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## Evolution of prokaryotic and eukaryotic virulence effectors

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Coevolutionary interactions between plants and their bacterial and eukaryotic pathogens are mediated by virulence effectors. These effectors face the daunting challenge of carrying out virulence functions, while also potentially exposing the pathogen to host defense systems. Very strong selective pressures are imposed by these competing roles, and the subsequent genetic changes leave their footprints in the extant allelic variation. This review examines the evolutionary processes that drive pathogen–host interactions as revealed by the genetic signatures left in virulence effectors, and speculate on the different pressures imposed on bacterial versus eukaryotic pathogens. We find numerous instances of positive selection for new allelic forms, and diversifying selection for genetic variability, which results in altered host–pathogen interactions. We also describe how the genetic structure of both bacterial and eukaryotic virulence effectors may contribute to their rapid generation and turnover.

### Addresses

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### Introduction

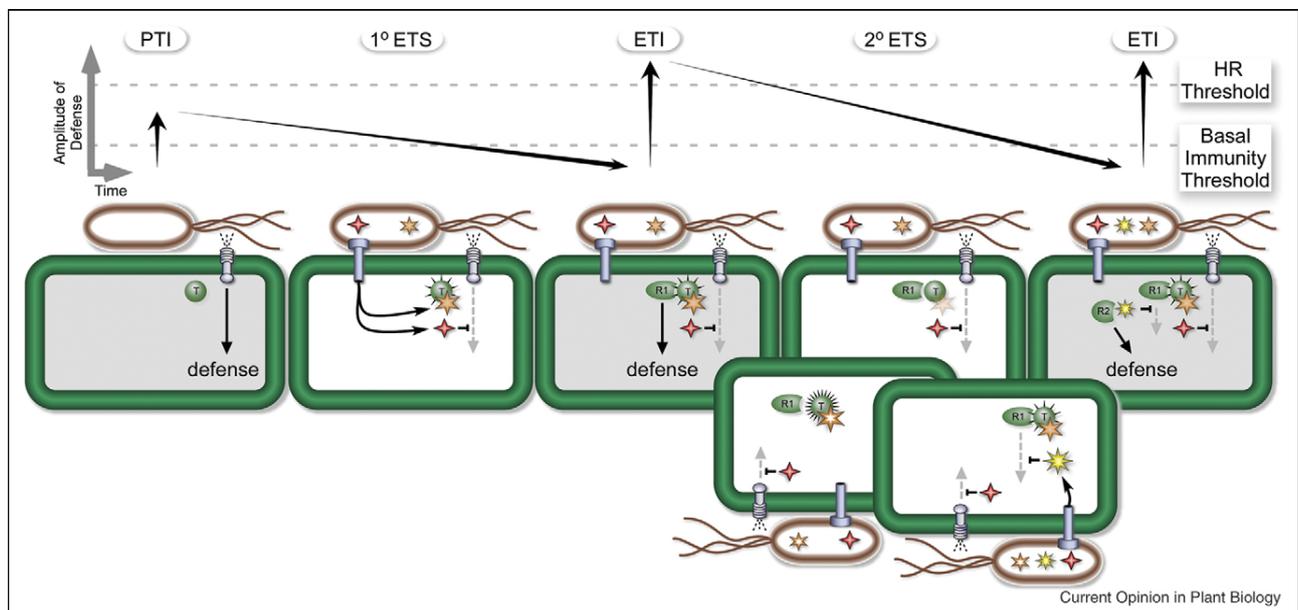
Pathogens and their hosts engage in a relentless and often deadly struggle. Each move made by one demands a commensurate move by the other, and a false move or slow response made by either one can literally mean death or dramatic loss of fitness. These interactions are mediated by diverse suites of virulence factors on the pathogen side, and surveillance and defense systems on the host side. Although martial analogies quite nicely describe host surveillance and defense systems, which can act both as weapons and targets, the analogy does not fit as well for pathogens. Virulence-associated effectors are certainly the primary weapons of pathogens, but they do not so much act as targets as tells. A tell is an action or object that betrays your intentions to your opponent.

Pathogens face the daunting challenge of invading hosts using virulence factors that are not only required for the suppression of the host immune system, but which can also directly inform the immune system of the presence of the pathogen. In this review, we will examine how the competing pressures of acting as weapons and tells drive the evolution of pathogen effectors.

