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The evolution of resistance genes in multi-protein plant resistance systems

Running head: Evolution of multi-protein resistance

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Summary

The genomic perspective aids in integrating the analysis of single resistance (*R*-) genes into a higher-order model of complex plant resistance systems. The majority of *R*-genes encode a class of proteins with nucleotide binding (NB) and leucine rich repeat (LRR) domains. Several *R*-proteins act in multi-protein *R*-complexes that mediate interaction with pathogen effectors to induce resistance signaling. The complexity of these systems seems to have resulted from multiple rounds of plant-pathogen coevolution. *R*-gene evolution is thought to be facilitated by the formation of *R*-gene clusters, which permit sequence exchanges via recombinatorial mispairing and generate high haplotypic diversity. This pattern of evolution may also generate diversity at other loci that contribute to the *R*-complex. The rate of recombination at *R*-clusters is not necessarily homogeneous or consistent over evolutionary time: recent evidence suggests that recombination at *R*-clusters is increased following pathogen infection, suggesting a mechanism that induces temporary genome instability in response to extreme stress.

DNA methylation and chromatin modifications may allow this instability to be conditionally regulated and targeted to specific genome regions. Knowledge of natural *R*-gene evolution may contribute to strategies for artificial evolution of novel resistance specificities.

Introduction

This review focuses on resistance (*R*-) genes, and does not discuss basal resistance involving recognition of conserved pathogen associated molecular patterns (PAMPs; reviewed in [1,2•]). *R*-genes were originally defined based on the genetic observation that a single *R*-gene signals a resistance response following infection of a pathogen carrying a specific avirulence gene; the gene-for-gene model conceptualizes *R*-proteins as receptors for specific, pathogen-produced elicitors.

While this genetic observation remains true, the cellular components that govern resistance responses are more complicated than simple gene-for-gene interactions. The products of plant and pathogen genomes commingle in common landscapes in the apoplast, at the cell membrane, in the cytosol, and within the nuclear compartment [2•]. Over evolutionary time, pathogens have evolved virulent effectors that modify, subvert, and exploit host proteins in order to gain access to the cell, suppress host defenses, modify metabolic processes, and produce compounds that benefit the pathogen. Hypothetically, each juncture of pathogen assault presents a simultaneous opportunity for detection of the pathogen infection, by evolution of *R*-proteins that "guard" host proteins

and recognize virulent modifications or effector-host interactions [3]. Subsequently, a pathogen may evolve to escape recognition, by alteration of binding specificity of the effector, evolution of novel host defense suppression, or simply by outright gene deletion. The plant genome may in turn evolve fortuitous compensations that restore recognition. Hence, pathogen effectors may have positive and negative impacts over the course of plant-pathogen coevolution, acting as two-edged swords that enable infection, but trigger resistance if recognized by the host. This dual role, depending on genetic context, is emphasized by the term (a)virulence gene. In this review, we discuss the implications of multi-protein resistance on the evolution of *R*-gene dependent plant resistance systems and describe mechanisms that may facilitate and regulate rapid evolution of *R*-clusters.

Resistance in the genomic context: multi-protein resistance complexes

R-proteins are expected to mediate pathogen recognition via several types of transient or constitutive interactions – including potential interactions with pathogen effectors, effector-host complexes, modified/unmodified host proteins, downstream defense signaling proteins, and/or adapter proteins that mediate binding, stabilize, or localize the *R*-protein – thereby forming multi-protein "*R*-complexes." The *R*-complex mechanism has been conceptualized as a "trigger" [4]: under normal conditions, intramolecular bonds and *R*-complex interactions stabilize the *R*-protein in an inactive conformation; following infection, pathogen effectors disrupt this stable conformation by virulent modification of host proteins or other interactions with the *R*-complex, thus activating *R*-protein signaling. Several lines of evidence support the "trigger complex" model: 1) The *R*-

