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Phenotypic diversification by gene silencing in *Phytophthora* plant pathogens

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Advances in genome sequencing technologies have enabled generation of unprecedented information on genome content and organization. Eukaryote genomes in particular may contain large populations of transposable elements (TEs) and other repeated sequences. Active TEs can result in insertional mutations, altered transcription levels and ectopic recombination of DNA. The genome of the oomycete plant pathogen, *Phytophthora infestans*, contains vast numbers of TE sequences. There are also hundreds of predicted disease-promoting effector proteins, predominantly located in TE-rich genomic regions. Expansion of effector gene families is also a genomic signature of related oomycetes such as *P. sojae*. Deep sequencing of small RNAs (sRNAs) from *P. infestans* has identified sRNAs derived from all families of transposons, highlighting the importance of RNA silencing for maintaining these genomic invaders in an inactive form. Small RNAs were also identified from specific effector encoding genes, possibly leading to RNA silencing of these genes and variation in pathogenicity and virulence toward plant resistance genes. Similar findings have also recently been made for the distantly related species, *P. sojae*. Small RNA "hotspots" originating from arrays of amplified gene sequences, or from genes displaying overlapping antisense transcription, were also identified in *P. infestans*. These findings suggest a major role for RNA silencing processes in the adaptability and diversification of these economically important plant pathogens. Here we review the latest progress and understanding of gene silencing in oomycetes with emphasis on transposable elements and sRNA-associated events.

Introduction

Oomycetes attacking plants. Numerous organisms have evolved the ability to invade and colonize plant tissue. Plant pathogens among the filamentous fungi and the fungus-like oomycetes can cause plant diseases that result in huge crop losses on a global scale, and threaten food security for millions of people. Over the past decade, the genomes of many of these pathogenic organisms have been sequenced, revealing unexpected diversity in genome sizes and overall genome organization. This new genomic information has also shed light on mechanisms influencing gene regulation, some of which are conserved in diverse organisms.

Oomycetes only superficially resemble fungi, in that they can form hyphae, but are classified as Stramenopiles, along with the brown algae and diatoms.[1] The genomes of at least 8 plant pathogenic oomycete species have now been sequenced.[2] The most notorious of these pathogens are *Phytophthora infestans*, which causes potato late blight and precipitated the Irish potato famine in the 1840s, and *P. sojae* that causes stem and root rot of soybean. The genomes of *P. infestans* (240 Mb) and *P. sojae* (95 Mb) are large compared with most fungal genomes known today.[3] These 2 genomes have experienced gene gain, gene family expansions and repeat-driven enlargements resulting in an overall genome structure of gene rich regions and gene sparse regions.[3,4] The gene rich regions are densely packed with genes that are often only separated by a few hundred base pairs, or may overlap in some instances. In contrast, the gene sparse regions are littered with transposable elements (TEs) and other repetitive sequences.[3]

Plant pathogens, including oomycetes, deploy a set of secreted proteins called effectors to suppress plant innate immune responses that result from recognition of conserved pathogen associated molecular patterns (PAMPs).[5] Thus, the ability of effectors to suppress plant defenses, alter host cells or gene function, can directly impact pathogen fitness. Effectors can contribute to pathogen fitness in a quantitative manner, such as for the NIP effectors from the barley pathogen *Rhynchosporium commune*.[6] Alternatively, individual effectors may be essential for full pathogenicity, with their deletion or silencing resulting in drastically reduced pathogenicity, such as for the Pep1 effector from *Ustilago maydis*.[7]

In a co-evolutionary scenario, plants have counteracted pathogen attack and evolved resistance (R) proteins to recognize specific pathogen effectors, and mount effective defense responses. Recognized pathogen effectors are termed avirulence (Avr) proteins. Pathogen genotypes that avoid recognition and are virulent on *R* gene-expressing plants may exploit variation in *Avr* gene sequences, gene copy number, or transcriptional inactivation.[8,9] It should be noted that *P. infestans* and *P. sojae* Avr proteins are named according to their cognate R proteins in potato and soybean, respectively. As such, some avirulence effectors have the same name, but are not orthologs. For example, PiAvr3a (recognized by the potato resistance R3a) is essential for pathogenicity

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in *P. infestans*,[10,11] but PsAvr3a in *P. sojae* (recognized by the soybean resistance Rps3a) is dispensable.[12] PiAvr3a and PsAvr3a do not share significant sequence similarity.

