Title: The Evolutionary History of Small RNAs in the Solanaceae

Short Title: Solanaceae Small RNA Evolution

Patricia Baldrich^{1,*}, Sébastien Bélanger^{1,*}, Shuyao Kong^{2,*}, Suresh Pokhrel^{1,2,*}, Saleh Tamim^{4,5,*}, Chong Teng¹, Courtney Schiebout^{1,6}, Sai Guna Ranjan Gurazada^{4,7}, Pallavi Gupta^{1,8}, Parth Patel^{4,9}, Mayumi Nakano¹, Ayush Dusia^{4,10}, Blake C. Meyers^{1,3,#}, and Margaret H. Frank^{1,2,#}

* Contributed equally to the work (in alphabetical order)

#co-corresponding authors: Blake Meyers (bmeyers@danforthcenter.org) and Margaret Frank (mhf47@cornell.edu)

1 - Donald Danforth Plant Science Center, St. Louis, MO 63132

2 - School of Integrative Plant Science, Cornell University, Ithaca, NY 14853

3 - Division of Plant Sciences, University of Missouri, Columbia, MO 65211

4 - Center for Bioinformatics and Computational Biology, University of Delaware, Newark DE 19711

5 - Current address: Genomics Research Center, AbbVie, North Chicago IL 60064

6 - Current address: Dartmouth College

7 - Corteva Agriscience, Wilmington, DE 19805

8 - Institute for Data Science & Informatics, University of Missouri, Columbia, MO 65211

9 - Current address: Johnson & Johnson

10 - Current address: VMware Inc.

Margaret H. Frank, Donald Danforth Plant Science Center, St. Louis, MO 63132;

Current: Cornell University, School of Integrative Plant Science, Ithaca, NY 14853

<https://orcid.org/0000-0002-8269-8240>

*Sébastien Bélanger, Donald Danforth Plant Science Center, St. Louis, MO 63132

<https://orcid.org/0000-0002-6755-8207>

32

33 *Saleh Tamim, Center for Bioinformatics and Computational Biology, University of
 34 Delaware, Newark DE 19711, Current: Genomics Research Center, AbbVie, North
 35 Chicago IL 60064. <http://orcid.org/0000-0002-6215-7041>
 36

37 Chong Teng, Donald Danforth Plant Science Center, St. Louis, MO 63132
 38 <https://orcid.org/0000-0001-9898-6442>
 39

40 *Patricia Baldrich, Donald Danforth Plant Science Center, St. Louis, MO 63132
 41 <https://orcid.org/0000-0003-4669-6632>
 42

43 *Suresh Pokhrel, Division of Plant Sciences, University of Missouri, Columbia, MO
 44 65211; Donald Danforth Plant Science Center, St. Louis, MO 63132
 45 <http://orcid.org/0000-0002-5323-1940>
 46

47 Blake C. Meyers, Division of Plant Sciences, University of Missouri, Columbia, MO
 48 65211; Donald Danforth Plant Science Center, St. Louis, MO 63132
 49 <https://orcid.org/0000-0003-3436-6097>
 50

51 *Shuyao Kong, Cornell University, School of Integrative Plant Science, Ithaca, NY
 52 14853 <https://orcid.org/0000-0002-3124-8899>
 53

54 Previous contributors:

55 Mayumi Nakano, Donald Danforth Plant Science Center, St. Louis, MO 63132
 56 <https://orcid.org/0000-0001-9317-4509>
 57

58 Ayush Dusia, University of Delaware, Newark DE 19711. current: VMware Inc.,
 59 <https://orcid.org/0000-0003-1310-8523>
 60

61 Pallavi Gupta, Institute for Data Science & Informatics, University of Missouri, Columbia,
 62 MO 65211. <https://orcid.org/0000-0003-0146-239X>

Courtney Schiebout, Donald Danforth Plant Science Center, current: Dartmouth College, <https://orcid.org/0000-0002-4830-8237>

Parth Patel, Center for Bioinformatics and Computational Biology, University of Delaware, Newark DE 19711, current: Johnson & Johnson, <https://orcid.org/0000-0002-0195-3933>

Sai Guna Ranjan Gurazada, Center for Bioinformatics and Computational Biology, University of Delaware, Newark DE 19711, Current: Corteva Agriscience, Wilmington, DE 19805. <https://orcid.org/0000-0002-3084-5683>

ONE-SENTENCE SUMMARY

We use over 255 publicly-available small RNA datasets to characterize the small RNA landscape for the Solanaceae family.

AUTHOR CONTRIBUTIONS

B.C.M and M.H.F. conceived the original research plan and supervised the analyses, and supervised and completed the writing; P.B., S.B., S.K., S.P., S.T., C.T., C.S., S.G.R.G, P. G. and P.P. performed the analyses, generated tables and figures, and contributed to writing of the article; M.N and A.D. provided technical assistance with data processing. M.H.F. agrees to serve as the author responsible for contact and ensures communication.

FUNDING INFORMATION

The work was supported with funding from NSF IOS awards 1842698, 754097 and 1650843, and USDA NIFA award 2019-67013-29010 to Meyers; NSF award 1523668 to M. Frank.

ABSTRACT

The Solanaceae or "nightshade" family is an economically important group that harbors a remarkable amount of diversity. To gain a better understanding of how the unique biology of the Solanaceae relates to the family's small RNA genomic landscape, we downloaded over 255 publicly available small RNA datasets that comprise over 2.6 billion reads of sequence data. We applied a suite of computational tools to predict and annotate two major small RNA classes: (1) microRNAs (miRNAs), typically 20-22 nt RNAs generated from a hairpin precursor and functioning in gene silencing, and (2) short interfering RNAs (siRNAs), including 24-nt heterochromatic siRNAs (hc-siRNAs) typically functioning to repress repetitive regions of the genome via RNA-directed DNA methylation, as well as secondary phased siRNAs (phasiRNAs) and trans-acting siRNAs (tasiRNAs) generated via miRNA-directed cleavage of a Pol II-derived RNA precursor. Our analyses described thousands of small RNA loci, including poorly-understood clusters of 22-nt siRNAs that accumulate during viral infection. The birth, death, expansion, and contraction of these small RNA loci are dynamic evolutionary processes that characterize the Solanaceae family. These analyses indicate that individuals within the same genus share similar small RNA landscapes, whereas comparisons between distinct genera within the Solanaceae reveal relatively few commonalities.

INTRODUCTION

Plant genomes encode thousands of small RNA (sRNA) loci that participate in a wide variety of processes, including developmental transitions, epigenetic patterning, biotic and abiotic stress responses, and local and long-distance communication. sRNAs are 18 to 24 nucleotide (nt) non-coding sequences that are loaded into Argonaute (AGO) proteins to direct transcriptional or post-transcriptional gene silencing in a sequence-specific manner. sRNAs can be divided into different classes based on their length, biogenesis, and mode of action. This study focuses on microRNAs (miRNAs) and short interfering RNAs (siRNAs).

MicroRNAs are typically 21- or 22-nt in length and originate from a self-complementary, single-stranded (ss)RNA molecule, a Pol II product, that forms a hairpin structure. This characteristic secondary structure is typically recognized by DICER-LIKE 1 (DCL1) and processed through two sequential cleavage events into a mature double-stranded (ds)RNA duplex; each of these mature miRNAs has the potential to be loaded into Argonaute effector proteins (Axtell et al., 2011).

Small interfering RNAs (siRNAs) can be divided into multiple categories: hairpin siRNAs, which are generated in a manner similar to miRNAs, heterochromatic siRNAs (hc-siRNAs), and secondary siRNAs. hc-siRNAs are the most abundant and diverse sRNA class in plants; they are typically 24-nt in length and originate from heterochromatic regions of the genome (e.g. repetitive regions and transposable elements). hc-siRNA precursors are transcribed by RNA Pol IV, converted into dsRNA by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), and finally recognized and cleaved by DCL3, generating mature duplexes of which individual sRNAs guide sequence-specific RNA-directed DNA methylation (RdDM) (Won et al., 2014). In contrast, secondary siRNAs are generated from coding and non-coding Pol II transcripts and require a miRNA “trigger” to initiate production. Secondary siRNAs include phased siRNAs (phasiRNAs), trans-acting siRNAs (tasiRNAs), and epigenetically activated siRNAs (easiRNAs). PhasiRNA production is typically initiated by a 22-nt miRNA trigger

that recognizes and directs the cleavage of ssRNA targets, which are then transformed into dsRNA by RDR6, and processed into “in phase” sRNAs via cleavage in a sequential manner by DCL4 and DCL5 (Fei et al., 2013; Chen et al., 2018b).

The evolutionary history of sRNA biogenesis pathways is complex. While the core pathways supporting the biogenesis of major sRNA classes are conserved across the land plants, lineage-specific instances of duplication, subfunctionalization, and gene loss often lead to complicated evolutionary relationships amongst AGO, DCL, and RDR family members. In the AGO family, the moss genome of *Physcomitrella patens*, for instance, contain three AGO genes (AGO1/4/7), while flowering plant genomes often contain 10 to 20 AGO family members (Axtell, 2018). The Arabidopsis genome, for example, contains ten AGO genes encoding proteins that can be broadly grouped into three clades: AGO1/5/10, AGO2/3/7/8, and AGO4/6/9 (Zhang et al., 2015). These individual AGO clades interact with distinct classes of sRNAs. For example, AGO1 predominantly binds 21 or 22-nt miRNAs, AGO4 binds 24-nt hc-siRNAs in the canonical RNA dependent DNA methylation (RdDM) pathway, while AGO2 binds 22-nt siRNAs that function in viral responses (Ma and Zhang, 2018). Additional AGOs have undergone further subfunctionalization in select species, for example, there are four AGO1 paralogs in rice, each of which has distinct miRNA binding affinities (Wu et al., 2009). There are also instances of *de novo* function arising within the AGO gene family. For example, maize AGO18b is a grass-specific AGO that functions the regulation of inflorescence meristem development and negatively regulates spikelet number on the tassel (Sun et al., 2018). Despite the functional importance of gene family expansion and contraction amongst key components of sRNA biogenesis machinery, relatively little characterization of the DCL, RDR, and AGO families has been carried out in the Solanaceae. Existing data focused on small subsets of Solanaceae species have uncovered lineage-specific expansion events within the AGO (Liao et al., 2020), RDR, and DCL families (Esposito et al., 2018). Given the abundance of genomic resources now available for the Solanaceae, a more comprehensive, cross-species characterization of these gene family members will help resolve questions with respect to the evolution of unique aspects of sRNA biogenesis within this family.