Although there is tremendous diversity in the nature and modes of action of virulence factors produced by bacterial and eukaryotic pathogens, recent studies are beginning to reveal that from an evolutionary perspective, and at a very gross level, these factors interact with the host immune system in a characteristic manner. These patterns have been very nicely summarized and formalized in the Jones and Dangl ‘zigzag’ model (Figure 1) [1•], which identifies specific stages of these interactions. Host–pathogen interactions are generally believed to start with the recognition of pathogen-associated molecular patterns (PAMPs, or more accurately microbe-associated molecular patterns, MAMPs), which are conserved, and therefore putatively, functionally constrained epitopes presented by pathogens and recognized by host–pathogen recognition receptors (PRRs) [2–4]. Well-known PAMPs include components of the bacterial flagella (the flag22 epitope of flagellin), harpins, cold shock proteins, lipopolysaccharides, peptidoglycans, and the elongation factor Tu (EF-Tu) [5–7]. The perception of PAMPs by PRRs triggers a basal defense response called *PAMP-triggered immunity* (PTI), which involves the induction of MAPK signaling cascades, calcium flux, production of nitric oxide and reactive oxygen species, and the activation of stress-specific WRKY transcription factors [7–9]. PTI-mediated basal immunity effectively restricts the growth of the vast majority of potential pathogens, and is probably largely responsible for the general health of most plants.

Pathogens may respond to PTI through the action of effector proteins. Bacterial effectors secreted through the type III secretion system (T3SS) provide us with the best examples of pathogen proteins evolved to actively suppress basal defense. The T3SS is one of the most vital virulence systems used by Gram-negative bacterial plant pathogens such as *Pseudomonas syringae*, *Ralstonia solanacearum*, *Xanthomonas*, *Pantoea*, and *Erwinia* species. This system enables pathogens to inject type III secreted effector proteins (T3SEs) directly into the cytoplasm of their hosts. Although the specific virulence function of most T3SEs has yet to be determined, it appears that many, if not most of them, act to suppress or disrupt host defense signal transduction, thereby inhibiting an effective defense response. Jones and

Figure 1



Generalized schema of host-pathogen coevolution. The top section illustrates the relative amplitude of the host defense response for each of the stage in the interaction as described in the Jones–Dangl zigzag model [1\*]: PAMP-triggered immunity (PTI), effector-triggered susceptibility (ETS), and effector-triggered immunity (ETI). The upper and lower dashed lines along the top indicates when the amplitude of response is sufficient to elicit an HR or basal defense response, respectively. Cartoons of plant cells and attacking bacterial pathogens are presented along the bottom. PTI is induced by the perception of PAMPs (indicated by the dashed lines coming off the bacterial flagella) by plant membrane bound receptors, ultimately resulting in a basal defense response. In 1° ETS, bacterial pathogens respond by secreting and translocating T3SEs into the plant cell to disrupt basal defense signaling pathways and/or target host virulence targets (T). In this case, the red, four-pointed T3SE is blocking basal defense signaling, while the orange, six-pointed T3SE is causing a modification to the virulence target T. ETI is a countering strategy in which plants respond to T3SEs through the action of resistance (R) proteins that either directly recognize T3SEs or monitor virulence targets for characteristic modifications caused by T3SEs. In this case the R1 resistance protein has detected a pathogen-associated modification to the virulence target T, and has induced an R protein mediated defense response. In 2° ETS, pathogens can respond to ETI in at least three distinct ways. They may either lose the T3SE that induced ETI, as shown by the faded six-pointed T3SE or modify the T3SE so that its action no longer is recognized by the R1 protein, which is illustrated here by the re-coloring of the six-pointed T3SE and the new modification to the virulence target. This can be achieved either through the mutational process or through the acquisition of a homolog via horizontal gene transfer. The third possibility is that another T3SE may actively suppress R1-induced defense signaling, as shown by the yellow, eight-pointed T3SE. Finally, plants may respond to these challenges and restore ETI through the action of additional R proteins. In this example, the R2 resistance protein triggers a defense response after directly recognizing the eight-pointed T3SE.

Dangl dub this suppression *effector-triggered susceptibility* (ETS). We further make the distinction here of 1° ETS, in which T3SEs specifically suppress basal defense signaling, as has been seen for the *P. syringae* T3SEs AvrRpm1, AvrRpt2, HopM, and AvrE to name just a few [4,10–12]. If successful, ETS swings the interaction back in favor of the pathogen and results in what we would perceive as a disease state.

