**Phytophthora** Have Distinct Endogenous Small RNA Populations That Include Short Interfering and microRNAs

Noah Fahlgren, Stephanie R. Bollmann, Kristin D. Kasschau, Josh T. Cuperus, Caroline M. Press, Christopher M. Sullivan, Elisabeth J. Chapman, J. Steen Hoyer, Kerrigan B. Gilbert, Niklaus J. Grünwald, James C. Carrington

1 Donald Danforth Plant Science Center, St. Louis, Missouri, United States of America, 2 Center for Genome Research and Biocomputing, Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon, United States of America, 3 Horticultural Crops Research Laboratory, USDA Agricultural Research Service, Corvallis, Oregon, United States of America

**Abstract**

In eukaryotes, RNA silencing pathways utilize 20-30-nucleotide small RNAs to regulate gene expression, specify and maintain chromatin structure, and repress viruses and mobile genetic elements. RNA silencing was likely present in the common ancestor of modern eukaryotes, but most research has focused on plant and animal RNA silencing systems. *Phytophthora* species belong to a phylogenetically distinct group of economically important plant pathogens that cause billions of dollars in yield losses annually as well as ecologically devastating outbreaks. We analyzed the small RNA-generating components of the genomes of *P. infestans*, *P. sojae* and *P. ramorum* using bioinformatics, genetic, phylogenetic and high-throughput sequencing-based methods. Each species produces two distinct populations of small RNAs that are predominantly 21- or 25-nucleotides long. The 25-nucleotide small RNAs were primarily derived from loci encoding transposable elements and we propose that these small RNAs define a pathway of short-interfering RNAs that silence repetitive genetic elements. The 21-nucleotide small RNAs were primarily derived from inverted repeats, including a novel microRNA family that is conserved among the three species, and several gene families, including Crinkler effectors and type III fibronectins. The *Phytophthora* microRNA is predicted to target a family of amino acid/auxin permeases, and we propose that 21-nucleotide small RNAs function at the post-transcriptional level. The functional significance of microRNA-guided regulation of amino acid/auxin permeases and the association of 21-nucleotide small RNAs with Crinkler effectors remains unclear, but this work provides a framework for testing the role of small RNAs in *Phytophthora* biology and pathogenesis in future work.

**Citation:** Fahlgren N, Bollmann SR, Kasschau KD, Cuperus JT, Press CM, et al. (2013) *Phytophthora* Have Distinct Endogenous Small RNA Populations That Include Short Interfering and microRNAs. PLoS ONE 8(10): e77181. doi:10.1371/journal.pone.0077181

**Editor:** Rogerio Margis, Universidade Federal do Rio Grande do Sul, Brazil

**Received:** July 21, 2013; **Accepted:** September 6, 2013; **Published:** October 21, 2013

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

**Funding:** This project was supported by the National Research Initiative Competitive Grants Program grant 2008-35600-18780 from the USDA National Institute of Food and Agriculture to NJG and JCC, the US Forest Service Pacific Southwest Research Station Sudden Oak Death research program to NJG, and the National Science Foundation grant MCB-1231726 and the National Institutes of Health grant AI043288 to JCC. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

*E-mail:* nik.grunwald@ars.usda.gov (NJG); JCarrington@danforthcenter.org (JCC)

† Current address: Department of Genome Sciences and Medicine, University of Washington, Seattle, Washington, United States of America

‡ Current address: Forage Genetics, International, West Salem, Wisconsin, United States of America

**Introduction**

*Phytophthora* species are a diverse group of filamentous, eukaryotic plant pathogens that are related to other stramenopile (heterokont) eukaryotes within the chloroalveolate super-group [1,2]. The stramenopile group includes golden-brown algae, diatoms, brown algae, and oomycetes. These diverse organisms, ranging from autotrophic algae to pathogenic, fungus-like oomycetes, were only recently grouped together based on phylogenetic analysis [1]. The oomycete lineage (non-photosynthetic stramenopiles, including the genus *Phytophthora*) was shown to be evolutionarily ancient. The oomycota lineage is estimated to have split from the photosynthetic stramenopiles in the ochrophyta lineage near the Proterozoic-Phanerozoic boundary ca. 570 million years ago [2]. The genus *Phytophthora* contains some of the most devastating plant pathogens and contains upwards of 100 formally described species [3]. *Phytophthora* species are capable of infecting nearly all eudicot plants and several monocot species, causing multibillion-dollar damage to crops, ornamental plants, and natural environments. *P. infestans* infection of potatoes and tomatoes causes late blight disease and is best known as the cause of the Irish potato famine [4,5]. *P. sojae* is an economically important pathogen of soybeans resulting in estimated losses of $1–2 billion annually worldwide [6]. *P. ramorum* causes sudden oak death and infects a large variety of ornamental nursery crops [7]. *P. ramorum* infections have afflicted trees in the western United States since the mid-1990s and more recently gained notoriety for causing massive landscape-scale dieback in plantations of Japanese larch in the United Kingdom [8]. The genomes of these three...
*Phytophthora* species have been sequenced [9,10], thus enabling discovery of the silencing machinery employed and the resulting small RNA expression patterns observed in this group. The fact that the oomycete lineage is ancient and basal to the stramenopile group provides an opportunity to elucidate the evolutionary history of the silencing machinery in the stramenopiles.

Eukaryotes use RNA-based pathways to regulate gene expression at the transcriptional and post-transcriptional levels. RNA silencing pathways utilize small RNAs (~20–30 nucleotides) to program effector protein complexes to recognize specific target nucleic acids in a sequence-dependent manner [11]. Target recognition by small RNA-effector complexes generally results in the suppression of activity of the target, either through degradation or recruitment of additional silencing factors. Generally, small RNAs are generated from double-stranded RNA through the activity of one or more Ribonuclease III (RNaseIII)-like enzymes [12]. Therefore, the conversion of single-stranded RNA into double-stranded RNA is a key step in RNA silencing pathways and occurs by intramolecular hybridization of self-complementary RNA to form a stem-loop (or hairpin) RNA, or by conversion of RNA into double-stranded RNA through the action of an RNA-dependent RNA polymerase (RDR). Several classes of small RNA are distinguished and include most notably the short-interfering RNAs (siRNA) and microRNAs (miRNA).