The Solanaceae harbors a remarkable amount of morphological and metabolomic diversity. The nightshade family includes staple crops (e.g. potato and eggplant), high-value crops (e.g. tomato, pepper, petunia, and tobacco), and emerging crop systems (e.g. ground cherry), making it the third most economically important plant family in the world. Moreover, several species in the Solanaceae family serve as popular model systems for studies of biological processes pronounced within this family, including fruit ripening (Klee and Giovannoni, 2011; Shinozaki et al., 2018), plant-pathogen interactions (Goodin et al., 2015; Pombo et al., 2020), and the production of specialized metabolites (Fan et al., 2019; Leong et al., 2019). These model systems are supported by high-quality reference genomes (Mueller et al., 2005; Potato Genome Sequencing Consortium et al., 2011; Bombarely et al., 2012; Tomato Genome Consortium, 2012; Qin et al., 2014; Bombarely et al., 2016) that have facilitated the generation and analysis of genomic-scale deep sequencing datasets within the family. There are currently more than 250 Solanaceae libraries, equating to over 2.6 billion reads of publicly-available sRNA data available in the NCBI short read archive (SRA). Previous work focused on specific aspects of sRNA biology within the Solanaceae have identified unique sRNA phenomena that are associated with the diverse biology of this family. One specific case is the miR482/miR2118 superfamily in Solanaceae, which also includes miR5300 members. In eudicots, this family of 22-nt miRNAs targets the encoded sequence of the P-loop motif of NLR disease resistance proteins (Shivaprasad et al., 2012), generating a secondary siRNA cascade with implications for basal innate immunity (Zhai et al., 2011; Shivaprasad et al., 2012). Evolutionary studies indicate that this superfamily originated prior to the split of the *Solanum* and *Nicotiana* genera (de Vries et al., 2015), and superfamily members have even been found in Gymnosperm species, indicating that this miRNA family has deep evolutionary origins (Xia et al., 2015).

Genomic resources that support cross-species analyses in land plants have grown substantially over the last two decades. There are now more than 200 fully-sequenced genomes covering major branches of the land plant phylogeny. In addition to the availability of plant genomes, innovations in RNA library construction and sequencing technologies, as well as algorithms and bioinformatic resources, have drastically

reduced experimental costs and facilitated the large-scale production of publicly-available sRNA sequence data. Two recent studies using publicly-available and newly generated sRNA datasets harvested from a broad range of land plant taxa have taken advantage of these resources and substantially improved the annotation of miRNA, phasiRNA and hc-siRNA loci in land plant genomes ([Lunardon et al., 2020](#); [Chen et al., 2019](#)). These studies both show that siRNA loci are minimally conserved across species, while mature miRNAs tend to be conserved across lineages ([Chen et al., 2019](#); [Lunardon et al., 2020](#)). There are numerous lineage- or species-specific miRNA sequences which correspond to young miRNAs ([Cuperus et al., 2011](#); [Chávez Montes et al., 2014](#); [Lunardon et al., 2020](#)). Although large-scale studies produce a massive amount of data and describe major evolutionary trends, they are often unable to capture lineage- and species-specific phenomena.

In this study, we applied a suite of computational tools to a large collection of publicly-available sRNA libraries to investigate the unique aspects of miRNAs and siRNA biology within the Solanaceae. We describe thousands of sRNA loci, some of which are unique to the Solanaceae. For example, we found that an annotated, Solanaceae-specific miRNA family is actually a MITE-derived siRNA, and we observed family-wide expression of *DCL2*-derived 21-nt phasiRNAs. Our analyses also revealed aspects of sRNA biology that are only conserved at the sub-family level, including a lineage-specific expansion of previously unidentified miRNAs in *Nicotiana*, a distinct split in phasiRNA production between the *Solanum/Capsicum* and *Nicotiana/Petunia* branches of the family, and distinct clusters of 22-nt sRNAs that are expressed in response to viral infection in tomato. Overall, our analyses indicate that individuals within the same genus share similar sRNA landscapes, whereas comparisons between distinct genera within the Solanaceae reveal relatively few commonalities.

MATERIALS AND METHODS

Evolution of small RNA biogenesis protein genes in the Solanaceae

We downloaded protein sequence files from Phytozome v13, the Sweetpotato Genomics Resource, and the SOL Genomics Network databases, and selected proteomes for *Arabidopsis thaliana*, two Convolvulaceae (*Ipomoea trifida* and *Ipomoea triloba*) and the seven Solanaceae species (*Nicotiana benthamiana*, *Nicotiana tabacum*, *Petunia axillaris*, *Petunia inflata*, *Capsicum annuum*, *Solanum lycopersicum* and *Solanum tuberosum*) for analysis (Supplemental Table 1). We used SonicParanoid v1.2.6 ([Cosentino and Iwasaki, 2018](#)) and OrthoFinder v2.3.11 ([Emms and Kelly, 2015](#)) to perform a gene orthology inference using default parameters. To identify orthologous groups related to NRP, RDR, DCL, DRB and AGO proteins, we used the reference protein IDs available in the UniProt database for *A. thaliana*. We then aligned the retrieved protein sequences using default parameters in MUSCLE v3.8.1551 ([Edgar, 2004](#)), and trimmed alignments with trimAL v1.4.rev15 applying the -gappyout option ([Capella-Gutierrez et al., 2009](#)). To infer phylogenetic trees we implemented the maximum likelihood method in IQ-TREE v1.6.12 with the following parameters: -alrt 1000 and -bb 1000 ([Minh et al., 2013](#); [Nguyen et al., 2015](#); [Kalyaanamoorthy et al., 2017](#)). We used iTOL v4 ([Letunic and Bork, 2019](#)) to visualize consensus trees and summarize orthologous protein genes for the five gene families investigated in each species (Supplemental Table 1). To investigate organ-specific expression for the DCL1, AGO1, and AGO2 paralogs identified for tomato, we downloaded expression data for *Solanum lycopersicum* from The Bio-Analytic Resource for Plant Biology ([Waese et al., 2017](#)).

Data collection, quality filters, and read processing

We downloaded raw sRNA seq and PARE libraries for *Solanum lycopersicum* (tomato), *S. tuberosum* (potato), *Nicotiana benthamiana*, *N. tabacum* (tobacco), *Capsicum annuum* (pepper), *Petunia axillaris*/*P. inflata* (petunia) from the NCBI SRA (<https://www.ncbi.nlm.nih.gov/sra>; detailed information regarding raw and processed libraries can be found in Supplemental Dataset 1. Briefly, we processed raw FASTQ reads using a Python script that performs quality filtering, trimming, adapter removal, and then converts reads into chopped tag count files (<https://github.com/atulkakrana/preprocess.seq> (Bolger et al., 2014)). We then mapped our reads to their respective reference genomes (Table 1) using Bowtie 1 (Langmead,

2010) allowing for zero mismatches. We applied two criteria for filtering out low-quality data: first, we excluded libraries with low mapping rates, and second, we removed libraries that lacked a significant accumulation of 21-nt and 24-nt reads based on size distribution plots (Supplemental Figure 1), indicating potential RNA degradation. We assembled species-specific expression databases using these final, high-quality, processed datasets (Nakano et al., 2020).

Solanaceae genome versions and functional gene annotations

We used the following reference genomes and their associated annotation files to build expression databases and perform the bioinformatic analyses described in this study: *Capsicum annum* Zunla-1 v2.0 (<http://public.genomics.org.cn/BGI/pepper/>) (Qin et al., 2014), *Petunia axillaris* and *P. inflata* (Bombarely et al., 2016), *Nicotiana tabacum* v4 (Edwards et al., 2017), *Nicotiana benthamiana* v 0.3 (Bombarely et al., 2012), *Solanum tuberosum* (Potato Genome Sequencing Consortium, 2011), and *Solanum lycopersicum* build 2.5 (Tomato Genome Consortium, 2012) (<ftp://ftp.solgenomics.net/genomes/>).

MicroRNA Predictions

We used three different software packages to obtain a robust prediction of microRNAs (miRNAs) across our Solanaceous model genomes: ShortStack (Axtell, 2013), miR-PREFeR (Lei and Sun, 2014), and miRPD (a version of miReap optimized for plant miRNA prediction; (Sun et al., 2013)). We standardized the minimum mapping coverage and size of hits across the three programs in order to control for dissimilarities in software performance. The vast majority of miRNAs fall between 20-22 nt in length so we narrowed our predictions to this 20-22 nt size range. Each program employs a different method for calculating coverage. In ShortStack we set the minimum coverage to 15, while in miR-PREFeR and miRPD we set the minimum coverage to 20. We then filtered out predicted miRNA precursors based on recently published guidelines for miRNA predictions (Axtell and Meyers, 2018). Briefly, we required that all miRNAs express both strands of the miRNA duplex (the mature miRNA and miRNA-star), and originate from a precursor that folds with a normalized minimum free energy (MFE) of < -0.2 kcal/mol/nucleotide. Notably, enforcing the expression of a complementary miRNA-

star sequence resulted in little to no filtering, so we applied a more stringent filter requiring that the abundance of the complementary miRNA-star reads were $\geq 10\%$ of the abundance of the mature miRNA.

We applied additional filtering for “novel” miRNAs that did not match miRBase entries (see “miRNA conservation across species,” below). To remove hairpin structures that might originate from MITEs and tRNAs, we filtered out novel miRNAs that aligned to our list of annotated transposable elements (TEs) and tRNA datasets for the Solanaceae (Supplemental Dataset 2). We found a surprising number of novel miRNAs in our *Nicotiana benthamiana* (hereafter, “benthi”) dataset which warranted manual curation. To investigate whether our novel benthi miRNAs originate from canonical precursor structures, we mapped our shortread data onto predicted miRNA precursors using structVis v0.4 (github: <https://github.com/MikeAxtell/structVis>), and removed novel miRNAs that showed non-canonical folding and/or miRNA/miRNA* expression. After these filtering steps we found the miRPD results to be highly inconsistent, producing unrealistically high numbers of predicted miRNAs for some of our species and zero predicted miRNAs for others. Thus, we decided to limit our analyses to the ShortStack and miR-PREFeR results. To create a consensus set of miRNAs for these two prediction programs, we collapsed precursors with overlapping coordinates from these two software programs into unique entries. Overall, we found that ShortStack was more conservative, predicting fewer miRNAs than miR-PREFeR; however, the miRNAs were also predicted with appreciable confidence and resulted in minimal post-prediction filtering. Our hybrid prediction approach in combination with stringent filtering allowed for sensitive, yet robust predictions across the species.

MicroRNA conservation across species

To facilitate cross species comparisons of conserved and novel miRNAs within the Solanaceae, we first clustered mature miRNA sequences for the predicted miRNAs (described above) into families by requiring pairwise sequence overlap of $\geq 75\%$. To annotate the miRNA families, we aligned mature miRNA sequences to the latest v22

miRBase database of all land plants requiring an 85% match, and provided new IDs for novel miRNA families (Kozomara and Griffiths-Jones, 2014; Kozomara et al., 2019). We tabulated all novel and previously annotated miRNA families across the Solanaceae in a master table that reports presence and absence for each miRNA family (Supplemental Dataset 3).