proteins MLA1 (from barley), and RPM1 and RPS2 (from Arabidopsis), which are each approximately 100 kDa, are associated with much larger protein complexes (approximately 1,000 kDa) under normal cellular conditions, and RPM1 redistributes to smaller complexes following pathogen infection, showing a dynamic shift towards co-elution with the virulence target RIN4 [4,5]. 2) Isolated domains of the potato Rx *R*-protein physically interact when co-expressed in potato lacking Rx, demonstrating that the domains form stable intramolecular bonds. Co-expression of the PVX coat protein elicitor causes these interactions to be disrupted, and results in HR signaling [6]. 3) Alterations that are expected to disrupt *R*-protein conformation – including mutations that may alter intramolecular bonds, truncations removing *R*-protein C-terminal domains, and artificial overexpression of *R*-genes (where oversaturation of the *R*-protein may stoichiometrically exceed *R*-complex proteins that stabilize the inactive conformation) – have all been shown to cause spontaneous resistance signaling in the absence of pathogens (reviewed in [4,7]). Self-oligomerization of *R*-proteins may also affect the conformation and stability of *R*-complexes, as in the product of the tobacco *N* gene, which provides resistance against TMV: N has recently been shown to oligomerize, apparently via binding at the TIR domain, in the presence of the TMV p50 elicitor [8•]. Given that a predicted truncated version of N, consisting of the TIR and NB domains but not the C-terminal LRR domain, is encoded by an alternatively spliced mRNA whose relative abundance increases compared to the full-length *N*-mRNA following TMV infection [9], and that the TIR domain is critical for p50 interaction [10•], oligomerization of alternate N proteins may be critical for stability of N and HR signaling. Such oligomerization also raises the possibility that *R*-proteins form

complexes with closely related homologs. In contrast to these documented *R*-complexes, other *R*-proteins, such as the tomato Eix [11], appear to function as classical gene-for-gene receptors and may not require other proteins to mediate elicitor binding, *R*-protein stabilization, or downstream signaling.

The evolution of resistance systems

Many *R*-genes are located in clusters which comprise several copies of homologous *R*-gene sequences arising from a single gene family (simple clusters) or colocalized *R*-gene sequences derived from two or more unrelated families (complex clusters), and may also contain unrelated single genes interspersed between the homologs. *R*-clusters range in size from two tandem paralogs to large complexes spanning several megabases. The largest *R*-clusters characterized to date include the maize *Rp1* cluster (~1-52 homologs per haplotype [12]), the lettuce *Dm3* (aka *RGC2*) cluster (~12-32 homologs per haplotype [13]), and the potato major late blight resistance (*MLB*) cluster (~45 homologs per haplotype; Kuang and Baker, unpublished data).

Genic and intergenic sequence repeats within *R*-clusters, generated by duplications and transposon insertions provide a structural environment that permits mispairing during recombination, giving rise to unequal crossovers and interlocus gene conversions [14••, 15]. Intergenic unequal crossover has the potential to place *R*-genes in new structural contexts that may alter expression, whereas intragenic mispairing generates chimeric genes that may encode novel functions. Both types of unequal recombination will also

result in altered gene copy number within the cluster (gene duplication on one chromosome and loss on the other) according to the number of genes present in the region between the mispaired recombination sites.

Sequence exchanges (unequal crossovers and/or gene conversions) have been documented in several *R*-clusters [13,16-22] and are associated with genic diversity, characterized by sequence shuffling and chimeric genes, and haplotypic diversity, characterized by a variable number of *R*-homologs within the cluster and a general loss of syntenic/orthologous relationships between haplotypes. Furthermore, unequal recombination, at the *Rp1* cluster and at the *Cf4/9* cluster, has been shown to generate novel *R*-haplotypes with resistance specificities that differ from either parent. Interestingly, similar clustering phenomena are seen at (a)virulence loci in multiple, evolutionarily distinct pathogen genomes [23•, 24•, 25]. This accumulated evidence indicates that *R*-clusters facilitate rapid evolution via recombinatorial mispairings, generating novel *R*-gene sequences which may encode altered specificities or have altered expression patterns.

The role of *R*-clusters in *R*-gene evolution is often conceptualized in terms of a gene-for-gene model, e.g., sequence shuffling of *R*-genes may generate novel specificities of an *R*-gene receptor for the pathogen elicitor. However, the revisioning of *R*-proteins as constituents of multiprotein *R*-complexes has important implications for *R*-gene evolution, namely, that the evolution of specificity and resistance depends on multiple proteins in the *R*-complex: *R*-genes do not evolve in isolation. Hence, selective evolution