Eukaryotic filamentous pathogens, such as fungi and oomycetes, also secrete a battery of effector proteins during the infection of plant hosts. Some of these secreted proteins can be taken up by a yet undefined host-encoded system [13] and translocated into the plant cytoplasm. Once inside the host cell, eukaryotic effectors function analogously to bacterial T3SEs, in that they are key virulence determinants that come into intimate contact

with host substrates and enable pathogen colonization by suppressing host immunity.

Although a growing list of T3SEs have been shown to suppress PTI, some bacterial T3SEs and eukaryotic effectors can also act as unintentional signals to the host, in other words, immune tells. The targeting and modification of host proteins by pathogen effectors, or the effectors themselves, can be perceived by host resistance (R) proteins that act as cellular sentries. These R proteins monitor the state of vulnerable host proteins, or for the presence of foreign proteins. The activation of plant R proteins upon recognition of pathogen effectors or modification of a host virulence target induces *effector-triggered immunity* (ETI), which is a secondary defense strategy characterized by the strengthening of the cell wall via callose deposition, the expression of pathogenesis-related

proteins, the release of oxidative radicals, and most dramatically, a programmed cell death response called the hypersensitive response (HR), which may entirely eradicate the pathogen from the host tissue [14,15]. A remarkable twist on these interactions was recently uncovered in the *Xanthomonas campestris* T3SE AvrBs3, which acts as a transcription factor for a plant cell size regulatory protein [16]. Plants have responded to this challenge by evolving an R gene that is also transcriptionally activated by AvrBs3, providing yet another route for plants to monitor pathogen effectors. ETI once again changes the outcome of the interaction from disease to resistance.

As is the case for PTI, pathogens are under strong selective pressure to avoid or suppress ETI signaling and move the interaction back in favor of the pathogen, bringing about 2° ETS. To do this, pathogens need to eliminate the signal(s) perceived by hosts — in effect, suppressing the tells. Assuming that a T3SE is directly or indirectly acting as an immune tell, 2° ETS can be achieved in a number of ways. The T3SE could be lost if its function is dispensable or redundant to that of another T3SE. The T3SE could be modified by the mutational process so that its structure or action is no longer detected by the surveilling R protein. Similarly, the T3SE could be replaced with a closely related or functionally equivalent homolog brought in through horizontal gene transfer. Finally, another T3SE may be acquired that suppresses the R protein signaling pathway leading to defense response. As discussed below, examples of the first three responses have all been observed in the HopZ family of *P. syringae* T3SEs [17•], while there are many examples of T3SEs that suppress the HR-associated defense response. For example, while the *P. syringae* T3SE AvrRpt2 has been shown to suppress PTI, it also can suppress ETI induced by the indirect interaction between the T3SE AvrRpm1 and its cognate R protein RPM1 via modification of the host protein RIN4 [18]. The *P. syringae* T3SE AvrPtoB requires its E3 ubiquitin ligase activity for HR-associated defense suppression [19]. Jamir *et al.* [20] screened 19 T3SEs and identified 7 that could completely or partially suppress HopA1 (formally HopPsyA) mediated HR.

The zigzag model nicely illustrates the dynamic nature of host–pathogen interactions. The winner in this arms race is determined by the speed and effectiveness of either pathogen perception via PTI or ETI, or defense suppression via ETS. Changes in the complement of pathogen T3SEs or host defense related proteins can dramatically alter these dynamics; therefore, there is intense selective pressure for these changes. We are just beginning to acquire data that permit us to reconstruct some of these dynamic evolutionary processes. As more comparative data become available, we have greater power to resolve the footprints left by the evolutionary processes driving these interactions.

### Evolution of bacterial effectors

The direct interaction of pathogen T3SEs with host substrates, especially the components of the host defense systems, subjects them to intense selective pressures. Examples are emerging, illustrating the importance of both vertical (descent with modification) and horizontal (recombination) evolutionary processes in response to these pressures. The distinction between these two can sometime be muddled, and in fact horizontal processes do not act independently of vertical processes; nevertheless, the relative balance between vertical and horizontal forces has a large impact on the source and nature of genetic variants molded by selection.