In plants and animals, and several other eukaryotic lineages, microRNAs are ~20–24-nucleotide RNAs that mediate silencing of target transcripts post-transcriptionally. Primary miRNA transcripts (pri-miRNA) are generally transcribed by RNA polymerase II and contain self-complementary regions that fold to form imperfect double-stranded stem-loop structures. In animals, pri-miRNAs are initially processed by the RNaseIII protein DROSHA, or by the spliceosome in the case of miitrons, to form precursor-miRNA (pre-miRNA). The miRNA and duplex strand (miRNA*) are released from the stem-loop RNA by the RNaseIII protein DICER (DCR) [13]. Plants lack a DROSHA homolog and instead DICER-LIKE1 (DCL1), a homolog of DCR, catalyzes both miRNA-processing steps [14]. The resulting miRNA/miRNA* duplex has 5′ monophosphates and 2-nucleotide, 3′ overhangs [15]. The final maturation step requires proper selection of the miRNA (guide strand) from the miRNA/miRNA* duplex by a member of the ARGONAUTE (AGO) protein family. AGOs select guide strands by one or more criteria including the miRNA 5′ end thermodynamic stability, 5′ nucleotide identity, miRNA/miRNA* duplex structure and in some cases other sequence-specific interactions [16]. AGO proteins loaded with miRNA are programmed to recognize and repress specific cellular target RNAs through AGO-mediated cleavage or translational repression [11]. MiRNA-guided target repression is an important regulator of developmental timing and patterning and response to biotic and abiotic signals [15,17].

Another diverse class of small RNAs, referred to broadly as siRNA, are common to animals, plants, and fungi [18,19]. Unlike miRNAs, siRNAs are generally produced from fully complementary RNA, generated through the activity of an RDR, or in some cases long, hairpin RNAs with nearly full complementarity. Biogenesis and function of siRNAs are diverse and include adaptive defense against viruses, maintenance of genome stability and chromatin structure, identification and suppression or elimination of transposable elements and duplicated DNA, and transcriptional and post-transcriptional control of gene expression [20,21,22,23]. In many lineages, the diversification of small RNA pathways has been accompanied by duplication and diversification of small RNA biogenesis factors (DCR/DCL and RDR) and effectors (AGO) [24].

Small RNAs have been studied in several pathosystems [25,26,27,28,29], but their role in regulating pathogen biology and host-pathogen interactions is poorly understood. Endogenous silencing pathways and differential expression of different classes of small RNAs during host-pathogen interaction remain to be characterized for most pathogens, including *Phytophthora* species. *Del, Rdr* and *Ago* genes were described in *P. infestans* [30], and several small RNA size classes between 21- and 32-nucleotides in length were described by deep sequencing in *P. infestans* [28], but further work is needed to characterize and assess the conservation of RNA silencing pathways in the *Phytophthora* genus. We used deep sequencing and comparative genomic approaches to analyze the endogenous small RNA populations of *P. infestans, P. ramorum* and *P. sojae*. A bimodal distribution of small RNA sizes was observed across all three species with peaks centered on 21- and 25-nucleotide long small RNAs. A bimodal distribution is suggestive of separate processing by the two DCL homologs in each species. The larger small RNA size class is predominantly associated with loci encoding transposable elements or other repetitive DNA while the shorter class was associated with several gene families, including Crinkler effectors and type III fibronectins, inverted repeats and a novel, conserved miRNA family. This work is a crucial step in elucidating the endogenous RNA silencing pathways in these important plant pathogens and provides novel insights into the silencing machinery in the stramenopiles.

**Results/Discussion**

**RNA Silencing Machinery in Phytophthora Species**

RNA interference (RNAi) is an established tool for studying gene function in *Phytophthora* species [31,32,33,34,35,36,37,38,39], which indicates that small RNA-based pathways exist in *Phytophthora*. In agreement with the observed activity of RNAi, homologs of the core eukaryotic RNA silencing machinery were identified in *P. infestans*, including one *Del*, one *Rdr*, and five *Ago* genes [30]. To determine if this complement of core silencing machinery is common among *Phytophthora* species, we used the genomic resources available for *P. infestans, P. sojae*, and *P. ramorum* to identify homologs in the three phylogenetically diverse species (see methods) [3,9,10]. Each *Phytophthora* genome encodes a single Rdr protein (Table 1). Phylogenetic analysis of the signature RNA-dependent RNA Polymerase (RdRP) domain suggests that the common ancestor of oomycetes (and possibly chromalveolates) had a single Rdr gene (Figure 1A). Two Del genes were found in *P. sojae* and *P. ramorum*, but *P. infestans* was previously reported to have a single Del gene [30], *PdDel1*, that is orthologous to *PdDel1* and *PdDel2* (Table 1). We hypothesized that *PdDel2* was either lost or was mistakenly excluded from the *P. infestans* genome assembly. To address the latter possibility, we searched for evidence of *PdDel2* in the whole-genome shotgun reads from *P. infestans* at the National Center for Biotechnology Information (NCBI) Trace Archive. We were able to assemble a 3962-nucleotide contig that contained a Del gene that was distinct from *PdDel1* and shared significant amino acid similarity to *PsDel2* and *PrDel2*, confirming that *P. infestans* has two Del genes (Figure 1B, Table 1). Del1 and Del2 from *Phytophthora* species and the alveolates *Tetrahymena thermophila* and *Paramecium tetraurelia* are phylogenetically distinct, suggesting an ancient divergence (Figure 1B).

Five Ago homologs and six probable Ago pseudogenes were identified from all three *Phytophthora* species (Table 1, Table 2). In a phylogenetic analysis of the DUF1783, PAZ and RNaseH-like PIWI domain, *Phytophthora* Ago proteins formed two deeply
branching clades (Figure 1C). One clade contained proteins encoded by the orthologous \textit{Agel} genes and \textit{PdAgo2}, an \textit{Agel} paralog (Figure 1C and D). The second clade contained four closely related groups of Ago proteins (Figure 1C). The \textit{Agel}, \textit{Ago4} and \textit{Ago5} groups are encoded by syntenic orthologs that are colocalized to the same approximately 100–200 kilobase pair
(Khp) region in each species (Figure 1D). *P.ago6* and two *PsAgo6* pseudogenes are also located in the *Ago3, Ago4* and *Ago5* region of *P. sojae* (Figure 1D, Table 2). The Ago1-5 proteins contain the conserved MID domain amino acids known to interact with small RNA guide strand 5’ phosphates [40,41], and contain the conserved PIWI domain metal-ion-coordinating residues (aspartic acid, aspartic acid, histidine) that are required for endonucleolytic cleavage activity (slicing) [42,43,44,45,46] (Figure 1E). In contrast, *PsAgo6* and *PsAgo7* encode proteins that lack one or more conserved catalytic residues in the PIWI domain (Figure 1E), and could represent a class that functions through a mechanism different from slicing. Alternatively, *PsAgo6* and *PsAgo7* may be pseudogenes generated by transposition events rather than slicing. The *PsAgo6* and *PsAgo7* pseudogenes may be involved in the generation of novel RNA silencing components that are higher than Ago proteins encoded by syntenic orthologs of *Ago1-5* and similar to the putative product of the *PsAgo5_1* pseudogene (Figure 1C). We conclude that the genomes of *P. infestans, P. sojae* and *P. ramorum* encode a highly conserved set of core RNA silencing components with two anciently diverged clades of Dcl and Ago genes.