The set of effectors utilized by *Phytophthora* species comprises a diverse collection of predicted secreted proteins. Some of these are presumed to be delivered to the apoplast i.e., the region between the plant cells where, for example, they act to block the action of secreted plant proteases and glucanases.[13] Evidence indicates that other effectors are translocated into host cells in order to target plant proteins and promote disease.[14,15] The latter group contains the extensively studied RxLR and Crinkler (CRN) classes, defined by their conserved translocation motifs.

In *Phytophthora* genomes, the genes encoding RxLR and CRN effectors and other candidate pathogenicity factors are predominantly located in the gene sparse, TE-dense regions. These are considered to be rapidly evolving compared with the gene dense regions, giving rise to a "2-speed genome,"[3] and to underlie the ability of *Phytophthora* species to rapidly evolve and overcome host resistance.[16,17] The complement of predicted secreted effectors found in these gene sparse regions has been termed the "plastic secretome".[18] It is thus likely that transposable elements may impact on evolution and expression of effectors in *Phytophthora*, and that this may also impact on pathogenic fitness.

Gene silencing in oomycetes. Gene silencing (also called RNA interference [RNAi], RNA silencing, quelling, co-suppression) is a master regulatory mechanism with diverse roles such as control of gene expression at transcriptional and post-transcriptional levels, and chromatin organization. Typically, silencing is initiated by double stranded RNA (dsRNA) molecules that are digested by the type III RNase called Dicer (DCR or DCL) into short 21 bp duplexes known as short interfering RNAs (siRNAs). The duplex is unwound and the antisense strand incorporated into an Argonaute (AGO) protein, which then binds to homologous mRNA and degrades it through the action of its PIWI (slicer) domain. These DCR and AGO enzymes form the core of RNA silencing pathways. Additional proteins are often associated with RNA silencing, such as RNA dependent RNA polymerases (RdR) and RNA helicases. RNA silencing may also connect to transcriptional silencing through cytosine methylation or histone modifications, involving proteins such as cytosine methyltransferase, histone methyltransferase, histone deacetylase, and chromodomain proteins.[19] Central players in RNA silencing are small RNAs (sRNAs), often ranging in size from 19–40 nt, and divided into different classes with diverse roles. RNA silencing processes and the different sRNA classes involved have been extensively studied in model species such as *Arabidopsis thaliana, Drosophila melanogaster, Caenorhabditis elegans* and *Schizosaccharomyces pombe*.[20,21] One of the key cellular roles of RNA silencing is to restrict the activity of transposable elements, thus maintaining genome stability.[21,22]

In oomycetes, a phenomenon termed internuclear gene silencing was discovered over a decade ago in *P. infestans*.[23] It was experimentally shown that transcriptional silencing of transgenic and endogenous copies of the *inf1* elicitin gene could be transmitted to a co-cultivated non-transgenic strain. Further, the effect was persistent even in non-transgenic homokaryotic progeny, despite the absence of an *inf1* transgene. Since then, the involvement of an unknown diffusible molecule that transmits internuclear gene silencing from transgenic *inf1*-silenced nuclei to wild-type nuclei has been debated. It is now assumed that sRNAs are the signal molecules for these events in *P. infestans*. In *P. infestans*, transcriptional silencing of genes most likely occurs via heterochromatin formation, involving histone modifications, but not cytosine methylation.[24-26] *P. infestans* is by far the most studied species within the oomycetes and is known to possess functional canonical gene silencing pathways, as in other eukaryotes[23,26-29] but has silencing components that display unusual protein domain organization. For example, *P. infestans* has a single Dicer-like (PiDCL1) enzyme that contains the expected dual RNaseIII domains, but is lacking other domains typically found in DCR proteins; evidence indicates that the RNA helicase domain that is common in other organisms is encoded separately. The diatom (also a stramenopile) *Thalassiosira pseudonana* has a similar DCL domain organization.[30] Similarly, *P. infestans* possesses a single RdR protein that also contains a DCR-like helicase domain, an organization only found in *Dictyostelium discoideum*. Five *P. infestans* genes encode 4 distinct AGO proteins with typical PAZ and PIWI domains. Small RNAs of approximately 21 nt have been associated with partial silencing in *P. infestans*,[27] and 40 nt sRNAs associated with TE silencing.[11] A subsequent study that involved sequencing sRNAs from 2 *P. infestans* isolates revealed distinct classes of sRNAs at 21, 25/26 and 32 nt. In that study, biogenesis of 21 nt sRNAs was shown to be PiDCL1-dependent, while longer sRNAs were PiAGO-dependent. Small RNAs have also been sequenced from *P. sojae* and, while an overall analysis of sRNAs was not presented in that study, sRNAs mapping to a transcriptionally silenced locus were predominantly 24/25 nt in size.[31]