Two studies have looked at the general patterns of descent and selection among multiple T3SE families within a bacterial species. Twenty-four *P. syringae* T3SE families were examined using nucleotide composition measures and phylogenetic approaches. Eleven of them were demonstrated to be recently acquired by horizontal transfer [21], while the remaining 13 appear to have been maintained since the early diversification of the species complex. Of the horizontally acquired T3SE families, four exhibit signatures of positive selection (where one beneficial allele selectively increases in frequency within a population at the expense of other allelic variants). Most of the T3SEs that are ancestral to *P. syringae* are genetically linked to the cluster of genes that encode the T3SS, which has been present in this species since its early diversification [22]. In addition, most of these ancestral families show polymorphism patterns consistent with purifying (negative) selection. These conclusions were supported by a microarray-based comparative genomic hybridization study of 53 T3SEs in 91 *P. syringae* strains. Approximately 66% of these T3SEs were present in at least 75% of strains, and nearly 20% were present in over 95% of strains [23], which is consistent with vertical transmission and the occasional loss of an effector in selected lineages.

A study by Pitman *et al.* [24••] revealed the rapidity in which pathogen T3SEs can evolve in response to pressures imposed by host defense. The bean pathogen *P. syringae* pv. phaseolicola 1302A carries the T3SE *hopAR1* (formerly *avrPphB*), which is recognized by the R protein, R3, resulting in a HR-associated defense response. Following infiltration and five serial passages of the bacteria in the resistant bean plants, water-soaked lesions developed, indicating a shift to a disease state rather than the previously seen defense response. Molecular analysis of the derived strain revealed that a ~106 kb genetic island had excised from the genome during passage. This island contains 100 predicted open reading frames, including the *hopAR1* T3SE, and the excision is mediated by the XerC integrase, which is located just inside the island. The expression of *xerC* is upregulated by 50-fold *in planta*, which may indicate that there is selective pressure for genomic flexibility during the infection process.

The *P. syringae* T3SE HopX (formerly AvrPphE) provides a compelling example of the importance of natural genetic variation in host–pathogen interactions [25]. Seven allelic variants of this T3SE were identified from various *P. syringae* pv. phaseolicola strains. Although the so-called wild-type allele of HopX induces an HR in bean plants carrying the R2 resistance protein, all of the alleles carrying nonsynonymous mutations (or a small insertion in one case) resulted in a loss of avirulence, thereby shifting the interaction from incompatible (defense) to compatible (disease).

Natural genetic variation generated by the action of transposable elements or related sequences also influences the outcome of host–pathogen interactions. For example, disruptions of the *X. campestris* pv. vesicatoria AvrBs1 T3SE [26], as well as the *R. solanacearum* T3SEs PopP [27] and AvrA [28] also result in a shift from incompatible to compatible states during the interaction with their plant hosts.

The human introduction of the R gene, Bs2, into commercially cultivated pepper and tomato varieties has resulted in the emergence and spread of new alleles of the corresponding *Xanthomonas axonopodis* pv. vesicatoria T3SE AvrBs2 [29]. Before the introduction of Bs2 into commercially cultivated varieties, all *X. axonopodis* pv. vesicatoria strains recovered from field samples carried wild-type (i.e. functional) *avrBs2*. The widespread use of the resistant pepper varieties appears to have selected for the emergence of new alleles of *avrBs2*, which now dominate the population. These new alleles are largely generated through the insertion and deletion of a 5bp repeat, as well as some SNPs. The spread of these new alleles have resulted in a selective sweep that reduced the overall genetic diversity in these populations [29].

A recent study of the HopZ family of *P. syringae* T3SEs reveals how both vertical and horizontal gene transfer shape the evolution of a single T3SE family, and how these changes can shift the balance of power from host to pathogen [17\*]. The members of the widely distributed HopZ T3SE family belong to the YopJ family of cysteine proteases/acetyltransferases found in both plant and animal pathogens [30,31]. The HopZ family comprises three major homology groups (named HopZ1, Z2, and Z3) that are widely distributed within *P. syringae*. A phylogenetic analysis of the HopZ1 homolog suggests that it is an ancestral T3SE in *P. syringae*, evolving at least three functional allelic classes (HopZ1a–c) and a number of degenerate forms through pathoadaptation. The HopZ2 and HopZ3 homologs, on the other hand, appear to have been recruited by *P. syringae* via horizontal transfer from other plant pathogenic bacteria. The introduction of the HopZ1 allele that is most similar to the ancestral allele into strains harboring alternate or degenerate alleles results in an R protein mediated defense response in

their respective hosts, which is not observed with the endogenous allele. All of the homologs seem to retain similar biochemical function, though they produce very different patterns of recognition in different hosts. Therefore, recognition of the ancestral HopZ T3SE by the host defense system may have selected for an allele not recognized by the host, or the replacement of the native T3SE with a functionally equivalent T3SE through horizontal transfer. In the case of the HopZ family, evolution by gene loss, pathoadaptation, and horizontal gene transfer all appear to have significantly influenced the interactions between the pathogens and their hosts, although the mechanism by which HopZ promotes virulence has yet to be elucidated.