Phytophthora have Distinct Small RNA Populations

The *P. infestans*, *P. sojae* and *P. ramorum* genomes provide a platform to study small RNA biogenesis and function in *Phytophthora* as they span the genus [3,9,10]. In plants, animals, fungi and other eukaryotes, high-throughput sequencing of small RNA is a valuable experimental tool for understanding small RNA biology [47]. In particular, analysis of small RNA length, diversity across the genome and co-occurrence with annotated genomic features is useful for classifying small RNA into distinct pathways (for example, see [27,48,49,50,51,52,53,54,55]). To assess the small RNA landscape of *P. infestans, P. sojae* and *P. ramorum*, we prepared small RNA libraries for sequencing from mycelium tissue. Individually prepared small RNA libraries from *P. sojae* and *P. ramorum* mycelia were sequenced on the 454 pyrosequencing platform [56] and yielded 34,784 and 59,314 reads, respectively. Two biological replicate samples prepared from *P. infestans* mycelium were sequenced on the Illumina 1G sequencing platform [57] and yielded a total of 9,932,843 reads. Small RNA sequences were parsed from adaptors and reads from each library that aligned without mismatches to its respective

| Group | Locus | Locus accession | Position |
|-------|-------|-----------------|----------|
| Rdr1  | P1Rdr1| PITG_10457      | Supercontig 18:1359241–1367325+ |
| Rdr1  | P2Rdr1| estExt_Genewise1Plus_C_3_140457 | Scaffold 3:2028009–2036275+ |
| Rdr1  | P3Rdr1| N/A            | Scaffold 67:79140–87321– |
| Dcl1  | P1Dcl1| PITG_09292      | Supercontig 14:1931317–1936311+ |
| Dcl1  | P2Dcl1| gm1.19145_g     | Scaffold 10:1947580–1952607– |
| Dcl1  | P3Dcl1| C_scaffold_46000013 | Scaffold 46:152941–157809 |
| Dcl2  | P1Dcl2| N/A            | Scaffold 42:583558–2586463– |
| Dcl2  | P2Dcl2| fgenesh1_pg.4.###_534 | Scaffold 43:164754–168440– |
| Ago1  | P1Ago1| PITG_04470      | Supercontig 54:3375249–4376242– |
| Ago1  | P2Ago2| PITG_0471      | Supercontig 54:3396571–4399354– |
| Ago1  | P3Ago1| Phys02.s_453613 | Scaffold 11:289545–292340– |
| Ago1  | PrAgo1| C_scaffold_37000014 | Scaffold 37:196057–199708+ |
| Ago1  | PsAgo1| PITG_01400      | Supercontig 2:1735937–1739823+ |
| Ago1  | PsAgo3| PITG_01443      | Supercontig 2:1512873–1515521– |
| Ago1  | PsAgo4| e_gw1.4.3046.1 | Scaffold 4:1590683–1593337– |
| Ago1  | PsAgo4| fgenesh1_pg.C_scaffold_18000159 | Scaffold 18:445984–449397– |
| Ago1  | PsAgo5| PITG_01444      | Supercontig 2:1518134–1520629– |
| Ago1  | PsAgo5| fgenesh1_pm.4.###_266 | Scaffold 4:1586191–1588686– |
| Ago1  | PsAgo5| C_scaffold_18000063 | Scaffold 18:453298–459311+ |
| Ago1  | PsAgo6| fgenesh1_pg.4.###_282 | Scaffold 4:1601071–1603176– |
| Ago1  | PrAgo7| N/A            | Scaffold 10:81401–85778+ |
| MIIR8788|pin-MIIR8788|N/A            |Supercontig 18:1064102–1064237– |
| MIIR8788|psj-MIIR8788a|N/A            |Scaffold 5:1772538–1777265– |
| MIIR8788|psj-MIIR8788b|N/A            |Scaffold 3:1777195–1777334+ |
| MIIR8788|pra-MIIR8788a|N/A            |Scaffold 67:2135112–213649– |
| MIIR8788|pra-MIIR8788b|N/A            |Scaffold 1330:23306–2443– |

1See file S2 for sequences.

2*P.ago1* and *P.ago2* are co-orthologs with *PrAgo1* and *PsAgo1*, therefore, the name *Ago2* was not assigned to any gene in *P. sojae* or *P. ramorum*. doi:10.1371/journal.pone.0077181.t001

Table 1. Dcl, Rdr, Ago and MIRNA genes in three Phytophthora species.
with 160,953 genomic matches and *P. infestans* distributed amongst transposons (Figure 2B). Small RNA read abundance was more be assigned to an annotated feature category overlapped retro-majority (84%) of small RNA genome alignments (loci) that could had 31,821 aligned reads (25,304 unique sequences) with 140,022 genomic matches, *P. ramorum* reads (16,609 unique sequences) with 54,692,467 genome were kept for further analysis.

The genome-wide small RNA size profile for each of the three species was bimodal with peaks centered on 21- and 23-nucleotide RNAs (Figure 2). The most abundant small RNAs were 25 nucleotides long, but 26-nucleotide small RNAs were nearly as prevalent (Figure 2A). In total, 18-29-nucleotide and 24-30-nucleotide small RNA were 30–44% and 36–70% of the total aligned reads in each species, respectively. In *P. infestans*, the majority (84%) of small RNA genome alignments (loci) that could be assigned to an annotated feature category overlapped retro-transposons (Figure 2B). Small RNA read abundance was more distributed amongst *P. infestans* features, relative to small RNA loci, with 16–33% of reads overlapping transposons, tandem repeats, protein-coding genes and retrotransposons (16%, 19%, 22%, and 33%, respectively), and less than 10% overlapping inverted repeats, helitron transposons and satellite repeats (7%, 3% and <1%, respectively) (Figure 2C). The proportion of small RNA loci and reads that overlapped each *P. infestans* feature category was approximately proportional to the total genome space occupied by each feature type. Therefore, to better understand the relative small RNA-generating activity of each type of feature, we normalized the feature small RNA size profiles to account for total feature length. *P. infestans* inverted and tandem repeats had the highest small RNA-generating activity per megabase pair (Mbp) (Figure 2D). In contrast, *P. infestans* transposable elements and protein-coding genes had 4.6–11.4 times fewer reads per Mbp than inverted repeats (Figure 2D). Therefore, while transposable element and protein-coding gene loci are the most widespread sources of small RNA, inverted and tandem repeat loci may be the most potent triggers of small RNA biogenesis.