Do TEs and silencing lead to diversification of pathogenicity in *Phytophthora*?

The expression of effector encoding genes has been most intensively studied in *P. infestans* and *P. sojae*. There are over 500 predicted RxLR effectors in *P. infestans* and 396 in *P. sojae*, many of which exist as members of small gene families.[3,32] In *P. infestans*, not all of these effector genes are expressed to detectable levels, and different genotypes express different complements of effectors.[33] This variation in effector expression provides a potentially important explanation for the pathogenic variability of these organisms, possibly as an adaptability strategy toward resistance genes in host plants. The molecular mechanisms underlying this variation in effector gene expression have not been determined, but recent studies have implicated RNA silencing.

An unknown in this scenario is comprehensive knowledge of precisely which effectors are essential for full pathogenicity in any oomycete pathogen. Knowledge of which effectors are essential, with either minor or major contributions to pathogenicity, will aid in understanding the impact of endogenous silencing and expression variation of effectors. This is unlike the situation in the bacterial plant pathogen, *Pseudomonas syringae* pv *tomato*, where the majority of type III secretion system effectors have

been deleted individually or collectively to assess effector redundancy and identify essential groups of effectors.[34,35] To date, in *P. infestans* only PiAvr3a and PiAvrblb2 have been demonstrated to have a role in infection[10,11,36] with PiAvr3a being essential for full pathogenicity. In *P. sojae*, PsAvr3b, PsAvh241, and PSR2 (PsAvh146) are the only RxLR effectors shown to be indispensable for pathogenicity.[37-39] However, as more oomycete effectors are being studied in more detail, their importance to these pathogens is being revealed.

In a study to determine if endogenous gene silencing impacted on effector expression, deep sequencing of sRNAs in *P. infestans* was performed (one weakly pathogenic isolate and one highly aggressive). This revealed that the majority of the sRNAs were derived from the abundant and diverse transposable elements that make up over 70% of the *P. infestans* genome. However, sRNAs were also identified that originated from genes encoding RxLR and CRN effectors.[29] These sRNAs were unlikely to be random mRNA degradation products, since no sRNAs mapped to any highly expressed single copy number genes (for example: actin, β-tubulin, and ubiquitin).[29] In the weakly pathogenic isolate, sRNAs were identified that originated from the gene coding for the essential effector PiAvr3a. Small RNA abundance was associated with a decrease or loss of transcript (silencing) for the homologous genes. Surprisingly, sRNAs originating from genes encoding CRN effectors were more prevalent, and exhibited a markedly narrow size distribution, being overwhelmingly 21 nt in length. The only other sequence type showing this restricted sRNA size distribution in *P. infestans* was endonuclease-containing helitron transposons (Helentrons).

A similar study in the soybean pathogen, *P. sojae*, revealed the involvement of sRNAs in endogenous silencing of the *PsAvr3a* effector gene, enabling that pathogen to avoid recognition by soybean Rps3a resistance. In *P. sojae*, at least 3 avirulence effectors may avoid resistance recognition in this manner.[12,40] These studies highlight the potential for *Phytophthora* species to vary their host range and level of aggressiveness through endogenous silencing of effector genes, mediated by sRNAs. The manner by which endogenous silencing of genes is initiated in *Phytophthora* is yet to be conclusively determined, but recent studies have revealed potential mechanisms by which this may occur.

We speculate that one possible mechanism leading to RNA silencing of effectors may be initiated by silencing of nearby TEs.