is expected to occur at other components of the *R*-complex that interact with the pathogen or stabilize the *R*-gene, and the formation of clusters may facilitate rapid evolution at these loci. This seems to be the case for the tomato *Pto*-*Prf* system, in which *Pto* was originally identified as an *R*-gene, though it encodes a serine/threonine kinase, rather than a stereotypical NBS-LRR *R*-gene [26]. Pto binds two unrelated *Pseudomonas syringae* pv *tomato* effectors, AvrPto and AvrPtoB, and depends on a stereotypical NBS-LRR protein, Prf, to signal resistance [27]. Hence, Pto may be better redefined as an effector target that is guarded by Prf, similar to the *Arabidopsis* proteins RIN4 and PBS1, which are virulently modified by *P. syringae* type III secreted effectors and guarded by *R*-proteins (modification of RIN4 is detected by RPM1 [28] and RPS2 [29], and modification of PBS1 is detected by RPS5 [30]; interestingly, PBS1, like Pto, is a serine/threonine kinase). Coimmunoprecipitation has confirmed that Pto and Prf interact and co-elute in a protein complex that appears to contain additional host proteins [31], providing evidence that Prf and Pto function in an *R*-complex. While the *Prf R*-gene is a single gene, *Pto* is located within a cluster of 5 kinase homologs, which, interestingly, contains *Prf* [32]. The *Pto* homologs seem to have experienced a complex history of plant-pathogen coevolution: one of the *Pto* cluster paralogs, *Fen*, has recently been shown to bind a mutated AvrPtoB, which has a disrupted C-terminal E3 ligase domain, and induce Prf-dependent HR [33••]. In contrast, wild-type AvrPtoB, carrying a functional E3 ligase domain, ubiquitinates Fen, resulting in Fen degradation accompanied by loss Fen-mediated resistance. AvrPtoB also has roles in suppressing basal defense [34], suggesting a multi-step evolutionary interplay: 1) Fen was an original virulence target of an AvrPtoB progenitor, and was "guarded" by Prf to trigger resistance; 2) The

AvrPtoB progenitor evolved E3 ligase function, suppressing effector-Fen interaction; 3) Evolution at *Pto*, the *Fen* paralogue, established recognition of AvrPtoB (and AvrPto) while avoiding E3 ligase targeting.

Pathogen effectors, including Avr2 (a cysteine protease targeting apoplastic Rcr3 required for *Cf2*-mediated resistance in tomato), AvrB (targeting RIN4 in *Arabidopsis*), AvrRpt2 (also targeting RIN4), and AvrPtoB, have been shown to interact with multiple host proteins in addition to the target that triggers resistance [35-37]. Amplification of effector targets in the host genome, as at the *Pto* cluster, may produce homologs that act as "decoys" [14••], retaining the ability to be targeted by the effector and mediate resistance via participation in *R*-complexes, but no longer encoding the function that allows the effector to subvert the host system. Hence, effector targets that participate in *R*-complexes may, in some cases, have been co-opted into purely recognitional roles.

Punctuated evolution in resistance systems

Comparative analysis of multiple haplotypes of the flax *N*, the lettuce *Dm3*, and the potato *MLB* (providing resistance against *Phytophthora infestans*) loci has revealed that the *R*-homologs at these clusters experience heterogeneous rates of evolution: distance tree analysis of sequences in these clusters shows that constituent homologs are grouped into clades, and that individual clades generally experience either "fast" or "slow" patterns of evolution, termed "type I" and "type II," respectively, by Kuang et al. [13] (Figure 1). For both types, sequence exchanges generally occur only between clade

members, with rare exceptions. However, paralogs in type I clades show high rates of sequence exchange and correspondingly may have high homology between paralogs and high haplotypic diversity (variable gene copy number, disruption of synteny), whereas paralogs in type II clades show infrequent sequence exchanges and retain orthologous relationships (higher homology between orthologs than between paralogs) and synteny. Physical mapping of the structurally complex *R1* and *MLB* late blight resistance loci has further shown that, while genes from different clades may be interspersed, regions of conserved synteny are physically distinct from regions that undergo high rates of sequence shuffling. Thus, it appears that the sequence exchanges characteristic of *R*-clusters may be confined to specific, highly variable regions, while other regions show a more "normal" pattern of evolution with few sequence exchanges and conservation of synteny.