*P. infestans* feature-specific small RNA size profiles were similar to the genome-wide profile: bimodal with peaks at 21- and 25-nucleotide RNAs (Figure 2A–D). Retrotransposons, transposons, helitrons and satellite repeats had similar size profiles where 24-30-nucleotide small RNAs were 3.5–5.7 times more abundant than 18-23-nucleotide small RNAs. For tandem repeats, 24-30-nucleotide small RNAs were only 1.1 times more abundant than 18-23-nucleotide small RNAs. In contrast, *P. infestans* inverted repeats and protein-coding genes were the only feature types for which 18-23-nucleotide small RNAs were more abundant than 24-30-nucleotide small RNAs. Based on the whole-genome and feature-specific small RNA size profiles, we hypothesized that *Phytophthora* species produce two small RNA size classes. To explore this possibility, we analyzed the size distributions of small RNA-generating loci using principal component analysis (PCA). Small RNA generating-loci were defined as segments of the *P. infestans* genome that contained mapped reads with up to 50 nucleotides of space between consecutive small RNAs. Segments were kept for further analysis if they contained at least one distinct pair of small RNAs that overlapped on opposite strands with 2-nucleotide 3’ overhangs, as expected for Dicer products [11]. We reasoned that segments that contained putative Dicer-duplexes were more likely to be bona fide small RNA-generating loci. For each of the 2,328 *P. infestans* segments that contained a Dicer-duplex pair (2,812,472 total reads), a proportional small RNA size profile was constructed, to remove abundance differences between segments, by calculating the proportion of small RNA reads for each size (20–27 nucleotides) relative to the total segment reads (Table A in File S1). PCA was done on the segments using a model that treated the proportion for each small RNA size (20–27 nucleotides) as an independent variable. The first principal component explained 30% of the variance and differentiated segments into two groups based on predominant small RNA sizes: 23-25-nucleotides versus 24-27-nucleotides (Figure 2E). The second principal component explained 15% of the variance and primarily differentiated segments based on the proportion of 23- and 26-nucleotide small RNA (Figure 2E). To further validate the size class differentiation, segments were clustered on the rotated data from the first and second principal components using the DBSCAN (density-based spatial clustering of applications with noise) algorithm [58]. DBSCAN clustering produced two clusters (Figure 2E). Cluster 1 contained 52 segments that produced primarily 21-nucleotide small RNAs (Figure 2F). Cluster 2 contained 2,141 segments that produced primarily 25/26-nucleotide small RNAs (Figure 2F). Clustering did not result in a noticeable differentiation between segments with different proportions of 25- and 26-nucleotide small RNA (Figure 2E). The PCA and clustering results are consistent with our hypothesis that *Phytophthora* species produce two primary small RNA sizes centered around 21- and 25-nucleotides.

Taken together, the size distributions, PCA and clustering analyses are all consistent with the presence of two size classes of *Phytophthora* small RNA. The presence of two small RNA size classes and two Dcl orthologs in each species raises the possibility that each Dcl produces RNAs of one of the two size groups, similar to the way that plants partition small RNA pathways with specialized DCL proteins [59]. Consistent with this model, RNAi knockdown of *Phdcl1* was recently shown to disrupt accumulation of 21-, but not 25/26-nucleotide small RNAs [28]. Additionally, *Phytophthora* Ago proteins may be partitioned into two or more functional groups that might stabilize 21- or 25/26-nucleotide small RNAs. Further analysis of RNA silencing-deficient lines will

### Table 2. Putative Ago pseudogenes.

| Locus | Parent | Locus accession | Position |
|-------|--------|----------------|----------|
| PsAgo5_ps | PsAgo5 | gm1.16110_g | Scaffold 7:1941866–1945020+ |
| PsAgo6_ps1 | PsAgo6 | fgenes1_pg.A._281 | Scaffold 4:1597730–1599169– |
| PsAgo6_ps2 | PsAgo6 | gm1.10550_g | Scaffold 4:1595135–1597461– |
| PrAgo1_ps1 | PrAgo1 | fgenes1_pg.C._scaffold_1073000001 | Scaffold 1073:6488–7803– |
| PrAgo1_ps2 | PrAgo1 | fgenes1_pg.C._scaffold_7000159 | Scaffold 7:706074–707466+ |
| PrAgo3_ps | PrAgo3 | gwEuk.107.27.1 | Scaffold 107:78418–78798+ |

doi:10.1371/journal.pone.0077181.t002
Figure 2. Classification of small RNA populations in three Phytophthora species. (A) Distribution of small RNA reads by size in P. infestans, P. sojae, and P. ramorum. (B–D) Distribution of small RNA in P. infestans at retrotransposon, transposon, helitron transposon, inverted repeat, tandem repeat, satellite repeat and gene loci. Graphs are color-coded by small RNA size based on the legend in (A). (E) Biplot of principal components 1 and 2 from the PCA of P. infestans small RNA-generating segments. Small RNA-generating segments are color-coded based on overlapping features. Segments included in Cluster 1 or Cluster 2 from DBSCAN clustering of segments based on rotated small RNA size profile data are outlined. (F) Proportion of small RNA reads corresponding to 20-27-nucleotide small RNA for each small RNA-generating segment in DBSCAN Cluster 1 (upper) or Cluster 2 (lower). Gray lines are small RNA size profiles for individual segments and black lines are the cluster average profiles.

doi:10.1371/journal.pone.0077181.g002
be required to confirm this hypothesis and identify any functional differences between the two small RNA size classes.