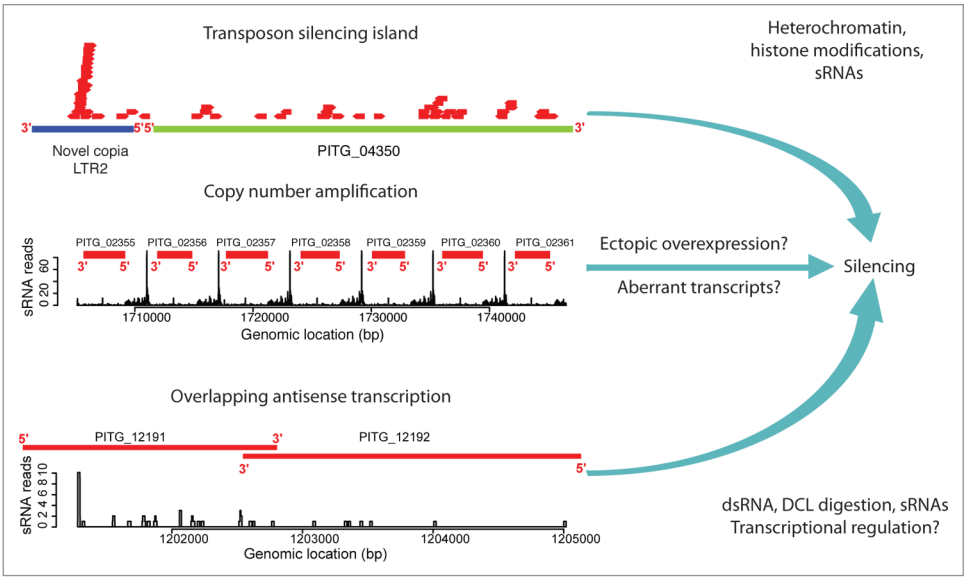

Figure 1. Initiation of pathways to endogenous RNA silencing of genes in *P. infestans*, using examples from sRNA sequencing.[29] In transposon silencing islands (top), heterochromatin formation may spread from a nearby transposon (blue) to also silence the neighboring RxLR effector gene, PITG_04350 (green). This involves histone modifications, and production of sRNAs (red arrows) via DCL, AGO and histone deacetylase. Silencing of amplified copies of endogenous genes (middle; red) may involve excessive transcription or formation of aberrant transcripts that are targeted for destruction via the RNA silencing pathway; genomic hotspots of aligning sRNAs indicate the involvement of DCL and AGO proteins. It is possible that one or more copies in the gene family array are expressed, while others may be silenced. Concurrent overlapping antisense transcription (bottom; red) may lead to formation of double stranded RNA (dsRNA) which can be processed into sRNAs, leading to mRNA destruction via AGO, transcriptional regulation or silencing. In middle and bottom models, gene position on the *P. infestans* genome sequence is shown below the gene model; vertical black bars indicate the abundance of aligning sRNAs. More detailed proposed models for sRNA biogenesis and silencing in *Phytophthora* can be found elsewhere.[17,29]

In *P. infestans*, transcriptional silencing is due to heterochromatin formation, and has been shown experimentally to impact on gene expression up to 600 bp from the silenced locus.[24] Further, a gene-TE transcriptional fusion can lead to the silencing of the introduced fusion construct in *P. infestans*, as well as the endogenous copies of the gene and TE.[11] Approximately half of the predicted 563 RxLR effectors in *P. infestans* are located within 2 kb of a transposon sequence, including the *PiAvr2*, *PiAvrBlb2*, *PiAvrBlb1*, and *PiAvr4* effector genes encoding avirulence proteins recognized by potato *R*-genes.[17] Mapping of sRNAs to RxLR effector genes, neighboring TEs, and the intervening sequences (up to 2 kb) revealed evidence for sRNAs derived from both sequences, and included sRNAs that mapped into the interval between both sequences, suggestive of silencing across the entire genomic region. We have named these regions TE-effector-silencing islands. To illustrate TE-effector-silencing islands in the *P. infestans* genome, an example is shown (**Fig. 1**) where the selected RxLR effector gene is located less than 350 bp from a transposon sequence. This distance is similar to that reported from *Drosophila*, where spread of silencing from transposons to active genes occurs, leading to transcript repression.[41] PITG_04350 has a *Copia LTR* retrotransposon only 50 bp from the 5' end. Interestingly, this effector gene and the transposon have been duplicated in the *P. infestans* genome (PITG_21984

is an identical copy). This example demonstrates the potential of RNA silencing to generate pathogenic variation in *P. infestans* through influencing the transcript levels from effector genes. Small RNAs that map to TE-effector-silencing islands vary in size class and show specific peaks at 21, 25–26 and 30 nt. This distribution of sRNA sizes is broadly similar to the size distributions seen for the total sRNAs mapping to the genome sequence.[29] Sequencing of sRNAs to greater depth than already performed will yield a more comprehensive overview of the contribution that this phenomenon may have in initiating endogenous silencing of effectors.