The mechanism underlying differentiation of conserved and variable regions within *R*-clusters has not been established. One contributing factor may be stochastic sequence changes [16], including mutations and transposon insertions, accruing to restrict mispairing between paralogs, leading to distinct lineages that may exchange sequence with clade members, but not with dissimilar members from other clades. An alternative, though not necessarily mutually exclusive, force contributing to the evolution of *R*-clusters may be inhibition of recombination. Recently, a series of studies demonstrated that recombination rates (detected by a transgene carrying two GUS fragments that recombine to generate an intact reporter) are elevated following pathogen stress [38], that this elevated recombination is induced by a systemic signal that can be transmitted in the

absence of the pathogen [39], and that increased recombination persists in subsequent generations in the absence of stress [40•]. Recombination rates are also altered in RAD51D and SNI1 mutants that positively and negatively regulate, respectively, the induction of systemic resistance, indicating that DNA accessibility, via chromatin modifications, may provide a mechanism to conditionally regulate both defense gene transcription and recombination in response to pathogen stress [41••]. Recently, stress-related methylation was analyzed by Boyko et al. [42•], who demonstrated that TMV infection in susceptible tobacco plants resulted in an approximately 6 to 8 fold increase in restructuring events detected in homologs of the LRR region of *N* homologues in the progeny of infected, versus non-infected, plants, whereas increased instability was not detected at other loci. This pathogen-induced increase in instability was correlated with a global increase in methylation, but a decrease in methylation specifically at *R*-homologs in the progeny of infected plants. Hence, chromatin modifications may repress sequence exchange at *R*-clusters in the absence of virulent pathogens, and variable patterns of methylation within *R*-clusters may contribute to heterogeneous rates of evolution.

The heritable "systemic recombination signal" (SRS) is reminiscent of patterns of systemic silencing mediated by small RNA pathways [43,44], and endogenous small RNA pathways regulate the establishment and inheritance of DNA and chromatin modifications by targeting DNA and histone methylation to specific DNA sequences [45]. Transcribed tandem duplications can generate small RNAs that establish chromatin modifications [46,47], and insertion of transposable elements (TEs), which are widely observed in *R*-clusters [15], may also change local methylation patterns via TE-derived

small RNAs [48,49]. Furthermore, stress-conditioned hypomethylation of *R*-clusters is likely to activate TEs, in addition to permitting recombination, providing an additional mechanism of *R*-cluster restructuring. Indeed, numerous TEs have been shown to be activated in response to stress [50,51].

Evidence for stress-induced rearrangement of *R*-clusters may give new conceptual insight into *R*-gene evolution, raising the possibility for a mechanism of punctuated evolution of (at least some) *R*-clusters. In the absence of pathogen pressure, recombination and transposon activity at *R*-clusters is expected to be inhibited, presumably by chromatin modification, such that sequence exchanges and therefore sequence homogenization is limited, *R*-gene paralogs diversify by point mutations, and functional *R*-genes and haplotypes are conserved; this pattern of evolution is similar to that described by the *Birth and Death Model* [52]. Following biotic stress from a pathogen that escapes host defenses, alleviation of methylation will result in increased recombination that persists for multiple generations, facilitating restructuring events including haplotypic gene duplication/loss and the generation of chimeric genes through sequence exchanges. Absence of stress, through the evolution of functional resistance, or through ecological separation from the pathogen, will restore genome stability. Critically, differential patterns of methylation may allow sub-regions of *R*-clusters to undergo persistent recombination in the absence of pathogen stress, thus generating *R*-gene sequence reservoirs that may encode latent specificities for pathogens that the plant genome has not yet encountered.

Chromatin modifications, lethal recombinations, and the extent of homology within *R*-clusters may all affect observed recombination rates, complicating the analysis of stress-conditioned recombination. Large-scale methylation assays [53•, 54], which quantify methylation by combining microarray analysis with DNA methylation and chromatin modification enrichment techniques, provide an excellent tool to specifically correlate observed recombination with methylation status. Whole-genome methylation data is publicly available for *Arabidopsis* [55••, 56••, 57••], providing a tool for the genome-wide analysis of methylation at *R*-clusters. In crop plants and wild species, several large *R*-clusters have been partially or completely sequenced. Development of microarrays for methylation assay of these *R*-clusters would extend methylation studies from the *Arabidopsis* model, allowing analysis of larger clusters that may more closely resemble genomic patterns in natural populations, and enabling analysis of the roles, if any, that resistance signaling and small RNA pathways play in regulating patterns of cluster methylation following pathogen infection.