**Genome-wide Distribution of Small RNA-generating Loci**

The feature types in Figure 2, other than inverted repeats, were associated with abundant 25/26-nucleotide small RNAs, relative to 21-nucleotide small RNAs. Furthermore, the relative proportion of feature types in Cluster 1 and 2 (21- versus 25/26-nucleotide small RNA-generating segments, Figure 2E) were significantly different (p<0.001, Fisher's exact test). Protein-coding gene and simple repeat segment repeats were 7.0 (95% confidence interval: 3.5–13.4) and 36.5 (95% confidence interval: 11.0–116.1) times more likely to generate predominantly 21-nucleotide small RNAs, respectively (p<0.001, Fisher's exact test). In contrast, transposable element segments were 3.2 (95% confidence interval: 1.8–6.0) times more likely to generate predominantly 25/26-nucleotide small RNAs (p<0.001, Fisher's exact test). The *P. sojae* genome (240 Mb) is more than twice as large as the *P. argaeae* and *P. ramorum* genomes (95 and 65 Mb, respectively) due to recent proliferation of transposable elements [9,10]. Expansion of the *P. sojae* genome was also uniform and resulted in a distinct genome architecture that consists of blocks of gene-dense and gene-sparse regions [9]. Genome-wide differences in gene density can be visualized by binning genes into a two-dimensional matrix based on the length of 5' and 3' intergenic border regions [9]. As shown previously [9], most genes in *P. sojae* are flanked by intergenic regions between 20 bp and 3 Kbp long (gene-dense regions, GDRs), while another set are flanked by at least one intergenic region that is 5 Kbp or larger (gene-sparse regions, GSRs and border regions, BRs) (Figure 3A, left panel). GDRs and GSRs are markedly different; genes that lack transposable elements in their flanking intergenic regions are primarily in GDRs (Figure 3A, right panel). Similarly, the proportion of intergenic space occupied by transposable elements was lowest in GDRs (median = 0%) and highest in GSRs and BRs (median = 22%) (Figure 3B). A similar pattern was observed for intergenic small RNAs. Intergenic segments from gene-sparse, transposon-rich regions had the highest density (reads per bp) of all small RNAs, and in particular, 25/26-nucleotide small RNAs (Figure 3C). In contrast, intergenic segments generally had low 21-nucleotide small RNA density and were distributed between both GDRs and GSRs (Figure 3C). Small RNA density within genes was more variable, but was generally highest for 25/26-nucleotide small RNAs (Figure 3D). Unlike intergenic small RNAs, intragenic 25/26-nucleotide small RNA density was more pronounced in genes from GSRs than BRs (Figure 3D). Genes generally had low 21-nucleotide small RNA density (Figure 3D), with the exception of a few gene families discussed below.

The distribution of 25/26-nucleotide small RNAs across the *P. sojae* genome coincides with the location of transposable elements and low gene density. Additionally, we observed enrichment of transposable element loci at 25/26-nucleotide-generating segments of the *P. sojae* genome. Therefore, we hypothesized that 25/26-nucleotide small RNAs function to repress transposable elements in *Phytophthora* species. In agreement with our observations, *P. argaeae* strains that contain naturally silenced alleles of the avirulence gene *Avr3A* were shown to have abundant 24-26-nucleotide small RNAs at the *Avr3A* locus [60]. Furthermore, heritable transgenerational silencing of *Avr3A* was demonstrated in crosses between silenced and non-silenced strains, suggesting an epigenetic mode of inheritance [60]. In other RNA silencing systems, transposable elements and other repetitive DNA are silenced through heterochromatin modifications [61]. In plants, 24-nucleotide small RNAs guide AGO proteins to target loci, resulting in DNA methylation and ultimately repressive chromatin modifications [22]. The mechanism by which 25/26-nucleotide small RNAs might silence targets is unknown, but previous work suggests that transcriptional silencing in *Phytophthora* is mediated through chromatin modifications and not DNA methylation [62,63].

**Distinct Small RNA Populations from Protein-coding Gene and Pseudogene Loci**

As described above, the majority of small RNAs in *P. sojae* are derived from transposable elements, repeats and other intergenic regions, but some genes were identified at small RNA-generating loci (Figure 2, Figure 3). To examine small RNA-generating loci in more detail, we identified 1,166 *P. sojae* genes and pseudogenes that overlapped at least 100 total small RNA reads. Gene annotations were updated using BLAST2GO [64], and genes were grouped by annotation and small RNA size profile (Figure 4A, Table B in File S1). Of the 1,166 genes, 875 genes (including 17 pseudogenes) overlapped predominantly 24-30-nucleotide small RNAs and 291 genes (including 54 pseudogenes) overlapped predominantly 18-23-nucleotide small RNAs (Figure 4A, Table B in File S1). The majority (53.8%) of the small RNA-generating genes were of unknown type (hypothesised and conserved hypothetical), and 90% of the genes in these categories were associated primarily with 24-30-nucleotide small RNAs (Figure 4A, Table B in File S1). Annotated families with four or more members in the small RNA-generating set included genes encoding 4 annexins (ANX), 9 aspartyl-t-RNA synthetases (AARS), 12 ATP-binding cassette (ABC) transporters, 163 crinkler effectors (CRN) (including 68 pseudogenes), 17 C48 family cysteine proteases (C48), 6 elicitors (ELI) (including 2 pseudogenes), 16 type III fibronectin (FN3), 4 M96 mating-specific proteins (M96) (including 1 pseudogene), 6 major facilitator superfamily (MFS) transporters, 25 methylene-tetrahydrofolate dehydrogenases (MTHFD), 11 P-type ATPases (P-ATPase), 10 polysaccharide lyases (PL), 11 RXLR (Arg-X-Leu-Arg, where X is any amino acid) effectors, 6 SET domain-containing proteins and 11 SpoU rRNA methyltransferases (Figure 4A, Table B in File S1). The small RNAs from *AARS*, *C48*, *M96*, *MTHFD*, *PL*, *RXLR*, *SET* and *SpoU* genes were predominantly 24-30-nucleotides long (60–85% of the total reads on average per family). Interestingly, five of the eight families (*C48*, *PL*, *RXLR*, *SET* and *SpoU*) are enriched in gene-sparse regions [65]. Given that 24-30-nucleotide small RNAs were enriched at transposable element loci, or in gene-sparse, transposable element-rich regions (Figure 2, Figure 3), genes that overlap 24-30-nucleotide small RNA-generating regions may represent loci that were captured by transposable element silencing machinery either in cis or in trans. The distribution of small RNAs within, upstream, and downstream of genes that overlapped predominantly 24-30-nucleotide small RNAs was similar to transposons and LTR retrotransposons where 25/26-nucleotide small RNA abundance was greatest within genes or transposable elements and continued, at a lower level, into the 500 bp upstream and downstream regions (Figure 4B). The higher 25/26-nucleotide small RNA abundance within genes and transposable elements, relative to flanking regions suggests that silencing is generally feature-specific. The appreciable 25/26-nucleotide small RNA density in the upstream and downstream flanking regions may arise from local spreading of silencing.