***PHAS* locus predictions and annotations**

We used three programs to identify *PHAS* loci: ShortStack (Axtell, 2013), PHASIS (Kakrana et al., 2017), and PhaseTank (Guo et al., 2015). We ran PHASIS and PhaseTank using the default parameters listed in the program manuals and filtered the results for high-confidence loci that were identified with a p-value of ≤ 0.001 . We ran ShortStack using the same parameters that were applied to our miRNA predictions. PhaseTank did not perform consistently across the analyzed genomes; it only reported *PHAS* loci for unassembled regions of the genomes. After we removed unassembled contigs, the program returned no results, so we decided to remove PhaseTank from our analysis pipeline. We merged the *PHAS* loci obtained from PHASIS and ShortStack into a single list for each species, and collapsed loci located within 400 nucleotides of each other into individual *PHAS* predictions (Supplemental Dataset 4).

We annotated coding versus non-coding *PHAS* loci using available CDS GFF files. To characterize non-coding *PHAS* loci we aligned our non-coding *PHAS* loci to the NCBI non-redundant (nr) database and annotated the loci with one of the top ten hits using default alignment parameters in BLAST (Altschul et al., 1990). To test whether coding *PHAS* loci were generated from orthologous genes across species, we constructed gene orthogroups for species with associated peptide fasta files. Finally, we linked these orthogroup predictions with annotation files for tomato, Arabidopsis, and pepper (Supplemental Dataset 5). The published pepper gff file lacks annotations, so we generated our own pepper annotation file by running a BLASTP search against the NCBI NR database and retrieving the best BLAST hit for each entry.

miRNA target predictions

We used sPARTA, a miRNA target prediction program to identify miRNA targets with the following specifications: -genomeFeature 0 -tarPred H -tarScore S (Kakrana et al., 2015). We applied a cut off score of ≥ 4 to filter for reliable miRNA-target interactions. *PHAS* loci are triggered by 22 bp miRNAs that have a 5' Uracil bias (Chen et al., 2010; Cuperus et al., 2010). We ran sPARTA with the -featureFile option to identify miRNAs from our predicted miRNA list that trigger *PHAS* locus production from our predicted PhasiRNA list. We applied a quality score of ≥ 4 to filter for PHAS locus triggers, prioritized miRNA triggers that start with Uracil (U), and selected miRNA triggers equal to 22 bp with the exception of miR390.

hc-siRNAs and transposable elements across SOL genomes

To analyze hc-siRNAs, we first ran RepeatMasker (<http://www.repeatmasker.org>) on each one of the analyzed genomes using the Viridiplantae RepBase database (<http://www.girinst.org/rebase>) as a library, to identify transposable elements (TE). Next, we used the identified TEs as a database to map sRNA reads using Bowtie (Langmead, 2010) with default parameters and categorized all 24-nt long reads that mapped to any of our identified TEs as hc-siRNAs. It was impossible to identify the specific origin of these sRNAs due to the repetitive nature of TEs, so we only considered TE superfamilies for this study. Genome-wide representation of hc-siRNAs was visualized by plotting Manhattan plots with the qqman R package (Turner, 2014). We plotted the data based on the genome mapping coordinate along the chromosome for each hc-siRNA and its relative abundance.

Identification of 22-nt clusters expressed in response to Geminivirus infection

To discover 22-nt sRNA clusters that may be linked to Geminivirus infection in tomato, we downloaded sRNA libraries for control and infected treatments from the TOMATO_sRNA_SOL Next-Gen DB (Nakako et al., 2020) and filtered for 22-nt reads. We tuned the parameters for 22-nt cluster identification using two fruit peel libraries (Tomato_S_7 and Tomato_S_8, cv. Ailsa Craig, Gao et al., 2015) and two leaf libraries (Tomato_S_50, cv. Moneymaker, and Tomato_S_53, line FL505, Bai et al., 2016). To

identify clusters that accumulate in response to Geminivirus infection, we analyzed two infected leaf libraries (Sly_leaves_t2, cv. Pusa Ruby, Saraf et al., 2015; Tomato_S_52, cv. Moneymaker, Bai et al., 2016) and their corresponding control libraries (Sly_leaves_t1 and Tomato_S_50, respectively). We applied the following analysis pipeline to identify 22-nt sRNA clusters: first, we ran ShortStack with the parameters tuned to yield the highest reproducibility between repeated runs, relatively high similarity between results from similar samples (e.g. leaf vs. leaf), and relatively high difference between results from distinct samples (e.g. leaf vs. fruit peel), without compromising the alignment rate (Johnson et al., 2016). This fine tuning resulted in the following parameter set: --mmap u --mismatch 1 --bowtie_m 3 --ranmax 2 --pad 125 --mincov 75rpm. Using these parameters, we ran ShortStack to identify all of the 22-nt sRNA clusters in the aforementioned four libraries. Next, we filtered out clusters that were shorter than 200 bp in length, and clusters that were inconsistently expressed in one infected-control sample pair, but not in the other. We applied a threshold of absolute log2 fold change greater than two in order to identify differentially expressed clusters between the Geminivirus infected and mock-treated samples.

Data visualization and representation

We visualized the 22-nt sRNA clusters using the transcriptome browser function in the TOMATO_sRNA_SOL Expression database (Nakano et al., 2006; Nakano et al., 2020; <https://mpss.meyerslab.org/>). We generated dot plots using the ggplot2 package (Wickham, 2016), heatmaps using gplots package (Warnes et al., 2019), and heatmaps of DCL2 and AGO1 paralog expression using the TBTools heatmap function (Chen et al., 2018a).

RESULTS

Evolution of small RNA biogenesis genes in the Solanaceae

sRNA biogenesis and function are regulated by proteins in the Nuclear RNA Polymerase (NRP), RNA-dependent RNA polymerase (RDR), Double-stranded RNA binding protein (DRB), Dicer-Like protein (DCL) and Argonaute protein (AGO) families.

To describe and interpret sRNA biogenesis and function in the Solanaceae, we performed a phylogenetic analysis of these proteins encoded in genomes of *Arabidopsis thaliana*, two Convolvulaceae outgroups (*Ipomoea trifida* and *Ipomoea triloba*) and seven Solanaceae species (*Nicotiana benthamiana*, *N. tabacum*, *Petunia axillaris*, *P. inflata*, *Capsicum annuum*, *Solanum lycopersicum* and *S. tuberosum*). In Table 2 we summarize the number of protein genes encoded for *NRP*, *RDR*, *DCL*, *DRB* and *AGO* gene families in each species. Two observations emerged from this table. First, some gene families expanded when comparing *Arabidopsis* to members of the Convolvulaceae and/or Solanaceae families. For instance, the number of genes expanded in the *AGO* family comparing *A. thaliana* (10 *AGOs*) to Convolvulaceae (13 *AGOs*) or Solanaceae for the genus *Petunia* (12 or 13 *AGOs*) and the genus *Solanum* (13 or 15 *AGOs*). A second observation was the appearance of missing gene annotations for specific gene families in certain species (e.g. *NRP* genes encoded in tomato and potato). Thus, we chose to focus on *AGO* and *DCL* gene families in all species. All annotated genes are detailed in Supplemental Table 1. Their evolutionary relationships are visualized in Figure 1 and Supplemental Figure 2.

AGO proteins are required to load sRNAs and to catalyze their functional activity. *AGO* proteins can either trigger the biogenesis of sRNAs, through the miRNA-directed cleavage of *TAS/PHAS* transcripts, or catalyze sRNA regulatory activity through transcriptional or post-transcriptional regulation. Our phylogenetic analysis identified an expansion of specific and distinct *AGO* genes for Convolvulaceae and Solanaceae species when compared to *Arabidopsis*. For example, we identified a Convolvulaceae-specific expansion for *AGO5* (to three copies) and *AGO7* (to two copies) that did not occur in the Solanaceae (Figure 1; Table 2). We also identified differential *AGO* gene family expansion and loss across the Solanaceae. For instance, we expected to find two copies of *AGO2/AGO3* (based on gene family membership in *A. thaliana*) but we only identified one homolog in *Capsicum*, *Nicotiana ssp.* and *Petunia ssp.*, and three in *Solanum ssp.* (Figure 1; Table 2). A common feature that we observed across the Solanaceae was a family-wide expansion of *AGO1*; depending on the particular species, we identified two to three *AGO1* paralogs within the Solanaceae (Figure 1; Table 2). To test whether this *AGO1* expansion coincides with evidence for

subfunctionalization, we downloaded and visualized public expression data for the two *AGO1* paralogs we identified in *Solanum lycopersicum* (Figure 1B; http://bar.utoronto.ca/eplant_tomato/). Interestingly, we observed a distinct expression pattern of both copies with one mainly accumulating during vegetative development (Solyc06g072300), while the other mainly accumulated in fruits during reproductive development (Solyc03g098280). This observation suggests that indeed, these *AGO1* paralogs are in the process of subfunctionalization in tomato. We also discovered that *AGO2* expanded into triplicate copies that are located in tandem on chromosome 2 in tomato (Figure 1A). Existing public data indicates that only one of the three *AGO2* copies is expressed, and this is predominantly during fruit development (Figure 1C). In brief, we discovered lineage-specific expansions of *AGO* subfamily members in the Convolvulaceae and Solanaceae. Public expression data for tomato indicates that these *AGO1* and *AGO2* paralogs may have subfunctionalized following duplication.

We annotated additional sRNA biogenesis genes, including *NRP*, *RDR*, *DRB* and *DCL* (Figure 2; Supplementary Figure 2; Supplementary Table 2), and discovered a subfamily expansion of *DCL2* in the *Solanum* (tomato and potato) and *Capsicum* (pepper) branch of the Solanaceae. For all three species, *DCL2* expanded through a tandem duplication on chromosome 11 (annotated as chromosome 12 for pepper). In tomato, *DCL2* underwent additional duplication events, expanding to three tandem duplicates on chromosome 11 and additional copy on chromosome 6 (Figure 2). To gain insights into the impact of gene expansion on function, we downloaded public expression data for tomato, and found that the *DCL2* copy on chromosome 6 (Solyc06g048960) is broadly throughout the whole plant and the three paralogs on chromosome 11 (Solyc11g008520; Solyc11g008530; Solyc11g008540) are specifically enriched in flowers and fruits (Figure 2B). We also observed much higher expression levels for two of the *DCL2* paralogs on chromosome 11 (Solyc11g008530; Solyc11g008540) relative to the broadly expressed copy of *DCL2* on chromosome 6 (Solyc06g048960). Together, these observations highlight a Solanaceae-specific expansion and putative subfunctionalization of *DCL2*.

