant pectin	[38]
β-1,4-endoxylanases	Xylanase IP	Arabinoxylan	[39,40]
Glucanase IP	β-1,3-endoglucanases	Fungal β -1,3-glucan	[29]
Allosamadin*	Chitinase/lysozyme	Fungal chitin / bacterial murein	[32,34,35**,37]

*Substrate mimic. Hydrolytic enzymes involved in the attack and defense of cell walls are shown in bold.

The substantial polymorphism manifest in comparisons between functionally distinct alleles or haplotypes at R-gene loci could reflect plant–pathogen arms races, with positive selection between alleles and the spread of different alleles in different populations. Alternatively, R-gene evolution, reflecting a coevolutionary outcome of diversifying selection, might explain positive selection within and between allelic haplotypes. Response to diversifying selection may cause turnover of allelic complex locus haplotypes, while the cost of resistance and persistence of gene-for-gene interactions act to maintain polymorphism. Under diversifying selection, R-gene loci might exhibit greater variation within populations and less differentiation between populations. The lettuce *Dm3* region, which carries resistance to lettuce downy mildew (*Bremia lactucae*) as well as root aphid and contains 24 R genes [26] responding to strong positive selection [8], shows substantial diversity within populations as well as population differentiation in lettuce wild relatives [27**]. Continued investigation of gene-for-gene variation within and between populations and species, and of gene-for-gene interactions in natural populations, promises to distinguish between these hypotheses.

Attack, defense and counter-attack

Antagonistic systems include the interaction of attack and defense enzymes and their corresponding inhibitors (Table 2), and toxins and their counteracting detoxifying enzymes. As yet only a few antagonistic systems have received evolutionary examination, and in each case there is evidence that positive selection affects their components. These data also provide mechanistic evidence for escalating attack and defense systems, but the species interactions that drive the escalations are unexamined.

Plants produce a variety of defense enzymes that attack the polysaccharides or peptidoglycans in pathogen cell walls. These glucanhydrolases are often part of the pathogenesis-related (PR) response, and include chitinases and β -1,3 endoglucanases. Glucanhydrolases are well-represented in the plant genome; with 75% of its genome sequenced, over 60 chitinases and β -1,3 endoglucanases have been identified in *A. thaliana*. Apart from direct protection by inhibiting pathogen growth, glucanhydrolases produce

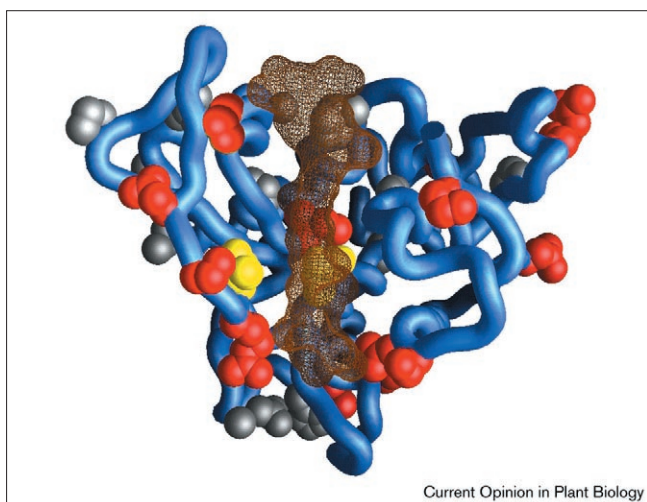
oligosaccharides that are elicitors of plant PR response and systemic-acquired resistance [28].

Pathogens can counteract these enzymes with various inhibitors. The recent characterization of protein soybean β -1,3-endoglucanase inhibitor protein (GIP) from *Phytophthora sojae* demonstrated that this GIP inhibited only one of two purified soybean endoglucanase isoforms, and failed to affect tobacco and endogenous *P. sojae* endoglucanases [29]. *P. sojae* also secretes additional inhibitors of other soybean β -1,3-endoglucanases. The recruitment of multiple, isoform-specific inhibitors suggests the escalation of defense against attack enzymes.

Chitinases hydrolyze chitin, a β -1,4-linked polymer of N-acetyl-D-glucosamine, and many are active on the murein of bacterial cell walls. Because chitin is embedded in a matrix of glucan fibers and complexed with both proteins and other carbohydrates [30], relatively few fungi are sensitive to chitinase alone. Many more are sensitive to chitinase in combination with β -1,3-endoglucanase [31–33], which degrades surrounding glucan polymers. Still other fungi appear insensitive to both enzymes, but their mechanisms of resistance, which may include inhibition of chitinase are unknown. A bacterial chitinase inhibitor, allosamadin, has been isolated and apparently acts as a structural analog of the proposed reaction intermediate [34].