Unlike most other features in the *P. sojae* genome, some genes encoding ANX, CRN, FN3 and P-ATPases overlapped small RNAs that were primarily 18-23-nucleotides long (64–95% of the total reads on average per family). In contrast to genes associated
with 25/26-nucleotide small RNAs, 18-23-nucleotide small RNAs, and in particular 21-nucleotide small RNAs, at ANX, CRN, FN3 and P-ATPase loci were more abundant on average and were almost exclusively located in the gene body (Figure 4B and C). In particular, CRN and FN3 genes produced large clusters of abundant 21-nucleotide small RNAs (Figure 4A, C). In the P. infestans genome, CRN genes and pseudogenes are found in several large gene clusters and are often grouped with CRN genes with the same domain architecture [9]. Of the 163 CRN genes and pseudogenes, 135 genes were associated with predominantly 18-23-nucleotide small RNAs, and 123 of these CRN genes were from or were found clustered with CRN with one of four domain architectures (Figure 5A). CRN groups that generate 21-nucleotide small RNAs were the DN5-type that were found primarily (14 of...
Figure 4. Small RNA from protein-coding gene and pseudogene loci in *P. infestans*. (A) Heatmap-based size profile of 18-30-nucleotide small RNA reads mapping to 1,166 *P. infestans* genes that overlapped at least 100 total small RNA reads. Gene annotation groups with at least four genes are labeled with alternating black and gray boxes. (B and C) Regional metaplots with average 18-27-nucleotide small RNA reads per position. X-axis positions are relative scale (0–100%) for each region. Genes from (A) with more 24-30- than 18-23-nucleotide small RNA reads, LTR retrotransposons and transposons are shown (B). Genes from (A) with more 18-23- than 24-30-nucleotide small RNA reads, crinklers and type III fibronectins are shown (C).

doi:10.1371/journal.pone.0077181.g004
A Conserved MIRNA Family in Phytophthora

To address the question of whether oomycetes have precisely-processed miRNAs similar to those in other eukaryotes, we designed a pipeline to identify candidate MIRNA genes using high-throughput small RNA sequencing data from P. infestans, P. sojae and P. ramorum. First, small RNA that mapped to transposable element or structural RNA loci were removed from further analysis. Second, 20-22-nucleotide small RNA sequences were filtered to limit further analysis to sequences with two or more reads and ten or fewer matches to the genome. The remaining foldback, the most abundant sequence was considered to be the putative miRNA precursor sequence. The remaining foldback sequences were also shown to be conserved across multiple species and to have a conserved secondary structure. The candidate MIRNA precursor sequences were then used to search the genome for putative target genes.

Conclusions

In plants, animals and fungi, duplication and diversification of genes that encode DCR/DCL, RDR, AGO and other accessory proteins has resulted in the evolution of distinct RNA silencing pathways [24]. We find that Phytophthora species have a conserved set of core RNA silencing machinery, including one RdR, two Dcl and four Ago proteins, with some species-specific duplicates. Our detailed analysis of small RNA sizes and distribution across the P. infestans genome, as well as small RNA sizes from P. sojae and P. ramorum, supports the presence of two primary, distinct small RNA size classes: 21- and 25/26-nucleotides. The 21- and 25/26-nucleotide small RNAs are not just distinct in size but are also generated from distinct features, with 25/26-nucleotide small RNAs predominantly associated with transposable elements and
Figure 5. Regions of \textit{P. infestans} containing clusters of \textit{CRN} and \textit{FN3} genes and pseudogenes. (A and B) Genomic regions containing \textit{CRN} and \textit{FN3} genes. The position of genes, transposable elements, \textit{CRN} genes (A) and \textit{FN3} genes (B) are shown. 18-23- and 24-30-nucleotide small RNA read density heatmaps are shown (see legend at the bottom of B).

doi:10.1371/journal.pone.0077181.g005

Figure 6. Computational and molecular characterization of \textit{Phytophthora MIRNA} genes. (A) Single-nucleotide resolution small RNA read density in regions containing \textit{pin-MIR8788}, \textit{pra-MIR8788a}, \textit{psj-MIR8788a} and \textit{pin-IR2758}. (B) \textit{MIR8788} foldback structures predicted with RNAfold [90]. Ribonucleotides are colored by base-pairing entropy. (C) T-coffee alignment of DNA sequences corresponding to the \textit{MIR8788} foldbacks. Alignment quality is shown as a heatmap. (D) Northern blot detection of the \textit{pin-miR8788} guide RNA and \textit{pin-IR2758} and \textit{Pi}-helitron-derived small RNA in \textit{P. infestans} mycelia tissue. Northern blot detection of serine-tRNA was included as a loading standard. (E) Summary of TargetFinder results for predicted target transcripts of \textit{miR8788}. (F) RLM-5' RACE validation of \textit{psj-miR8788}-guided cleavage of \textit{AAAP} transcripts. Arrows indicate the detected cleavage position with the number of clones supporting cleavage out of the total tested. The representative structure of \textit{AAAP} transcripts is shown with the \textit{AAAP} domain and miRNA target site highlighted.

doi:10.1371/journal.pone.0077181.g006
21-nucleotide small RNAs associated with inverted repeats and several gene families. These results are largely in agreement with Vetukuri et al. [28] except that we do not provide evidence for or against a 32-nucleotide size class, as these RNAs were not present in our data sets. Taken together, we propose that Phytophthora have at least two distinct small RNA pathways that may be partitioned by Rdr, Dcl and Ago diversification as well as small RNA size. In this model, we propose that 25’/26-nucleotide small RNAs define a siRNA pathway that targets transposable elements and other repetitive features and silences target loci through an epigenetic mechanism. Besides a role for genome defense and evolution of genome structure, epigenetic regulation of pathogenicity factors has important implications for the evolution of isolates and host range [60].