Mining publicly-available sRNA data for the Solanaceae

We selected seven core species in the Solanaceae (tomato, potato, pepper, ‘petunia_ax’ *Petunia axillaris*, ‘petunia_in’ *P. inflata*, tobacco, ‘benthi’ [*Nicotiana benthamiana*]) for our cross-species analyses of sRNA loci. These species were selected based on the availability of fully sequenced genome assemblies and publicly-available sRNA data. Notably, we excluded eggplant from this study due to the paucity of publicly-available *S. melongena* sequence data. In total, we analyzed over 2.6 billion sRNA reads from 255 different libraries. By far, tomato and potato were the most extensively sampled, having 84 and 73 high-quality publicly available libraries, respectively, while tobacco was the least sampled species (represented by only 9 libraries on the NCBI SRA; Supplemental Dataset 1). The depth of sequencing also varied quite substantially across libraries. This was largely due to the differences in sequencing technologies. In line with previous studies, we counterbalanced this heterogeneity in sequence depth by merging all high-quality libraries together and treating them as independent reference sets (Lunardon et al., 2020). Most of these reference sets included organ atlas samples that represent expression patterns from diverse organ systems and developmental stages. The total number of genome aligned reads for each species varied from just over 70-million reads for *Petunia axillaris* and tobacco to more than 650 million reads for tomato (Supplemental Dataset 1).

MicroRNA predictions indicate an expansion of novel miRNAs in *Nicotiana* species

We identified previously annotated and novel miRNAs across the Solanaceae using two software programs: ShortStack and miR-PREFeR (Axtell, 2013; Lei and Sun, 2014). After filtering for high-quality predictions based on hairpin folding and the presence of both strands of the microRNA duplex (Axtell and Meyers, 2018), we created a consensus set of miRNAs with assigned annotations based on $\geq 85\%$ sequence similarity to miRBase v22 (Supplemental Dataset 3). Overall, our analyses predicted between 124 and 440 miRNAs across the Solanaceous species (Figure 3; Supplemental Dataset 3). The depth of sequence data did not correlate strongly with the number of miRNAs that we predicted for a given species. For example, pepper (330)

and benthi (440) had the highest number of predicted miRNAs by far, even though these species are only supported by a moderate depth of sequence data relative to tomato, which only had 194 predicted miRNAs (Supplemental Dataset 3). This indicates that our miRNA predictions were generally not constrained by sampling.

We were surprised to find more than 400 predicted miRNAs in the benthi genome. To gain higher confidence in these benthi predictions, we manually filtered the benthi miRNA precursors based on the folding and expression of their predicted precursors using SructVis (Supplemental Datasets 3 & 6; <https://github.com/MikeAxtell/strucVis>). Even after manually filtering, we had a substantial number of predicted miRNAs for benthi (368), indicating that these predictions reflect a real lineage-specific miRNA expansion in *Nicotiana* (Figure 3A). In agreement with this conclusion, we also identified a relatively high number of miRNAs in tobacco (194), despite having the lowest depth of sequence data for this species. Notably, less than half of the miRNAs that we identified in either of the two *Nicotiana* genomes could be annotated using miRBase, indicating that miRNA expansion in *Nicotiana* was likely driven by the evolution of “young” lineage-specific miRNAs.

We also uncovered a substantial expansion of predicted miRNAs in pepper (330), which is congruent with previous miRNA predictions for this species (Seo et al., 2018; Taller et al., 2018). In contrast, we predicted relatively few miRNAs for both of the petunia genomes (124 - *P. inflata* and 126 - *P. axillaris*). This reduced number of predicted miRNAs in petunia may simply reflect a lack of sample diversity for these species; publicly-available sRNA-seq datasets for petunia are predominantly composed of floral samples (Supplemental Dataset 1). Indeed, a previous analysis using combined miRNA prediction software and a homology based search for known miRNAs uncovered about 140 miRNAs for both petunia genomes, indicating that our *de novo* predictions on their own are missing putative miRNA loci. Increasing the diversity of sRNA samples would likely improve miRNA annotations for this genus (Bombarely et al., 2016). Finally, we only identified a moderate number of predicted miRNAs in the Solanum genus (potato =

160, tomato = 194), despite having a substantial amount of sequence data (>80 sRNA libraries) for tomato. *Solanum* is by far the best characterized genus in the Solanaceae with regard to sRNA biology. Given that our predictions for this genus are similar to those from previous studies, we are likely approaching saturation for *Solanum*-specific miRNA predictions (Tomato Genome Consortium, 2012; Zhang et al., 2013; Cardoso et al., 2018; Zuo et al., 2020; Deng et al., 2021).

miRNA families are conserved within genera

To gain insight into miRNA conservation across Solanaceous species, we collapsed all predicted miRNAs into clusters using a 75% sequence similarity cutoff, and annotated the clusters with miRBase (v22 all land plant miRNAs (Kozomara et al., 2019); Supplemental Dataset 7). This resulted in 911 clusters; 392 of which are present in two or more species, 172 that are conserved at the genus level (i.e. *Solanum*-conserved, *Petunia*-conserved, and *Nicotiana*-conserved), and only 12 that are conserved across all seven species in this study (Supplemental Dataset 7; Figure 3B-C). The 12 conserved clusters can be further condensed into a handful of known miRNA families based on annotations that are broadly conserved across the land plants (e.g. - miR156/157, miR164, miR169/miR399, miR171, miR399, miR165/166/168, and miR172, miR482/miR2118 (Axtell and Meyers, 2018). We designated clusters that could not be annotated with miRBase as “novel.” The majority of these novel clusters appear to be newly-evolved, species-specific miRNAs, while the majority of clusters that are conserved across multiple species (present across ≥ 3 species) aligned with annotated miRNAs in miRBase. In agreement with previous studies showing that lineage-specific miRNAs tend to be weakly expressed relative to conserved miRNAs, we found that the expression of conserved miRNAs was significantly higher on average relative to novel miRNAs (Supplemental Figure 3; benthi p-value = 0.00057903, pepper p-value = 1.98548E-08, potato p-value = 1.97921E-06, tomato p-value = 2.27413E-07, petunia p-value = 2.39254E-05, tobacco p-value = 5.89638E-08; (Axtell et al., 2007)).

Solanaceae-specific miRNA family miR5303 is derived from MITE loci

Among the most conserved miRNA groupings, we identified three clusters containing a Solanaceae-specific miRNA, miR5303 (Gu et al., 2014). With the exception of petunia, the miR5303 family was present in relatively high copy numbers across the Solanaceous genomes (7 to 11 miRNAs in each species; Supplemental Dataset 4). It is rare for miRNAs to be present in such high copy numbers; however, our findings are congruent with a previous study that identified a high number of miR5303 copies in tomato, potato, eggplant, and pepper (Gu et al., 2014). To correctly identify miR5303 family members, we first aligned all miR5303 variants in miRBase v22 (Figure 4), and found that most of the variants are 24-nt in length, which again, is atypical for a bonafide miRNA family (Meyers et al 2008; Fig 4A). We identified nine miR5303 variants that are 21-nt in length in our tomato predictions that have $\geq 75\%$ similarity with miR5303 miRBase entries, and found that six of these tomato miRNAs clustered into a family containing a conserved 5' - UUUUG consensus sequence (highlighted by yellow rectangles in Fig 4B). To further characterize the function of this family, we performed a miRNA target prediction analysis using all coding sequences in the tomato genome as putative targets. We found that miR5303 family members are predicted to target thousands of genes in the tomato genome, which again, is unusual for a miRNA family (Supplemental Dataset 8). Given the presence of 24 nucleotide mature miRNA variants that are reminiscent of 24-nt siRNAs, and the perfect/near perfect folding of miR5303 precursor sequences that are often formed by inverted repeats (Figure 4C), we decided to test whether miR5303 actually originates from miniature inverted-repeat transposable elements (MITEs) in Solanaceae genomes. Notably, MITEs exist in high copy numbers, can form hairpin sequences that are structurally similar to miRNAs, and are known to generate 24-nt siRNAs (Kuang et al., 2009). To test whether miR5303 family members originated from MITEs, we blasted precursors of miR5303 from tomato against a plant MITE database (Chen et al, 2013) and found that they align perfectly with annotated MITEs, with the miR5303 mature miRNA variants mapping to the terminal inverted repeat (TIR) ends of the MITEs (Figure 4D). We therefore conclude that miR5303 is not a miRNA, but a MITE.

Identification of 21-nt *PHAS* loci support genus-level conservation of phasiRNA biology

We applied two bioinformatic tools: PHASIS (Kakrana *et al.* 2017) and ShortStack (Axtell 2013), to assemble a list of 21-nt *PHAS* loci across the Solanaceae. To classify coding versus non-coding phasiRNAs we merged *PHAS* locus coordinates with gene annotation files for each species. Some of the lesser studied Solanaceae genomes have incomplete annotations which resulted in an overprediction of non-coding loci for petunia, pepper, and benthi. To correct this imbalance, we used BLAST to align all non-coding *PHAS* loci to the nr database (Altschul *et al.*, 1990), and manually annotated loci that aligned to protein-coding, rRNA, and non-coding RNA sequences (Figure 5; Supplemental Dataset 4). *PHAS* loci are typically triggered by 22-nt miRNAs that have a 5' Uracil bias (Chen *et al.*, 2010; Cuperus *et al.*, 2010). To identify putative triggers for 21-nt phasiRNA production, we ran target predictions using our list of mature 22-nt miRNAs plus miR390 as triggers and predicted *PHAS* loci as targets (Supplemental Dataset 9).

PhasiRNAs are predominantly generated from transcripts of genes encoding disease resistance-related NB-LRRs, pentatricopeptide repeat (PPR) proteins, MYB transcription factors, and trans-acting siRNA (*TAS*) loci (Liu *et al.*, 2020; Shivaprasad *et al.* 2012). We identified 2-3 times more *PHAS* loci in *Solanum* (tomato and potato) and *Capsicum* (pepper) species than *Nicotiana* and *Petunia* (Figure 5). The majority of these *Solanum/Capsicum* loci were derived from disease resistance-related genes (Figure 5A); this list includes *TAS5*, a recently discovered *NB-LRR* locus that functions in pathogen response in tomato (Canto-Pastor *et al.*). In contrast, the *Nicotiana* and *Petunia* genomes expressed relatively few disease resistance related phasiRNAs (Figure 5A). We also found a substantial number of non-coding loci that we decided to examine in further detail with the goal of uncovering novel *TAS* loci within the Solanaceae. As a quality control, we confirmed that *TAS3*, a deeply-conserved non-coding phasiRNA, was detected in all of our species datasets (Supplemental Dataset 4; (Xia *et al.*, 2013)). Most notably, we discovered a previously uncharacterized, non-coding locus in both tomato and potato (Figure 5B). This locus is >80% conserved between the two species (Figure 5C) and generates highly abundant 21-nt siRNAs,

some of which putatively target genes encoding MYB transcription factors (Figure 5D). We performed a BLAST alignment between the tomato *PHAS* locus and all other non-coding *PHAS* loci to determine whether this locus exists outside of *Solanum*. This search failed to uncover any significant alignments, indicating that this novel phasiRNA evolved after *Solanum* split from other genera in the Solanaceae (Särkinen et al., 2013).