Amplified copy number or overlapping genes as triggers of gene silencing in *Phytophthora*. Many genes in *Phytophthora* genomes exist as multi-copy gene families. The location of effector genes in rapidly evolving genome regions may also be associated with gene amplifications. In the *P. infestans*, *P. sojae*, and *P. ramorum* reference genome sequences, many of the RxLR effector genes have been duplicated, giving rise to families of closely related paralogs.[3,12] Furthermore, copy number variations have been identified between different *P. infestans* isolates for RxLR effector and other genes.[33] In our earlier study, we identified genomic "hotspots" for sRNAs arising from the whole genome, among which were instances of gene amplifications organized in arrays of the repeated gene sequences (Vetukuri, Whisson, Dixelius, unpublished) such as for copies of PITG_02355 found in a gene dense region (**Fig. 1**), and PITG_16517 in a gene sparse region. These observations also agree with previous experimental evidence focused on optimizing gene silencing protocols in *P. infestans*, which had noted that silencing was most reliably initiated in transgenic lines where transgene integration had occurred in arrays or otherwise elevated transgene copy number.[27] It is therefore possible that extensive duplication of genes may lead to silencing of all copies.

Endogenous RNA silencing in other organisms can result from overlapping antisense transcription, leading to formation of dsRNA, and subsequent sRNAs.[42] In *P. infestans*, we identified instances in the gene rich regions of the genome where predicted genes had potential for overlapping transcripts, and these were found to be sRNA hotspots. An example of antisense overlapping transcription, supported by expressed sequence tags, include gene pairs PITG_12191/12192 (**Fig. 1**), PITG_05805/05804, and PITG_14066/14067 (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html). It remains to be shown if both genes become silenced or only one, or if this is a strategy used by *Phytophthora* to accurately regulate expression of genes required at different lifecycle stages; neighboring genes in *P. infestans* are typically not co-expressed.[43]

Transgenerational inheritance of silencing. In genetic studies to determine the mode of inheritance of avirulence in *P. sojae* or *P. infestans*, it is usually assumed that avirulence (R-Avr recognition) behaves as a dominant allele. However, in both of these *Phytophthora* species, virulence toward specific resistances is occasionally found to be dominant, and either no segregation or skewed segregation of avirulence:virulence occurs.[31,44-47] This has been best characterized in *P. sojae*, where *PsAvr1a*, *PsAvr3a* and *PsAvr1c* may segregate as dominant alleles in some crosses, but virulence is dominant and may fail to segregate in other crosses.[31,46-49] These contraindicative results were difficult to explain when first observed. However, a recent study in *P. sojae* has revealed that the underlying cause of dominant inheritance of virulence toward specific resistances is due to transgenerational inheritance of gene silencing.[31] *PsAvr3a* is an avirulence gene located in a highly polymorphic region of the *P. sojae* genome displaying copy number variation and polymorphic gene expression among different isolates. Isolates which accumulate detectable transcripts of *PsAvr3a* are avirulent on soybean plants carrying the cognate *Rps3a* resistance gene, whereas isolates lacking *PsAvr3a* mRNA are virulent. Small RNAs were detected in isolates lacking *PsAvr3a* mRNA, indicating that silencing of *PsAvr3a* transcript in *P. sojae* leads to evasion of host immune surveillance. All progeny in F_2 and F_3 generations of the cross between avirulent and virulent isolates were virulent and lacked *PsAvr3a* transcripts, including those not carrying the original silenced allele. Although transferred through sexual crosses, this is a similar observation to internuclear gene silencing, described previously in *P. infestans*.[23] The precise conditions that initiate heritable silencing of effectors remain to be determined, but may involve TE proximity or gene duplication as described earlier.