Artificial evolution of resistance

In natural populations, it seems that resistance is achieved in part by maintaining high levels of diversity at *R*-clusters and generating novel *R*-genes through rapid evolution, rather than by evolving any single gene that is particularly durable. Heavy crop breeding and artificial selection erase this genetic diversity. However, it may be possible to mimic natural evolution in a laboratory setting. The first successful *in vitro* artificial evolution of a beneficial *R*-gene was recently achieved by random PCR mutagenesis of the LRR

region of the potato *Rx* gene, expanding its range of specificity to provide resistance against additional PVX strains and also against the distant popular mosaic virus [58••]. Alternatively, *in vitro* DNA shuffling may provide a superior method for artificial evolution. Using this technique, Bernal et al. [59] fragmented four paralogs of the tomato *Pto* gene and reannealed the fragments to generate a library of *Pto* homolog chimeras, similar to chimeras produced by natural sequence exchanges, and retrieved 56 non-redundant combinatorial clones that interacted with AvrPto in Y2H. The study focused on dissecting *Pto* functional domains, rather than generating novel specificities; however, shuffling has previously been used to enhance protein performance [60], and has the advantage of recombining natural sequence polymorphisms which may constitute functional domains. Novel *R*-specificities have also been generated *in planta* in crosses between diverse *Rp1* haplotypes in maize [17] and Cf4/9 haplotypes in tomato [61]. Pathogen stress may increase the rate of recombination at *R*-clusters, potentially facilitating the evolution of novel specificities over multiple generations of exposure to a virulent pathogen. A conceptually similar approach in the *Pseudomonas syringae* pv. *phaseolica* pathogen involved recovering bacteria from leaves undergoing HR in resistant *Phaseolus vulgaris* L. bean and reinnoculating into uninfected plants [62•]. After passing through multiple plants under stress conditions, a new pathotype emerged which had undergone genome rearrangements leading to a loss of the avirulence gene recognized by the bean host. *In vitro* shuffling techniques and *in planta* maximization of diversity and recombination may provide plausible methods for generating novel *R*-genes against pathogens that have overcome the resistance specificities present in a given plant population.

Conclusion

R-complexes may be symptomatic of the complexity of plant-pathogen coevolution, in which surveillance systems have evolved to monitor virulent modifications of host components. Genome shuffling may produce subtle changes in both host and pathogen components of *R*-complexes, altering binding and conformational stability and leading to quantitative changes in resistance phenotypes, as well as qualitative, resistance/susceptibility phenotypes. Haplotypic diversity and gene loss/gain may also change the constituents and specificities of *R*-complexes. We expect that the genomic perspective will facilitate the identification and evolutionary analysis of other components of *R*-complexes, and we look forward to detailed analysis of genic and intergenic regions in *R*-component clusters, particularly to gain insight into structural features such as chromatin modifications that may affect, and even regulate, the pattern of cluster evolution.

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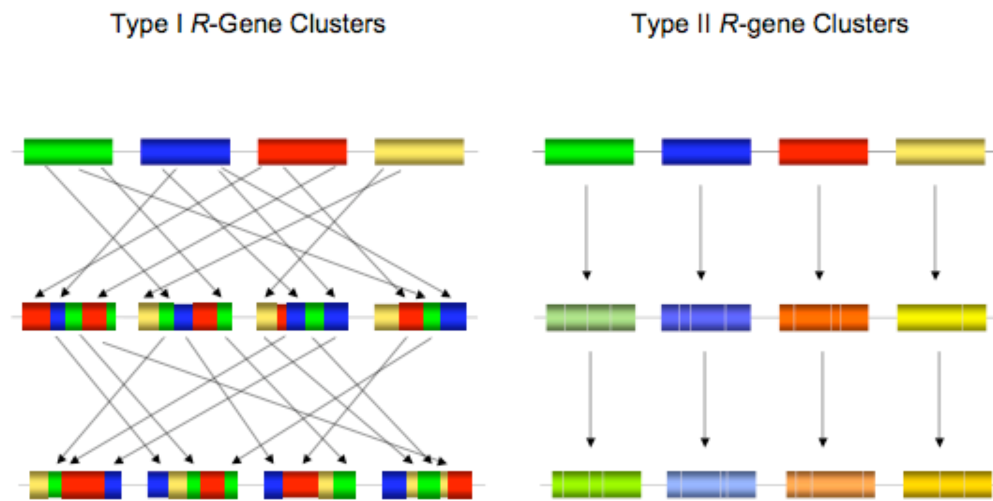

Figure. 1. “Fast evolving” Type I resistance genes are characterized by frequent sequence exchanges between paralogues that obscure orthologous relationships, whereas “slow evolving” Type II resistance genes rarely experience sequence exchanges between paralogues and maintain orthologous relationships. Black arrows represent changes accrued over evolutionary time. Point mutations are shown as vertical white lines.