As mentioned above, PPR-derived *PHAS* loci are abundantly expressed in other species. In our analysis, we only identified two PPR-related *PHAS* loci in the Solanaceae: one in tomato (Solyc10g076450) and one in potato (PGSC0003DMG400020556). We did, however, find strong evidence for another, well-described siRNA mechanism for the transcriptional regulation of PPR genes, in which non-coding TAS-like-1/2 (TASL-1/2) loci are triggered by miR7122, generating 21-nt products that in turn target, and likely silence, PPR genes (Xia et al., 2013). We identified TASL1/2 and its associated miR7122 trigger in both *Nicotiana* species (bentham and tobacco). As demonstrated in other species, major 21-nt products from these TASL loci target PPR genes (Supplemental Figure 4). Surprisingly, we found no evidence for TASL1/2 and its miR7122 trigger in other Solanaceae genera. Given the conservation of this locus in other eudicots outside of the Solanaceae family, we hypothesize that miR7122-TASL1/2 is conserved in *Nicotiana* and was independently lost from the other genera in the nightshade family (Xia et al., 2013).

To investigate whether *PHAS* loci are generated from orthologous genes across species, we constructed gene orthogroups and searched for conserved *PHAS* loci within these orthogroupings (Supplemental Dataset 6). Overall, this analysis yielded surprisingly few orthologous *PHAS* loci within the Solanaceae. We did, however, discover that *DICER-LIKE2* (*DCL2*) is a broadly present phasiRNA-producing locus expressed in all of the species in our study (Figure 6). Interestingly, this *PHAS* locus has been previously reported in the Fabaceae, where it is triggered by different miRNAs in medicago and soybean, indicating convergent evolution of phasiRNA production at *DCL2* (Zhai et al., 2011). A *DCL2* *PHAS* locus has also been identified in tomato (Canto-Pastor et al. 2019); however, this is the first instance of its widespread presence across the nightshade family. As reported earlier in the results, *DCL2* has expanded

through tandem duplications that are shared in the tomato, potato, and pepper genomes (Figure 2). We found the genomic regions containing these tandem *DCL2* paralogs to be structurally conserved on *Solanum* chr11/*Capsicum* chr12 (Figure 6A). Recently, a *DCL2*-dependent miRNA (miR6026) was validated as a trigger for phasiRNAs derived from the *DCL2* locus in tomato (Wang et al., 2018), creating a negative feedback loop on *DCL2*. To investigate whether the same miRNA triggers initiate *DCL2* phasiRNA production in the rest of the Solanaceae, or, as in the Fabaceae (Zhai et al., 2011), independent triggers evolved at this locus, we looked for shared miRNA-*PHAS* locus interactions. We identified a conserved target site with a strong target score for miR10533 in tomato, potato, pepper, and petunia (Figure 6B). Like miR6026, miR10533 is also dependent on *DCL2* for its biogenesis (Wang et al., 2018). However, unlike in the Fabaceae, where phasiRNA production proceeds immediately after the miRNA target site (Zhai et al., 2011), *DCL2* phasiRNAs are produced both upstream and downstream of the miR10533/miR6026 target sites in the Solanaceae (Figure 6A). This imprecise initiation of phasing either indicates that there are additional miRNA triggers for this locus, or we are seeing the product of trans-triggering from 22-nt siRNAs that are generated by neighboring *DCL2* paralogs (Figure 6B).

hc-siRNAs and transposable elements across SOL genomes

Transposable elements (TEs) are recognized as one of the major evolutionary forces that can shape genomes, via mutations and genome rearrangements. Presumably as protection from the possible detrimental consequences generated by TEs, plants have evolved a mechanism using 24-nt heterochromatic siRNAs (hc-siRNAs) that methylate and repress TEs (Ahmed et al., 2011; Wang et al., 2013). In order to analyze the hc-siRNA landscape in Solanaceae, we first identified TEs in all the available genomes. We observed that TEs represent between 25% (in petunia) and 48% of the genome (in benthi) (Supplemental Figure 5). As expected, the majority of TEs belong to an abundant LTR retrotransposon family, with most LTRs mapping specifically to Gypsy/Ty3 and Copia/Ty1 elements (Galindo-González et al., 2017) (Supplemental Figure 5A). Previous work mapped an expansion of LTRs in the Pepper genome; our results indicate that this observation applies broadly across the Solanaceae, and is

likely a generalized signature of Solanaceous genomes (Park et al., 2012; Vicient and Casacuberta, 2017). After identifying TEs we analyzed the number of 24-nt reads that originate from each of the transposon superfamilies. Due to the repetitive nature of TEs, we did not attempt to further classify the sRNA reads into TE subfamilies. We observed that most of the sRNA reads mapped to retrotransposons, including LTR and non-LTR TEs (Supplemental Figure 5B). However, we were surprised to find that a large portion of hc-siRNAs also mapped to SINE elements. SINE elements comprise a unique subset of TEs; they are transcribed by RNA polymerase III, do not encode proteins, and are widespread in almost all multicellular eukaryotic genomes, with the exception *Drosophila* (Kramerov and Vassetzky, 2011). Our discovery of SINE-derived hc-siRNAs indicates that similar to LTRs, the spread of non-autonomous SINE elements is repressed via hc-siRNA-directed methylation.

Next, we analyzed the distribution of hc-siRNAs across Solanaceous genomes. We focused this analysis on tomato, pepper, potato and tobacco, which all have chromosome-scale assemblies. As expected, we found relatively high accumulation of hc-siRNAs along the telomeric ends of chromosomes; these hc-siRNAs promote telomere methylation in an RdDM-dependent manner (Vrbsky et al., 2010). We also observed a striking lack of hc-siRNAs across the entire potato genome (Figure 7). This result is similar to a previous observation described in strawberry, where, as in potato, asexual cloning is the primary means of propagation. Clonally propagated strawberry accumulates very low levels of DNA methylation relative to sexually propagated plants (Niederhuth et al., 2016). In the same way, we postulate that depleted hc-siRNA levels in potato could be caused by repeated rounds of asexual propagation. Further studies comparing sexually versus asexually propagated potato varieties would help resolve the connection between mode of reproduction and whether circumventing meiosis over multiple generations leads to degeneration of RdDM-directed methylation.

Discovery of 22 nucleotide clusters expressed in response to Geminivirus infection

In an exploratory analysis, we found a cluster of 22-nt sRNA reads mapped to a 4.8 kb region in the *S. lycopersicum* genome, and that this cluster is present in a ToLCV-infected sample of *S. lycopersicum* cv. Pusa Ruby and a TYLCV-infected sample of cv. Moneymaker, but absent from the respective mock-treated controls (Figure 8, Moneymaker data not shown). This cluster of 22-nt sRNA expression contains a *DISEASE RESISTANCE PROTEIN* (Solyc05g005330.3.1) and *ABSCISIC ALDEHYDE OXIDASE 3* (Solyc05g005333.1.1). To investigate whether there are additional 22-nt clusters that are specifically expressed in response to ToLCV/TYLCV infection, we applied a genome-wide analysis for clusters of 22-nt sRNAs. Using the aforementioned two infected and two control samples, we identified 1,521 22-nt sRNA clusters in total, 390 of which passed our filtering criteria (Supplemental Figure 6 and Supplemental Dataset 10). Twenty two of the clusters were induced by more than 4-fold in both cultivars in response to viral infection (Supplemental Figure 6B and Supplemental Table 2). Apart from the cluster shown in Figure 6A & B, one prominent example is the two clusters shown in Figure 6C & D, which are induced by ToLCV infection in cv. Pusa Ruby by approximately 1,000-fold and induced by TYLCV infection in cv. Moneymaker by approximately 10,000-fold. These clusters overlap with three gene copies of *EIN3-BINDING F-BOX PROTEIN 1* (Solyc05g008700.2.1, Solyc05g008720.1.1, and Solyc05g008730.1.1), one *MYZUS PERSICAE-INDUCED LIPASE 1* (Solyc05g008710.1.1), and one *RNA-DEPENDENT RNA POLYMERASE* (Solyc05g008740.1.1). Further studies are needed to test whether these Geminivirus-induced 22-nt sRNA clusters have a functional role in plant defense, or are potentially induced by the viral pathogen.

DISCUSSION

The Solanaceae family is widely used as a model for studying the evolutionary diversification of specialized metabolism (Fan et al., 2019), morphology (Koenig and Sinha, 2007; Martinez et al., 2020), fruit development (Klee and Giovannoni, 2011), and biotic and abiotic stress tolerance. In this study, we took advantage of the numerous publicly-available sRNA datasets that have been generated for the family to explore

unique aspects of sRNA biology within the Solanaceae. We found several features that are shared amongst diverse Solanaceous species including a Solanales-wide duplication of sRNA biogenesis machinery, a previously-annotated Solanaceae-specific miRNA family that we show is actually a MITE-derived miRNA-like siRNA, and DCL2-derived 21-nt phasiRNAs that are broadly expressed across the diverse taxa in our study. We also found several phenomena that evolved within sub-family lineages of the Solanaceae. We discovered a *Nicotiana*-specific expansion of novel miRNAs, a distinct split in the number and types of phasiRNAs that are produced in the *Capsicum/Solanum* versus the *Petunia/Nicotiana* branches of the family, a dramatic depletion of 24-nt hc-siRNAs in asexually propagated potato, and 22-nt siRNA clusters that accumulate in response to viral infection in tomato. Below, we discuss some of the evolutionary implications for Solanaceae-wide and sub-family specific sRNA phenomena that we discovered in this study.