### Evolution of eukaryotic effectors

Positive selection has been observed in many eukaryotic effectors [32\*\*,33\*\*,34–36]. A sophisticated genomic analysis of three oomycete plant pathogens, *Phytophthora sojae*, *Phytophthora ramorum*, and *Hyaloperonospora parasitica* by Win *et al.* [37\*] revealed diverse and largely unique collections of effectors with the conserved RXLR translocation signal. Genetic patterns consistent with positive selection were detected in 70 out of 99 effector families, with the strongest signals of selection observed in the C-terminal domain. The divergence of these effector repertoires and the presence of degenerated effector genes indicate that RXLR effectors have undergone a relatively rapid birth and death evolution, which likely strongly influences their ability to evolve new host specificity or retain virulence to the existing hosts [37\*]. Genes encoding these effectors were also found to be clustered in the genome. For example, 30 out of 72 families are clustered in regions of under 100 kb in length. Similar clustering has also been observed in the maize smut pathogen *Ustilago maydis*, where ~20% of the predicted secreted proteins are arranged in 12 clusters [38]. Interestingly, plant R genes are frequently found to occur in clusters as well [39]. Clustering of both host R genes as well as pathogen effectors may reflect the importance of recombination in giving rise to novel variants in these two rapidly evolving classes of genes [37\*].

Selection has also been observed in four *avr* genes (*Avr2*, *Avr4*, *Avr4E*, and *Avr9*) from *Cladosporium fulvum*, the causal agent of tomato leaf mold [36]. In compatible interactions, *Avr4* binds to chitin and protects the pathogen from plant chitinases [40]. However, these effectors trigger an HR in tomato plants that carry the cognate R proteins, Cf-2, Cf-4, Cf-4E, and Cf-9, respectively. An analysis of between 67 and 81 alleles of these four effectors revealed an average of ~0.3% nonsynonymous variation (based on the number of segregating sites), and absolutely no synonymous genetic variation [36]. Although not explicitly calculated in the publication, a comparison can be made between two measures of genetic variation; *theta*, which is based on the number

of segregating sites, and  $\pi$ , which is the pairwise nucleotide diversity. In all four cases it appears that  $\theta$  is either substantially larger, or of the same magnitude as  $\pi$ , indicating that these effectors have more than likely experienced positive selection, as opposed to diversifying selection (where multiple alleles are maintained in a population because of selection for diversity). Finally, the authors found substantial reticulations in a phylogenetic analysis of these effectors [36]. These genealogical incongruencies are indicative of recombination, and support an important role for genetic exchange in introducing and reassorting the genetic variation segregating in these populations.

In an escalatory coevolutionary arms race, if the diversification of effector genes is an adaptive response driven by host defense-imposed selective pressure, the reciprocal selection should act on the corresponding plant *R* genes and lead to diversification of the *R* genes. Eukaryotic effectors provide clear examples where the diversification of the pathogen effectors is matched by the diversification of the host *R* genes. For example, high levels of amino acid polymorphism were identified at both the *ATR13* locus in *H. parasitica* and its corresponding *R* gene *RPP13* in *Arabidopsis thaliana*. *RPP13* alleles differ in their resistance to various pathogen isolates, indicating that the *ATR13* and *RPP13* polymorphism are probably maintained through diversifying selection driven by the antagonistic interplay between *A. thaliana* and *H. parasitica* [32<sup>••</sup>,41].