Recent work strongly suggests that arms races involving class I chitinase have taken place. A comparison of K_a and K_s for 22 class I chitinases in the genus *Arabidopsis*, as well as in paralogs within tobacco and rice, provides clear evidence of accelerated amino-acid substitution [35**]. Applying maximum-likelihood models of codon evolution to the *Arabidopsis* chitinase phylogeny confirmed that positive selection has occurred and identified fifteen individual positively selected amino-acid sites. Surprisingly, these sites were found disproportionately often around the active-site cleft where they are likely to affect substrate binding or catalysis, or binding of inhibitors (Figure 1). These observations suggest an arms race in which one or more pathogens produce chitinase inhibitors, while the plant evolves chitinases with decreased affinity for those inhibitors. Chitinases have also evolved other specialized functions, such as producing the

Figure 1



Structural model of class I chitinase with bound hexaNAG substrate (net surface) showing the location on the carbon backbone of positively selected (red) and neutral (gray) substitutions and catalytic glutamates (yellow). Nearly all positively selected substitutions occur on the cleft side of the enzyme, and a disproportionate number occur within the cleft, suggesting evolution in response to protein and/or substrate mimic inhibitors [35••]. Figure prepared by AM Dean.

correct size or type of chitin oligomers for eliciting defense or nodulation responses [36,37].

Pathogens produce their own glucanhydrolases, including polygalacturonase (PG), pectin lyase, pectin methyl-esterase, carboxymethylcellulose and xylanase, that facilitate invasion by dismantling the host cell wall. Inhibitor proteins of fungal pectin lyases have been isolated from sugar beet, and pectin lyase inhibitor protein concentration has been shown to correlate with resistance to root rot caused by *Rhizoctonia solani* [38]. Most recently, xylanase inhibitor proteins (IPs) effective against *Aspergillus niger* and *Trichoderma viride* were detected and isolated from wheat and related cereals [39,40]. Polygalacturonase inhibitor proteins (PGIPs) have been studied in detail. PGIPs defend plants by directly interfering with host cell-wall degradation, and by preventing complete degradation by PG of pectic oligomers that act as defense elicitors. Stotz *et al.* [41••] provide an evolutionary analysis of 19 fungal PGs and 22 dicot PGIPs. Simple comparisons of K_a and K_s detected evidence of positive selection for several citrus PGIPs. Applying the more powerful likelihood models of codon evolution to phylogenies of 19 fungal PGs, 11 dicots PGIPs and four legume PGIPs revealed that these proteins had all undergone positive selection. The adaptive turnover of alleles in both host and pathogen components of this system indicates an arms race, but again, the interacting species are unknown.

Stotz *et al.* [41••] also pinpointed nine amino-acid residues in PGIP that are predicted to sustain adaptive substitutions. The functional importance of two of these sites was con-

firmed in PGIP-1 and PGIP-2 of *Phaseolus vulgaris* using directed mutagenesis [42••]. PGIP-1 inhibits *A. niger* PG, whereas PGIP-2 inhibits *A. niger* and *Fusarium moniliforme* PGs. A single amino-acid substitution in PGIP-1 conferred the ability to inhibit *F. moniliforme* PG, but was not identified as positively selected by Stotz *et al.* Two other single amino-acid substitutions reduced PGIP-2 affinity for *A. niger* PG and *F. moniliforme* PG. These two substitutions were also positively selected, suggesting that they may change frequently to confer new PGIP specificity. These results strongly suggest that adaptive diversification of bean PGIPs is driven by interaction with multiple pathogens.

Conclusions

Molecular genetic analysis of plant–pathogen interactions reveals many layers of antagonistic coevolution. Investigation of molecular evolution at these various levels reveals diversifying selection and the selective maintenance of variation in the information race, and positive selection at the interfaces of attack and defense. Arms races may bring only transient benefits; molecular evolutionary examples of non-arms race outcomes identify genes that might confer durable resistance in applied settings. Thus, the molecular genetics of plant–enemy interactions presents many models for understanding adaptation at the molecular level.

The overlaying of many molecular interfaces of antagonism clearly indicates that escalation occurs in coevolutionary arms races, especially when positive selection and the costs of resistance and virulence are considered. However, in order to fully understand the implications of molecular adaptation for plant–pathogen arms races, evolutionary studies of plant and pathogen interacting genes must integrate molecular genetics with coevolutionary ecology. Coevolutionary theory is an extremely active area of research; hypotheses for coevolutionary outcomes can be formulated for qualitative versus quantitative resistance, the genetic architecture of defense, diffuse versus pairwise species interactions, and metapopulation dynamics. Molecular data are ripe for coevolutionary models to be used to analyze the substitution rate and pattern of polymorphism for a given gene. Finally, disease ecology and population genetic structure must be characterized in genetic model organisms such as *A. thaliana* and in agricultural species' wild relatives. Thus, the molecular genetics of plant–enemy interactions can be a model for understanding antagonistic coevolution.

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