Expansion and subfunctionalization of sRNA biogenesis machinery within the Solanaceae

In our reconstruction of sRNA biogenesis machinery gene trees, we uncovered lineage-specific expansions of *AGO1*, *AGO2* and *DCL2* gene families. While *AGO1* increased in copy number across the Solanaceae, *AGO2* and *DCL2* specifically expanded in the *Solanum*, and *Solanum/Capsicum* branches of the family, respectively, through tandem duplications. The tandem *DCL2* paralogs have been previously characterized to function as part of the tomato viral defense response ([Wang et al., 2018](#)), discussed in further detail below. Using public expression data for tomato, we identified distinct expression patterns in vegetative and reproductive organs for *DCL2* paralogs, with enriched abundance in reproductive organs, suggesting that these gene paralogs are undergoing subfunctionalization. Recent studies show that 24-nt phasiRNAs are produced only in reproductive organs of angiosperms, while 21-nt phasiRNAs are produced in both vegetative and reproductive tissues (Xia et al, 2019, Pokhrel et al., 2020). The 24-nt reproductive phasiRNAs are triggered by miR2275 in most of the angiosperms while in Solanaceous species, the miRNA trigger remains unknown (Xia et al, 2019, Pokhrel et al, 2021). Given that *DCL2* was shown to process

endogenous 22-nt miRNAs, and that the expression of some *DCL2* paralogs peak in reproductive organs, it would be interesting to functionally test whether this *DCL2* paralog expressed in reproductive tissues specifically processes a 22-nt miRNA that triggers reproductive 24-nt phasiRNA biogenesis.

Solanaceae-wide miR5303 cluster is derived from MITEs

Our cross-species miRNA predictions and clustering revealed 12 family-wide clusters of miRNAs. While the majority of these clusters are associated with deeply-conserved land plant miRNA families that are shared outside of the Solanaceae, we investigated the Solanaceae-specific cluster annotated as miR5303 (Mohorianu et al., 2011). We discovered that this family exhibits many features that are atypical of miRNAs. For example, related miR5303 sequences in miRBase include both 21-nt and 24-nt mature sequences and most of the Solanaceae genomes harbor relatively high copy numbers of miR5303 isomiRs that putatively target thousands of genes based on our miRNA-target prediction analysis. We found that, similar to a previous study on MITE-derived miRNA-like siRNAs, mature miR5303 “miRNA” sequences align perfectly to the terminal inverted repeat (TIR) region of Solanaceae MITEs. (Kuang et al., 2009). How did our miRNA prediction pipeline lead to the identification of MITE-derived sRNAs? MITEs have near perfect hairpin folding and express sRNAs within mature miRNA size ranges, and thus fit many of the criteria used to predict novel miRNA precursors. It has been proposed that MITEs may contribute to the *de novo* evolution of miRNAs. However, this model is somewhat controversial, as MITEs tend to express imprecise sRNAs that are variable in both length and sequence, and thus are more likely the products of siRNA rather than miRNA biogenesis machinery (Cui et al, 2017). Even so, the conspicuous presence of miR5303-related sRNAs exclusively in the Solanaceae is intriguing, and further investigation into putative functional roles for these siRNAs could be interesting.

***DCL2* is a broadly expressed *PHAS* locus that may control viral responses across the Solanaceae**

In a previous study focused on mechanisms of viral resistance in tomato, paralogous copies of *DCL2* were shown to process a 22-nt miRNA (miR6026) that has dual

functions, participating in antiviral responses and creating a self-regulating feedback loop by triggering *DCL2* phasiRNA production (Wang et al., 2018). In this study we identified *DCL2* as a widely-expressed *PHAS* locus that is present in all seven Solanaceous species in our study. Contrary to previous characterization of this locus, we predicted that another 22-nt miRNA, miR10533, triggers phasiRNA production in *Solanum*, *Capsicum*, and *Petunia* genera. Similar to miR6026, miR10533 targets disease resistance-related genes in addition to *DCL2* (*PHAS* target predictions table). Based on the accumulation patterns of *DCL2* phasiRNAs, neither miRNA appears to be an obvious trigger, although target mimic experiments with miR6026 showed reduced *DCL2* phasiRNA production (Wang et al., 2018). We identified the miR6026 site more than 250 bp outside of our predicted phasiRNA production region, whereas miR10533 is sandwiched within the phasiRNA register (Figure 6). The lack of an obvious trigger suggests two possibilities: 1) there may be another, as yet unidentified miRNA trigger, and/or 2) as hypothesized by previous authors, the 22-nt sRNA products generated from the *DCL2* paralogs may be cross triggering one another in *cis*, creating multiple phasiRNA initiation sites (Wang et al., 2018). We have likely saturated miRNA predictions for tomato, given the depth of sRNA sequence data available for this species, and thus it is unlikely that we are simply missing an additional trigger for *DCL2* phasiRNA production. Consistent with the second hypothesis, we observed weaker phasing scores across most of the *DCL2 PHAS* loci, indicating that cross-locus regulatory interactions trigger phasiRNA production across *DCL2* loci.

DCL2 PHAS loci have also been identified in two different Fabaceae models, soybean and medicago, where they are triggered by independent miRNAs (Li et al., 2010; Zhai et al., 2011). Our study, in combination with this previous work, shows that phasiRNA target-trigger interactions around *DCL2* have evolved multiple times within and across eudicot families. This evolutionary hotspot for phasiRNA production fits into a model in which the expression of sRNA biogenesis machinery is regulated via self-triggered phasiRNA production. Depending on the species, different components of sRNA biogenesis machinery are processed into *PHAS* loci. In soybean, for example, *SGS3* is targeted for phasiRNA production (Song et al., 2011), and in Arabidopsis, *AGO1* and

DCL1 are both processed into *PHAS* loci (Xie et al., 2003; Vaucheret et al., 2004). Why do plants use post-transcriptional regulation to control siRNA biogenesis machinery? One potential theory is that by regulating sRNA biogenesis machinery at the post-transcriptional level, plants can maintain a standing pool of transcripts for sRNA biogenesis that can be rapidly released and translated in response to pathogens.

Lineage-specific expression of miRNAs and phasiRNAs

One of our goals in this study was to identify miRNAs that are uniquely conserved within the Solanaceae; however, as discussed above, we demonstrate that miR5303, the only Solanaceae-specific “miRNA family” that we were able to predict in all of our study species, is actually a MITE-derived siRNA. Rather than uncovering broadly conserved miRNAs, we discovered numerous instances of lineage-specific miRNAs, with the most pronounced example coming from our representative species for *Nicotiana*. Even after stringent filtering, we predicted over 350 miRNAs in the benthi genome, with more than half of these comprising previously unannotated, lineage-specific miRNAs. Congruent with previous observations, we show that these lineage-specific miRNAs are generally expressed at lower levels than deeply conserved family members (Supplemental Figure 3) (Axtell et al., 2007). There are, however, a handful of newly annotated miRNAs that are abundantly expressed (>1,000 rpm; Supplemental Figure 3). Given the ease of agrobacterium infiltration in benthi, followup studies using transiently expressed target mimic knockdowns and PARE sequencing may provide new insight into the targets and putative functions of these rapidly-evolving *Nicotiana*-specific miRNAs. One bottleneck that we encountered in this analysis was a lack of deep sRNA sequence data for tobacco. Despite the lack of sampling in tobacco, we still predicted more than 150 miRNAs for this genome, which is similar to the number of miRNAs that we found in the tomato genome, despite having almost 10 times more sRNA sequence data for tomato. We are likely looking at the tip of the iceberg when it comes to miRNA discoveries in tobacco, and we predict that deeper sequencing in tobacco and related *Nicotiana* species will facilitate big gains when it comes to miRNA discovery in the Solanaceae.

In a reverse trend, our genome-wide 21-nt phasiRNA predictions uncovered relatively few *PHAS* loci for *Nicotiana* and *Petunia* species compared to the *Solanum/Capsicum* branch of the family (Figure 5A). A distinct lack of *PHAS* loci encoding NB-LRRs in *Nicotiana* and *Petunia* accounts for one of the biggest differences in the number of *PHAS* loci between these two branches of the family. We also discovered two strongly expressed non-coding *PHAS* loci that are conserved at the sub-family level. One is conserved in both benthi and tobacco, and was previously described to function in the post-transcriptional regulation of PPR genes (Supplemental Figure 4) (Xia et al., 2013). The other locus is uncharacterized, and specific to tomato and potato (Figure 5B). We discovered that this new *PHAS* locus produces a prominent 21-nt siRNA that putatively targets MYB transcription factors (Figure 5D). It would be interesting to test whether this *Solanum*-specific *PHAS* locus really does function in regulating the expression of MYB transcription factors. PhasiRNA production is triggered by miRNAs. Therefore, it is logical that in addition to discovering lineage-specific miRNAs, we also found lineage-specific *PHAS* loci across the Solanaceae.

Repressed hc-siRNA expression reflects a history of asexual propagation in potato

Our genome-wide hc-siRNA analysis revealed a low abundance of 24-nt TE-associated siRNAs in potato. We hypothesize that this relative depletion in potato is caused by the asexual means of propagation that is common for this crop. Epigenetic marks are faithfully copied during mitosis; however, during meiosis, they are reset by RdDM-based repatterning, which involves TE-derived hc-siRNAs directing DNA methylation (Daxinger et al., 2009; Schoft et al., 2009; Mosher and Melnyk, 2010; Slotkin et al., 2009; Verhoeven and Preite, 2014). Asexual propagation circumvents meiosis, and thus this RdDM-based resetting of epigenetic marks is avoided (Verhoeven and Preite, 2014), and over many rounds of asexual propagation DNA methyl-cytosine marks and their associated hc-siRNAs may become depleted. In line with this hypothesis, a related study investigating genome-wide DNA methylation patterns in diverse species found a stark depletion in CHH cytosine methylation in strawberry, another asexually propagated species (Niederhuth et al., 2016). However, there are limitations to our

conclusions regarding depleted hc-siRNAs in potato. These limitations include imperfect sampling, notably, the majority of our potato samples are from vegetative tissues (although this is true for most of the species in this study; Supplemental Dataset 1), and insufficient information about the source tissue that was used to generate these public datasets, particularly information regarding how many generations of asexual reproduction the source tissue underwent prior to sampling. Since sRNA-derived TE silencing gradually accumulates over multiple generations, a well-devised study comparing long-term asexually versus sexually propagated source tissue would help resolve whether levels of RdDM are directly impacted by asexual propagation, and whether these hc-siRNAs could be reactivated during reproductive development.

22-nt clusters expressed in response to viral infection in tomato

Recent work in Arabidopsis uncovered 22-nt siRNAs that are expressed in response to environmental stress, and are capable of impacting global translational repression, including the translation of their cognate genes. Moreover, this translational repression is correlated with an inhibition of growth and activation of stress-response pathways (Wu et al. 2020). In this study, we identified similar 22-nt siRNA clusters in tomato that are expressed in response to viral infection. Whether these siRNAs are also involved in attenuating global translation and growth so that more resources can be allocated to antiviral response remains to be seen. Several of the clusters that we identified are generated from genes involved in ABA production and ethylene signaling, indicating that these 22-nt siRNA clusters may coordinate genomic responses to pathogen attack by fine tuning the expression of ABA and ethylene pathways. Future work investigating whether this response is initiated by the plant in order to activate defense mechanisms or perhaps is hijacked by the virus in order to silence antiviral responses would provide insight into the evolutionary function of these distinctive siRNAs.