Additionally, we propose that 21-nucleotide small RNAs define at least a second pathway that regulates targets by post-transcriptional control. The presence of a second pathway for small RNA biogenesis is further supported by the observed ancient divergence of Dcl1 and Dcl2, and the deep split in the Ago tree separating Ago1/2 from the other Agos, reflecting independent evolution. Some 21-nucleotide-generating loci, such as CRN and FX3, appear to be siRNA and likely are dependent on Rdr activity. In contrast, other 21-nucleotide small RNAs may be generated from hairpin RNA (e.g. MIR8788) without the need for Rdr activity. It is unclear what the function of 21-nucleotide small RNAs is, but given the large number of P. infestans CRN genes associated with 21-nucleotide small RNAs they may be important components of pathogenicity, host range or variability of effector repertoire between isolates.

Materials and Methods

Phytophthora Strains and Growth Conditions

Mycelium was grown from the sequenced strains of three Phytophthora species; Phytophthora ramorum Pr-102, Phytophthora sojae P6497 and Phytophthora infestans T30-4 [9,10]. Phytophthora isolates were maintained on cleared 10% V8 agar medium (100 mL V8 juice; 1 g CaCO3; 30 mg/L β-sitosterol (EMD Chemicals, Incorporated); 15 g agar; 900 mL deionized water) in a 20°C incubator in the dark [69]. Mycelia were grown in V8 broth (V8 agar medium without agar) on a rotating shaker in flasks and collected after 7 days for P. sojae and P. ramorum and after 4 days for P. infestans.

Total RNA Preparation

Total RNA was isolated as described previously [49]. Briefly, mycelium samples were frozen in liquid nitrogen, ground into a fine powder and homogenized in Trizol (1 g:10 mL, Life Technologies). After adding 2 mL chloroform per 10 mL Trizol, samples were mixed, incubated at room temperature for 5 minutes and centrifuged at 8400 g for 10 minutes. Two additional chloroform extractions were done before RNA was precipitated in 0.7 volumes isopropanol for 20 minutes at room temperature and spun for 30 minutes at 8400 x g. Minimally dried pellets were resuspended in 200 μL 0.1X TE, extracted 2 times with phenol:chloroform:isoamyl alcohol (50:49:1), and once with chloroform. Total RNA was precipitated with 5 M ammonium acetate and ethanol at −80°C overnight, spun at 12000 x g for 30 minutes at 4°C, resuspended in 100 microliters 0.1X TE and quantitated by Nanodrop (Thermo Scientific).

Small RNA Library Construction and Sequencing

Small RNA libraries for P. ramorum and P. sojae were prepared for sequencing on the 454 platform using 5’-ATCTGATGGCGUCUGAUA-3’ and 5’-ATCTGATGGCCACUGUAUA-3’ for P. sojae and P. ramorum, respectively, with strain-specific bases bolded, as described previously [49]. P. infestans libraries were prepared for sequencing on the Illumina 1 G platform as described previously [70] except that no RNA spike-in standards were added and no barcoded adaptors were used.

Small RNA Data Processing

Sequencing reads were parsed to remove adaptors and collapsed to a unique set with read counts. Parsed sequences from each species were aligned to their respective genome using BOWTIE v0.12.8 [71] with settings that limit the results to perfect matches only (–f –v 0 –a –S). Aligned small RNAs were stored in Sequence Alignment/Map (SAM) format and were accessed using SAMTOOLS [72]. The annotated genomes for P. sojae (v3.0) and P. ramorum (v1.1) were obtained from the Joint Genome Institute (JGI) [6]. The annotated genome for P. infestans was provided by the Broad Institute [9]. Where noted, reads were repeat-normalized by dividing the read counts by the total number of locations in the genome the small RNA mapped to.

Identification of Rdr, Dcl and Ago Homologs

Putative homologs of the small RNA biogenesis and effector genes Rdr, Dcl, and Ago were located using tBLASTn [73]. Plant and animal homolog protein sequences were used as the query against both transcript and genome databases from P. sojae, P. ramorum, and P. infestans [9,10]. For Pdr2, MEGALAST [73] was used to align Pdr2 to the P. infestans whole-genome shotgun reads at the NCBI Trace Archive (File S2), CAP3 [74] was used to assemble the whole-genome shotgun reads into a 3962 bp contig (File S2). In cases where BLAST results from transcript and genome databases overlapped at the same locus, the annotated gene model was used and is reported in Table 1. PDr2 and genomic BLAST results for PDr1, PsAgo3 and PsAgo7 did not have corresponding gene models so GENSCAN [75] and FGENESH [76] were used to predict gene structures (File S2). Conserved domains were annotated using the PFAM database [77] for the complete set of proteins identified. Proteins were filtered based on the presence of conserved domain architecture specific to the gene families: Rdr proteins were required to contain an RNA-dependent RNA polymerase domain [78], Ago proteins were required to contain a DUF1785, PAZ and PIWI domain [79], and Dcl proteins were required to contain two RnaselIII domains [80]. Six Ago genes had one or more missing or truncated domain and were considered pseudogenes (Table 2). Gene names were assigned to orthologs and paralogs based on phylogenetic and syntenic analysis (see below) and to preserve previously published nomenclature [28,30] (Table 1).

Phylogenetic Analysis

Phylogenetic analysis was done using the amino acid sequences of the RdRP domain, RnaselIII-RnaselIII domains region and DUF1785-PAZ-PIWI domains region. PaAgo6-ps1, PaAgo6-ps2, PaAgo6-ps1, PrAgo1-ps1 and PrAgo1-ps2 were not included in the Ago phylogenetic analysis because each had missing or truncated DUF1785, PAZ or PIWI domains. For each family, protein multiple sequence alignments were built with MAffT [81]. The aligned sequences were imported into Molecular Evolutionary Genetics Analysis (MEGA v5.05) [82] and trimmed to exclude positions that contained a gap in any sequence. Maximum Likelihood inference with RAxML v7.3.0 [83] was used with a GAMMA model of rate heterogeneity and the LG substitution matrix to build phylogenetic trees using the majority-rule consensus criterion. Support for clades was obtained using 1,000
bootstraps. Arabidopsis thaliana RDR and DCL proteins were included as outgroups for the Rdr and Dcl analyses, respectively, and T. gondii Ago1 was included as the outgroup for the Ago analysis. Consensus trees were drawn with DENDROSCOPE 3.2.4 [84]. Synteny analysis of Ago1-5 genes was done using the JGI Genome Portal and VISTA Point browser [85, 86].