The best example of parallel molecular diversification in *R* and *Avr* proteins driven by the coevolutionary arms race is probably from the flax–flax rust pathosystem. In the flax rust, *Melampsora lini*, diversifying selection has resulted in 12 allelic variants in the effector gene *AvrL567* in 6 strains. Five of these alleles are able to avoid the recognition from the cognate *R* proteins, L5, L6, and L7 in flax [33<sup>••</sup>]. Yeast two-hybrid experiments elegantly demonstrated that accumulated amino acid changes in *AvrL567* alleles directly affect protein–protein interactions between the effector and its corresponding *R* proteins, suggesting that *AvrL567* sequence diversity, driven by the *R* gene-imposed selective pressures, is associated with different recognition specificity. Interestingly, all five of these alleles maintain a conserved protein structure and stability, indicating the retention of the yet unknown virulence function of these alleles [33<sup>••</sup>].

### Different evolutionary strategies for bacterial and eukaryotic pathogens

An interesting dichotomy emerges when comparing effectors from bacteria and eukaryotes. The majority of eukaryotic effectors are directly recognized by *R* proteins, and diversifying selection seems reasonably common. By contrast, the majority of bacterial effectors are indirectly

recognized by *R* proteins, and positive selection is more common. In the former case the effector itself is recognized, while in the latter case the effector function is recognized. For example, both *P. syringae* T3SEs *AvrRpt2* and *AvrRpm1* target and modify the *A. thaliana* protein *RIN4* [42,43]. These effectors are indirectly recognized by the *R* proteins *RPS2* and *RPM1*, respectively, if either *R* protein detects a yet to be characterized modification to *RIN4*.

Unlike bacterial effectors, indirect recognition is relatively rare in eukaryotic effector–*R* protein interactions. One example of such recognition is *Avr2* of *C. fulvum*, which is indirectly recognized by its cognate *R* gene, *Cf-2* in tomato. *Cf-2* perceives the binding and inhibition of a tomato cysteine protease *Rcr3* by *Avr2* and triggers defense responses [44,45]. Almost all polymorphisms identified in *Avr2* involve insertions/deletions or frameshift mutations, which generally result in truncated nonfunctional alleles [36,46]. It is worth noting that these nonsense mutations have the potential to revert once the selection pressure has changed, and therefore, degenerate alleles may act as a genetic reservoir for the future.

It is interesting to speculate that plants may have different strategies for dealing with bacterial versus eukaryotic pathogens. Bacterial pathogens have the potential to evolutionarily outpace their plant hosts because of their short generation times and ready access to genetic variation via horizontal gene transfer. Plants may have adapted to this challenge by monitoring the state of potential pathogen targets rather than by directly tracking the pathogen effectors themselves. This recognition of ‘pathogen-induced modified self’ [1<sup>•</sup>] dramatically simplifies the manner in which host surveillance systems operate. Instead of having to track every evolutionary shift made by pathogen, hosts can simply monitor their own systems, and activate defenses once an alarm is triggered. In response, bacterial pathogens could rapidly turnover effectors once their effectiveness is compromised, which is consistent with the highly variable repertoires of T3SEs observed [23,47].

Eukaryotic pathogens, on the other hand, are essentially playing by the same evolutionary rules as their plant hosts, as they do not have dramatically higher rates of evolution or horizontal gene transfer [48]. Plant *R* proteins may therefore be more capable of keeping pace with effectors from eukaryotic pathogens, and may take the direct approach of physically interacting with the effector. If an effector required for the disease process is recognized by plant *R* protein, eukaryotic pathogens may have little choice but to respond by evolving a diversity of allelic variants through mutational changes, and selecting for those that are able to escape recognition while still retaining their required virulence functions.

These arguments lead to the prediction that the evolution of bacterial effectors will largely be driven by positive selection, or by the selection for the relatively rapid turnover of T3SE through gene loss and/or acquisition via horizontal gene transfer. In contrast, the evolution of eukaryotic effectors will largely be driven by diversifying selection, in other words, the generation and maintenance of multiple alleles. We might also expect stronger or more prevalent diversifying selection in *R* proteins that recognize eukaryotic effectors than those detecting bacterial effectors.