CONCLUSION

In conclusion, while genera within the Solanaceae share unifying features in the evolutionary history of their sRNA biology, there are many lineage-specific phenomena that are not broadly shared across the family. This conclusion is important as it provides

valuable information on the appropriateness of a given model species for providing generalized insights into sRNA biology. Simultaneously, we encountered limitations due to disparate sampling within the Solanaceae. For example, while sample data exist across developmental stages, environments, and genotypes to support investigations into tomato sRNA biology, there is a lack of publicly-available resources to study tomato's sister species, eggplant (Chapman, 2019). Similarly, our understanding of sRNA biology for *Petunia hybrida* is largely limited to reproductive samples. Given the sub-family phenomena that we identified in this study, we suggest that delving into under-sampled taxa rather than increasing sampling in well-studied species will yield a greater return on investment when it comes to discovering new aspects of sRNA biology within the Solanaceae.

LARGE DATASETS

Supplemental Dataset 1 – SRA Sample Processing
 Supplemental Dataset 2 – Solanaceae TEs
 Supplemental Dataset 3 – miRNAs Annotated by Species
 Supplemental Dataset 4 – phasiRNAs Annotated by Species
 Supplemental Dataset 5 – Solanaceae Orthogroups
 Supplemental Dataset 6 – Benthi structVis Filtering
 Supplemental Dataset 7 – Solanaceae miRNA Families
 Supplemental Dataset 8 – Tomato miRNA Target Predictions
 Supplemental Dataset 9 – PHAS Locus miRNA Interactions
 Supplemental Dataset 10 – Table of 22-nt sRNA Clusters

ACKNOWLEDGEMENTS

We thank Rui Xia for generating the annotation file that we used for the pepper (*Capscium annuum*) genome, and Feray Demirci and Deepti Ramachandrani for assistance with aspects of data processing. The work was supported with funding from NSF IOS awards 1842698, 754097 and 1650843, and USDA NIFA award 2019-67013-29010 to Meyers; NSF award 1523668 to M. Frank.

TABLES

Table 1: Reference genomes and software used for bioinformatic analyses

Species	Genome Version	File Source	Analyses
<i>Arabidopsis thaliana</i>	Araport11	Phytozome v13	MUSCLE, IQ-TREE, OrthoFinder
<i>Ipomoea triloba</i>	NSP323_V3 (Wu et al., 2018)	Sweetpotato Genomics Resource	MUSCLE, IQ-TREE, OrthoFinder
<i>Ipomoea trifida</i>	NSP306_V3 (Wu et al., 2018)	Sweetpotato Genomics Resource	MUSCLE, IQ-TREE, OrthoFinder
<i>Capsicum annum</i>	Zunla-1 v2.0 (Qin et al., 2014)	http://public.genomics.org.cn/BGI/pepper/	Expression DB, OrthoFinder, miR-Prefer, ShortStack, PHASIS, MUSCLE, IQ-TREE
<i>Petunia axillaris</i>	V1.6.2 (Bombarely et al., 2016)	ftp://ftp.solgenomics.net/genomes/	Expression DB, miR-Prefer, ShortStack, PHASIS, MUSCLE, IQ-TREE
<i>Petunia inflata</i>	V1.0.1 (Bombarely et al., 2016)	ftp://ftp.solgenomics.net/genomes/	Expression DB, miR-Prefer, ShortStack, PHASIS, MUSCLE, IQ-TREE
<i>Nicotiana tabacum</i>	V4.5 (Edward et al. 2017)	ftp://ftp.solgenomics.net/genomes/	Expression DB, miR-Prefer, ShortStack, PHASIS, MUSCLE, IQ-TREE
<i>Nicotiana benthamiana</i>	V0.3 (Bombarely et al., 2012)	ftp://ftp.solgenomics.net/genomes/	Expression DB, miR-Prefer, ShortStack, PHASIS

<i>Nicotiana benthamiana</i>	V0.4.4 (Bombarely et al., 2012)	ftp://ftp.solgenomics.net/genomes/	MUSCLE, IQ-TREE
<i>Solanum tuberosum</i>	ITAG v1.0 (Potato Genome Sequencing Consortium et al., 2011)	ftp://ftp.solgenomics.net/genomes/	Expression DB, miR-Prefer, ShortStack (miRNA prediction)
<i>Solanum tuberosum</i>	v3.4	ftp://ftp.solgenomics.net/genomes/	MUSCLE, IQ-TREE, Orthogroups
<i>Solanum tuberosum</i>	V4.04 (Hardigan et al., 2016)	http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml	PHASIS, ShortStack (PHAS locus prediction)
<i>Solanum lycopersicum</i>	V3.2	ftp://ftp.solgenomics.net/genomes/	Expression DB, miR-Prefer, ShortStack, PHASIS,
<i>Solanum lycopersicum</i>	V2.4 (Tomato Genome Consortium, 2012)	ftp://ftp.solgenomics.net/genomes/	MUSCLE, IQ-TREE
<i>Mimulus guttatus</i>	JGI v2.0 (Hellsten et al., 2013)	https://phytozome.jgi.doe.gov/pz/portal.html#!bulk?org=Org_Mguttatus	miR-Prefer, ShortStack, PHASIS,

Table 2: Summary of annotated AGO, DCL, DRB, NRP and RDR family members in Solanaceous and outgroup genomes.

Specie	Ploidy*	AGO	DCL	DRB	NRP	RDR
<i>Arabidopsis thaliana</i>	2n = 2x = 10	10	4	7	3	6
<i>Capsicum annuum</i>	2n = 2x = 24	11	5	4	5	5
<i>Ipomoea triloba</i>	2n = 2x = 30	13	4	7	5	6
<i>Ipomoea trifida</i>	2n = 2x = 30	13	4	7	4	5

<i>Nicotiana benthamiana</i>	2n = 4x = 38	18	4	7	6	7
<i>Nicotiana tabacum</i>	2n = 4x = 48	21	8	7	6	10
<i>Petunia axillaris</i>	2n = 2x = 14	12	4	3	3	4
<i>Petunia inflata</i>	2n = 2x = 14	13	4	5	3	5
<i>Solanum lycopersicum</i>	2n = 2x = 24	13	7	3	2	6
<i>Solanum tuberosum</i>	2n = 2x = 24	15	5	5	1	8

*The ploidy was assessed using the Kew Royal Botanical Gardens database (<https://cvalues.science.kew.org>)

FIGURE LEGENDS

Figure 1: Solanaceae-specific diversification of the AGO family highlights expansion and subfunctionalization of subfamily members. Phylogenetic tree of proteins encoded by the AGO gene family in the Solanaceae (A). Clades of AGO subfamily members are drawn with distinct colors. Yellow boxes highlight groups of AGO genes that have expanded in Solanaceae. Red circles indicate AGO homologs in tomatoes. Heatmaps show the relative expression of AGO1 and AGO2 from public data (B). Orthologous protein groups were identified using OrthoFinder and SonicParanoid. Protein alignments and phylogeny analysis were completed using MUSCLE and IQ- TREE. Phylogenetic tree was illustrated using iTOL.

Figure 2: Phylogenetic analysis of DCL family members encoded in the Solanaceae highlights a *Solanum/Capsicum*-specific expansion of DCL2. Phylogenetic tree of protein sequences indicating DCL subfamily members in the Solanaceae (A). Subclades of the DCL family are drawn with distinct colors and yellow boxes highlight groups of DCL2 that expanded in *Capsicum* and *Solanum* genera. Red circles indicate tomato paralogs for DCL2. Heatmaps show the relative abundance of these tomato DCL2 paralogs in diverse samples (B). Protein orthologous groups were identified using OrthoFinder and SonicParanoid. Protein alignments and phylogeny analysis were done using MUSCLE and IQ- TREE. Phylogenetic tree was visualized using iTOL.

Figure 3: MicroRNA predictions across Solanaceae support a lineage-specific expansion

of miRNAs. Venn diagrams of total miRNAs predicted by miRPrefer (red), Shortstack (yellow), and both programs (orange) for each species (A). Upset plots showing novel (B) and known (C) miRNAs that are shared across Solanaceae species. For each Upset plot: the bottom left panel shows the entire size of each set as a horizontal histogram, the bottom right panel shows the intersection matrix, and the upper right panel shows the size of each combination as a vertical histogram. The red line marks miRNAs common to all species, the purple line marks miRNAs common to both *Petunia* species, the orange line marks miRNAs common to both *Solanum* species, the green line marks miRNAs common to both *Nicotiana* species, and the blue and yellow lines mark benthi- and pepper-specific miRNAs, respectively.

Figure 4: miR5303 aligns to MITE terminal inverted repeat (TIR) sequences. The

previously-annotated Solanaceae-specific miRNA family, miR5303, exhibits many features that disqualify it as a bonafide miRNA. The majority of the miR5303 miRBase entries include mature sequences that are 24-nt in length, indicating that these sRNAs are likely derived from siRNA producing loci (A). miR5303 miRBase entries are highly variable in their sequence; we identified a consensus sequence to isolate true miR5303 family members (highlighted in the yellow box) (A). We filtered out miRNAs > 22-nt in length; however, our predicted miR5303 family members that are 21 or 22 nt follow the same 5' consensus sequence (UUUUG) as the miRBase entries (B). The predicted precursors of miR5303 family members have near perfect hairpin folding, indicating that these loci are likely inverted repeats, rather than real miRNA precursors (C), and BLAST alignment of miR5303 family members to the plant MITE database shows perfect/near perfect alignment of the precursors to MITE entries, with the mature miRNA sequences aligning to the TIR sequences (D).

Figure 5: Genus-specific trends of 21-nt PHAS loci within the Solanaceae. *Solanum*

(tomato and potato) and *Capsicum* (pepper) genera produce 2-3 times more PHAS loci and predominantly express disease resistance-related coding loci, relative to *Nicotiana* (tobacco and benthi) and *Petunia* genera (A). The raw number of loci for each category in (A) is shown in white. All of the species express non-coding PHAS loci (orange). Two previously unidentified non-coding TAS-like loci identified on chromosome 5 in tomato (left) and potato (right) show strong phasing (B) and 83% cross-species sequence conservation (C). An abundant 21-nt

product from this novel non-coding *PHAS* locus has sequence complementarity to MYB TFs (D).

Figure 6: *DCL2* phasiRNA-producing loci are conserved across Solanaceous species.

DCL2 paralogs in pepper, tomato, and potato, and orthologs in petunia, benthi, and tobacco all produce 21-nt phasiRNAs (A). We identified conserved miRNA target sites for miR10533 and miR6026 in pepper, tomato, potato, and petunia (red and yellow arrow respectively denotes cleavage sites) that has a strong target score, and may be a conserved mechanism for triggering *DCL2 PHAS* locus production in these species (B).