Statistical Analyses

All statistical tests were done using R v2.15 [87]. Principal component analysis was done by the scalar value decomposition method on centered and scaled data with the procomp function (stats package). For PCA, the size profiles for P. infestans small RNA-generating segments were normalized to remove abundance differences between segments but maintain the relative contribution of each size class to the overall segment profile. For each segment, the relative contribution of each small RNA size class between 20- and 27-nucleotides was calculated by dividing the total size class reads by the total reads for the segment. DBSCAN clustering [58] was done with the dbscan function (fpc package [58]) using a minimum cluster size of 30 and a maximum distance (eps) of 0.3. Fisher’s exact test was done with the fisher.test function (stats package).

Identification and Analysis of MIRNA Genes

A computational pipeline was used to identify putative miRNA from among the Phytophthora small RNA datasets. First, small RNA arising from transposable elements and structural RNA loci were removed. Second, only 20-22-nucleotide small RNAs with two or more reads and ten or fewer matches to the genome were considered. Third, an initial foldback assessment was done with small RNA-flanking sequence (1 Kbp) using EINVERTED (-gap 12 -threshold 10 -match 3 -mismatch -4) [89]. Small RNAs located within EINVERTED alignment results were analyzed further, and overlapping loci were consolidated. Fourth, secondary structure analysis was done using RNAfold [90] on consolidated loci where at least 95% of the small RNA reads were from one strand. The most abundant small RNA from each locus had to be located in a stem region and was considered a miRNA if a small RNA matching the predicted miRNA* was sequenced in our datasets.

To assess possible biases in our computational pipeline, we independently analyzed the P. infestans small RNA libraries using the ShortStack pipeline v0.4 [69]. Aligned small RNA reads in SAM format were compressed, sorted and indexed in BAM format using SAMTOOLS. Prep_bam.pl v0.1.1 from ShortStack was run with default settings except that the small RNA-flanking sequence (1 Kbp) using EINVERTED (-gap 12 -threshold 10 -match 3 -mismatch -4) [89]. Small RNAs located within EINVERTED alignment results were analyzed further, and overlapping loci were consolidated. Fourth, secondary structure analysis was done using RNAfold [90] on consolidated loci where at least 95% of the small RNA reads were from one strand. The most abundant small RNA from each locus had to be located in a stem region and was considered a miRNA if a small RNA matching the predicted miRNA* was sequenced in our datasets.

To assess possible biases in our computational pipeline, we independently analyzed the P. infestans small RNA libraries using the ShortStack pipeline v0.4 [69]. Aligned small RNA reads in SAM format were compressed, sorted and indexed in BAM format using SAMTOOLS. Prep_bam.pl v0.1.1 from ShortStack was used to prepare the BAM file for the ShortStack pipeline. ShortStack was run with default settings except that the small RNA size range was set to 18-26 nucleotides (-dicermin 18-26).

miRNA Target Prediction

Targets for miR8788 were predicted for P. sojae, P. ramorum and P. infestans transcripts using TargetFinder v1.6 (http://carringtonlab.org/resources/targetfinder) [67] with a score cutoff of 5 (-c 5).

Northern Blot Analysis

Total RNA (10 μg) from P. infestans mycelium tissue was resolved by denaturing 17% polyacrylamide-urea gel electrophoresis. For size standards, 21- and 24-nucleotide synthetic RNA markers (5’-pUGUGGGCCAGAGGUGUUCGU-3’ and 5’-pUGUGGGCCAGAGGUGUUCGU-3’ respectively) were also resolved. RNA was transferred to a positively charged nylon membrane (Nytran SuPerCharge, Whatman) by semi-dry electrotransfer. RNA was UV-crosslinked to the membrane twice at 1200 Joules. DNA probes for psso-miR8788 (5’-CGGGCGGTGTTACGCTTGGTAG-3’) and serine-tRNA (5’-CTGTGAGATTCGAACTCACGC-3’) were endlabeled by phosphorylation with γ32P-ATP and Optikinase (Amersham). Membranes were incubated with hybridization solution (PerfectHyb Plus, Sigma) at 38°C for 1 hour and then incubated with probes at 38°C overnight.

Supporting Information

Figure S1 MIR8788 processing in P. infestans, P. ramorum and P. sojae. MIR8788 foldback diagrams for P. infestans (A), P. ramorum (B) and P. sojae (C). miR8788 guide strands and miR8788* strands are highlighted in each foldback. Proportion of small RNA reads for the entire foldback are plotted as stacked bar graphs. Small RNAs are color coded by size.

File S1 Supplemental tables for small RNA-generating segments, genes and inverted repeats. (XLSS)

File S2 Manually annotated gene sequences. (DOCX)

Acknowledgments

We thank Karan Fairchild and Kim Henslee for excellent technical support, and the Oregon State University Center for Genome Research
and Bioinformatics for use of the bioinformatics computer cluster. The P. 
spp. v3.0 sequence data were produced by the US Department of Energy
Joint Genome Institute http://www.jgi.doe.gov in collaboration with the
user community. Mention of trade names or commercial products in this
manuscript are solely for the purpose of providing specific information and
do not imply recommendation or endorsement.

References

1. Baldauf SL (2003) The deep roots of eukaryotes. Science 300: 1703–1706. doi:
10.1126/science.1085534.
2. Brown JW, Sorhannus U (2010) A molecular genetic timescale for the
rise and fall of the Phyllanthus infestans lineage that triggered the Irish potato
c alte. 2: e00731. doi: 10.7554/eLife.00731.
3. Blair JE, Coffey MD, Park SY, Geiser DM, Kang S (2008) A multivirovirus
phylodynamics and retrotransposon movement in soybean and model
oys. Mol Plant Pathol 8: 1–8. doi: 10.1111/j.1364-3733.2006.00373.x.
4. Fy WE (2008) Phytolysis: the pathologist and the (R) gene: destroyer. Mol Plant
Pathol 9: 395–402. doi: 10.1111/j.1364-3733.2007.00465.x.
5. Yoshida K, Schuenemann VJ, Cano LM, Pais M, Mishra B, et al. (2013) The
small RNA populations in Phytolysis: plasmid and retrotransposon movement in
small RNA pathways. Nat Rev Genet 8: 884–896. doi: 10.1038/nrg2179.
6. Tyler BM (2007) A molecular genetic timescale for the
ancestors. EMBO J 23: 578–587. doi: 10.1002/ebc.20017.
7. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
8. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
9. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
10. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
11. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
12. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
13. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
14. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
15. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
16. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
17. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
18. Elkahy E, Kuhn CD, Tocci A, Haase AD, Greene EM, et al. (2012) The
structure of the human argonaute-2 in complex with miR-20a. Cell 150: 100–110.
doi: 10.1016/j.cell.2012.05.029.