The sRNAs that aligned to *PsAvr3a* were 24–26 nt long, in agreement with one of the abundant size classes of sRNAs mapped to RxLR effectors in *P. infestans*. We hypothesize that this sRNA size class may regulate heterochromatin formation in association with other proteins involved in gene silencing pathways. A similar class of sRNAs is part of the "double lock" mechanism that controls TEs in *Arabidopsis*, along with DNA and histone methylation.[50] In plants, a DCL produces these 24 nt sRNAs, and triggers transcriptional gene silencing via RNA-dependent DNA methylation (RdDM).[51] Thus, it can be speculated that 24–26 nt sRNAs may also guide transcriptional gene silencing in oomycetes. Transgenerational epigenetic inheritance mediated by sRNAs was recently reported from *C. elegans*, where it was shown that offspring of strains exposed to dsRNA had direct chromatin modifications at the target site and generated more sRNAs in subsequent generations.[52-54] Moreover, the silenced state can be maintained in the progeny and transmitted across generations even in the absence of the original dsRNA trigger. This form of sRNA mediated transgenerational epigenetic inheritance is dependent on the nuclear RNAi pathway, including the Argonaute NRDE-3 (Nuclear RNAi Defective). Similar pathways exist in *Drosophila* and *Arabidopsis*, and we assume that similar mechanisms might operate in *Phytophthora*. By gaining an understanding of parallel silencing mechanisms across different kingdoms, we can infer the conserved functional roles that sRNAs have in epigenetic inheritance, and determine the impact of endogenous gene silencing in economically important plant pathogens such as *Phytophthora*.

Future Prospects

There remains much to be determined about how RNA silencing functions in oomycetes, ranging from biogenesis of specific classes of sRNAs, to identifying why specific gene copies become

silenced while others remain active. The first evidence of how TE expression produces gene-regulating sRNAs impacting both developmental and epigenetic processes in *Arabidopsis* and *Drosophila*[55,56] will most likely initiate new studies focused on the epigenetic, evolutionary, and transcriptional importance of TEs in other organisms. To date, much of the focus of oomycete molecular biology has been on *Phytophthora* plant pathogens. The recent publication of the genome for *Saprolegnia parasitica*, an oomycete pathogen of fish, which has a more compact genome with relatively few TEs,[57] will facilitate studies to determine if endogenous gene silencing has a role in its evolutionary potential.

Transposon derepression in the sudden oak death pathogen, *Phytophthora ramorum*, is associated with a reduction in pathogen fitness.[58] In *P. infestans*, with its extensive collection of TEs, much of the RNA silencing is apparently directed at controlling TEs and thereby keeping the genome functionally intact. However, the rapidly evolving effector genes that are crucial for the pathogenic success of *Phytophthora* are also predominantly located in TE and repeat rich regions, from where they may be more readily duplicated and undergo sequence diversification. The trade-off is that some effectors may become inadvertently silenced through proximity to silenced neighboring TEs, or through becoming overly duplicated. Thus, the pathogenic success of *Phytophthora* species may be a balance between allowing enough genome instability to drive effector evolution, while maintaining potentially disruptive TEs in a sufficiently silent state.

Much of our current understanding of oomycete effector functions derives from bioinformatics predictions from genome sequences and heterologous expression systems which provide relatively little experimental evidence regarding the importance of specific effectors for pathogenicity. This is partly due to the difficulties of making stable gene knockouts in *Phytophthora* species that are diploid during most of their life-cycle. To gain a better understanding of *Phytophthora* pathogenicity, and the variation in this trait due to endogenous gene silencing of effectors, it will be crucial to experimentally determine which effectors are essential or functionally redundant. To date, sRNA sequencing projects have only examined few isolates of 2 *Phytophthora* species. The future challenge will be to examine sRNA populations in more diverse pathogen genotypes to determine: (1) which effectors may undergo endogenous silencing without loss of pathogenicity; (2) whether endogenous silencing via sRNAs is responsible for only a subset of all effectors being expressed; and (3) the extent to which sRNAs may contribute to variation in gene expression and pathogenicity. The activity and impact of endogenous gene silencing of effectors in *Phytophthora* plant pathogens provides a hitherto unexplored layer of complexity to genomic analyses, and additional potential for pathogenic variability to interact with plant immune systems.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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