### The origin of pathogen effectors

Effectors of both bacterial and eukaryotic pathogens are typical modular, multidomain proteins [19,49–56]. Both bacterial T3SEs and oomycete RXLR effectors have an upstream regulatory domain, an N-terminal secretion domain, and one or more C-terminal functional domains. A recent study examining T3SE diversity showed that many T3SEs can trace their origins to a shuffling process called terminal reassortment, in which N-termini or C-termini of T3SEs, which often contain important functional modules, are reassorted in a manner analogous to exon shuffling [57<sup>\*\*</sup>]. This process most commonly involves the fusion of the promoter and 5' portion of a T3SE, which encodes all of the regulatory domains required for type III dependent expression, secretion, and translocation, to a new genomic region. If this fusion produces a novel effector that confers a selective advantage to the pathogen, it may be retained. If the fusion does not confer an advantage, it will probably be rapidly lost. This process, which can occur through either the movement of modules via nonhomologous recombination or the joining of modules via deletion, appears to be an important means of generating novel effectors with new virulence functions or conferring new host specificities. An analysis of the T3SEs from 23 pathogenic and mutualistic bacterial species revealed that on average 24%, and as many as 32% of all T3SE families contain an effector that is a chimera of either two known T3SEs, or a T3SE and another gene, or have an apparently truncated form composed of just an effector N terminus — a so-called orphaned effector terminus (ORPHET). The number of chimeric effectors and ORPHETs in a species is significantly correlated with the number of T3SEs carried by that species. Furthermore, the proportion of chimeras and truncations found among T3SEs is significantly greater than that seen among non-T3SEs, with an average of 24% for T3SEs and 7% for non-T3SEs. An example of terminal reassortment includes the *P. syringae* HopW T3SE family. The N terminus of three *P. syringae* HopW1 T3SE alleles (one from Pph1448A and two from PmaES4326) and the HopAE1<sub>P<sub>sy</sub>B728a</sub> T3SE are highly similar through the region that includes the T3SS secretion and translocation signal. The similarity among these sequences also

includes the upstream region encompassing the ribosome-binding site for all four alleles, and hrp box promoter for HopW1-2<sub>P<sub>ph</sub>1448A</sub>, HopW1-2<sub>P<sub>ma</sub>ES4326</sub>, and HopAE1<sub>P<sub>sy</sub>B728a</sub>. Interestingly, the hrp box promoter and flanking region of HopW1-1<sub>P<sub>ma</sub>ES4326</sub> is very similar to that of the unrelated T3SE HopD1<sub>P<sub>to</sub>DC3000</sub>, although these two proteins share no other similarity. HopW1-2<sub>P<sub>ph</sub>1448A</sub> and HopW1-2<sub>P<sub>ma</sub>ES4326</sub> are both very short proteins (ORPHETs of 94aa), while HopW1-1<sub>P<sub>ma</sub>ES4326</sub> and HopAE1<sub>P<sub>sy</sub>B728a</sub> are full-length proteins (774aa and 914aa, respectively) that share absolutely no similarity beyond the N terminus.

Terminal reassortment can also influence bacterial virulence and host specificity. For example, the *P. syringae* T3SEs HopD1<sub>P<sub>to</sub>DC3000</sub> and HopAO1<sub>P<sub>to</sub>DC3000</sub> are N-terminal chimeras with very different C termini. The C terminus of HopAO1 has tyrosine phosphatase activity, which is responsible for the suppression of the innate immune response of *Nicotiana benthamiana* [58,59]. This region is not found in HopD1, and no tyrosine phosphatase activity has been ascribed to this effector; therefore, terminal reassortment may have given rise to T3SEs that may act in a host-specific manner.

Terminal reassortment is probably of greater importance to the origin of T3SEs because of their inherent modularity, their crucial and central role in host–pathogen interactions, and their frequent association with mobile elements and bacteriophage, which may facilitate their movement. Given the structural analogy between oomycete RXLR effectors and bacterial T3SEs it will be interesting to see whether a similar process also plays a role in the generation of eukaryotic effectors.

### Conclusion

Bacterial and eukaryotic effectors provide fascinating insights into how dynamic evolutionary processes directly influence every aspect in pathogen–host interactions — from ecological interactions to molecular mechanisms. As we move beyond the mindset of type sequences and model strains to a more sophisticated understanding of the extent, role, and significance of natural variation, we begin to appreciate how genetic diversity reflects and responds to the intense selective pressures imposed by coevolutionary arms race between host and pathogens. From a mechanistic perspective, an appreciation of this diversity provides immediate insight into molecular basis underlying virulence functions and recognition specificity. Evolution has already done the hard work of identifying residues and domains of proteins that are functionally crucial or vulnerable. With the appropriate sampling of natural diversity and analytical tools, we can trace the genetic signatures left by these evolutionary processes, and use these to guide our experimental design, thereby achieving a more complete understanding of host–pathogen interactions.

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