Figure 7: hc-siRNA expression across chromosomes in Solanaceae.

Normalized read counts for 24-nt TE-associated hc-siRNAs are plotted across the assembled chromosomes for tobacco, tomato, potato, and pepper. The x-axis represents the chromosome number, alternating between grey and black, and the y-axis represents the average number of reads at each chromosome position. The average read number was calculated based on the number of available libraries for each species. As expected, the majority of hc-siRNAs map to telomeric ends. Unexpectedly, potato accumulates a low proportion of 24-nt hc-siRNAs relative to total sRNAs.

Figure 8: Tomato genome produces 22-nt sRNA clusters in response to geminivirus infection.

sRNA reads mapped to two genomic regions in an infected-control sample pair are shown. The central red and blue boxes represent gene models from the 3.0 version of *S. lycopersicum* genome, and shaded boxes represent repetitive sequences. Dots represent sRNA reads, color-coded by length, mapped to the genomic region, with abundance represented by the position along the Y axis. Hollow dots are multi-mappers, and solid dots are unique-mappers. (A) and (C) are from a control, mock-infected sample (Sly_leaves_t1) and (B) and (D) are from the corresponding virus-infected sample (Sly_leaves_t2). (A) and (B) show the genomic region Ch05:268043..272842. (C) and (D) show the genomic region Ch05:2979082..2987879.

REFERENCES

Ahmed I, Sarazin A, Bowler C, Colot V, Quesneville H (2011) Genome-wide

1071 evidence for local DNA methylation spreading from small RNA-targeted sequences
1072 in Arabidopsis. *Nucleic Acids Res* 39: 6919–6931

1073 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment
1074 search tool. *J Mol Biol* 215: 403–410

1075 Axtell MJ (2018) The small RNAs of *Physcomitrella patens* : Expression, function
1076 and evolution. *Annual Plant Reviews online* 113–142

1077 Axtell MJ (2013) ShortStack: comprehensive annotation and quantification of small
1078 RNA genes. *RNA* 19: 740–751

1079 Axtell MJ, Meyers BC (2018) Revisiting Criteria for Plant MicroRNA Annotation in
1080 the Era of Big Data. *The Plant Cell* 30: 272–284

1081 Axtell MJ, Snyder JA, Bartel DP (2007) Common functions for diverse small RNAs
1082 of land plants. *Plant Cell* 19: 1750–1769

1083 Axtell MJ, Westholm JO, Lai EC (2011) Vive la différence: biogenesis and evolution
1084 of microRNAs in plants and animals. *Genome Biol* 12: 221

1085 Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina
1086 sequence data. *Bioinformatics* 30: 2114–2120

1087 Bombarely A, Moser M, Amrad A, Bao M, Bapaume L, Barry CS, Bliet M, Boersma
1088 MR, Borghi L, Bruggmann R, et al (2016) Insight into the evolution of the
1089 Solanaceae from the parental genomes of *Petunia hybrida*. *Nat Plants* 2: 16074

1090 Bombarely A, Rosli HG, Vrebalov J, Moffett P, Mueller LA, Martin GB (2012) A draft
1091 genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe
1092 biology research. *Mol Plant Microbe Interact* 25: 1523–1530

1093 Canto-Pastor A, Santos BA, Valli AA, Summers W, Schornack S, Baulcombe DC
1094 Enhanced resistance to bacterial and oomycete pathogens by short tandem target
1095 mimic RNAs in tomato. doi: 10.1101/396564

1096 Capella-Gutierrez S, Silla-Martinez JM, Gabaldon T (2009). trimAl: a tool for
1097 automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics*
1098 25:1972-1973.

1099 Cardoso TC de S, Alves TC, Caneschi CM, Santana DDRG, Fernandes-Brum CN,
1100 Reis GLD, Daude MM, Ribeiro THC, Gómez MMD, Lima AA, et al (2018) New
1101 insights into tomato microRNAs. *Sci Rep* 8: 16069

1102 Chapman MA (2019) Introduction: The Importance of Eggplant. In MA Chapman,
1103 ed, *The Eggplant Genome*. Springer International Publishing, Cham, pp 1–10

1104 Chen C, Chen H, He Y, Xia R (2018a) TBtools, a Toolkit for Biologists integrating

1105 various biological data handling tools with a user-friendly interface. *bioRxiv* 289660

1106 Chen C, Zeng Z, Liu Z, Xia R (2018b) Small RNAs, emerging regulators critical for
1107 the development of horticultural traits. *Horticulture Research* 5: 6–8

1108 Chen H-M, Chen L-T, Patel K, Li Y-H, Baulcombe DC, Wu S-H (2010) 22-
1109 Nucleotide RNAs trigger secondary siRNA biogenesis in plants. *Proc Natl Acad Sci*
1110 *U S A* 107: 15269–15274

1111 Cosentino S, Iwasaki W (2018). SonicParanoid: fast, accurate and easy orthology
1112 inference. *Bioinformatics*. 35(1):149-51.

1113 Cuperus JT, Carbonell A, Fahlgren N, Garcia-Ruiz H, Burke RT, Takeda A, Sullivan
1114 CM, Gilbert SD, Montgomery TA, Carrington JC (2010) Unique functionality of 22-nt
1115 miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in
1116 *Arabidopsis*. *Nat Struct Mol Biol* 17: 997–1003

1117 Daxinger L, Kanno T, Bucher E, van der Winden J, Naumann U, Matzke AJM,
1118 Matzke M (2009) A stepwise pathway for biogenesis of 24-nt secondary siRNAs
1119 and spreading of DNA methylation. *EMBO J* 28: 48–57

1120 Deng K, Yin H, Xiong F, Feng L, Dong P, Ren M (2021) Genome-wide miRNA
1121 expression profiling in potato (*Solanum tuberosum* L.) reveals TOR-dependent
1122 post-transcriptional gene regulatory networks in diverse metabolic pathway. *PeerJ*
1123 9: e10704

1124 Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accu- acy and
1125 high throughput. *Nucleic Acids Res* 32: 1792–1797

1126 Emms DM, Kelly S (2015). OrthoFinder: solving fundamental biases in whole
1127 genome comparisons dramatically improves orthogroup inference accuracy.
1128 *Genome biology* 16: 157.

1129 Esposito S, Aversano R, D'Amelia V, Villano C, Alioto D, Mirouze M, Carputo D
1130 (2018) Dicer-like and RNA-dependent RNA polymerase gene family identification
1131 and annotation in the cultivated *Solanum tuberosum* and its wild relative *S.*
1132 *commersonii*. *Planta* 248: 729–743

1133 Fan P, Leong BJ, Last RL (2019) Tip of the trichome: evolution of acylsugar
1134 metabolic diversity in Solanaceae. *Curr Opin Plant Biol* 49: 8–16

1135 Fei Q, Xia R, Meyers BC (2013) Phased, Secondary, Small Interfering RNAs in
1136 Posttranscriptional Regulatory Networks. *Plant Cell* 25: 2400–2415

1137 Galindo-González L, Mhiri C, Deyholos MK, Grandbastien M-A (2017) LTR-
1138 retrotransposons in plants: Engines of evolution. *Gene* 626: 14–25

1139 Goodin MM, Zaitlin D, Naidu RA, Lommel SA (2015) *Nicotiana benthamiana*: Its
1140 History and Future as a Model for Plant–Pathogen Interactions. *Molecular Plant-*

1141 Microbe Interactions 2015: 28–39

1142 Gu M, Liu W, Meng Q, Zhang W, Chen A, Sun S, Xu G (2014) Identification of
1143 microRNAs in six solanaceous plants and their potential link with phosphate and
1144 mycorrhizal signaling. *J Integr Plant Biol* 56: 1164–1178

1145 Guo Q, Qu X, Jin W (2015) PhaseTank: genome-wide computational identification
1146 of phasiRNAs and their regulatory cascades. *Bioinformatics* 31: 284–286

1147 Hardigan MA, Crisovan E, Hamilton JP, Kim J, Laimbeer P, Leisner CP, Manrique-
1148 Carpintero NC, Newton L, Pham GM, Vaillancourt B, et al (2016) Genome
1149 Reduction Uncovers a Large Dispensable Genome and Adaptive Role for Copy
1150 Number Variation in Asexually Propagated *Solanum tuberosum*. *Plant Cell* 28:
1151 388–405

1152 Hellsten U, Wright KM, Jenkins J, Shu S, Yuan Y, Wessler SR, Schmutz J, Willis
1153 JH, Rokhsar DS (2013) Fine-scale variation in meiotic recombination in *Mimulus*
1154 inferred from population shotgun sequencing. *Proc Natl Acad Sci U S A* 110:
1155 19478–19482

1156 Kakrana A, Li P, Patel P, Hammond R, Anand D, Mathioni SM, Meyers BC (2017)
1157 PHASIS: A computational suite for de novo discovery and characterization of
1158 phased, siRNA-generating loci and their miRNA triggers. *bioRxiv* 158832

1159 Kalyaanamoorthy S, Minh BQ, Wong TK, von Haeseler A, Jermiin LS (2017).
1160 ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature*
1161 *methods*. 14:587.

1162 Klee HJ, Giovannoni JJ (2011) Genetics and control of tomato fruit ripening and
1163 quality attributes. *Annu Rev Genet* 45: 41–59

1164 Koenig DP, Sinha NR (2007) Genetic Control of Leaf Shape. *Encyclopedia of Life*
1165 *Sciences*. doi: 10.1002/9780470015902.a0020101

1166 Kozomara A, Birgaoanu M, Griffiths-Jones S (2019) miRBase: from microRNA
1167 sequences to function. *Nucleic Acids Res* 47: D155–D162

1168 Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence
1169 microRNAs using deep sequencing data. *Nucleic Acids Res* 42: D68–73

1170 Kramerov DA, Vassetzky NS (2011) Origin and evolution of SINEs in eukaryotic
1171 genomes. *Heredity* 107: 487–495

1172 Kuang H, Padmanabhan C, Li F, Kamei A, Bhaskar PB, Ouyang S, Jiang J, Buell
1173 CR, Baker B (2009) Identification of miniature inverted-repeat transposable
1174 elements (MITEs) and biogenesis of their siRNAs in the Solanaceae: new
1175 functional implications for MITEs. *Genome Res* 19: 42–56

1176 Langmead B (2010) Aligning short sequencing reads with Bowtie. Curr Protoc
1177 Bioinformatics Chapter 11: Unit 11.7

1178 Lei J, Sun Y (2014) miR-PREFeR: an accurate, fast and easy-to-use plant miRNA
1179 prediction tool using small RNA-Seq data. Bioinformatics 30: 2837–2839

1180 Leong BJ, Lybrand DB, Lou Y-R, Fan P, Schillmiller AL, Last RL (2019) Evolution of
1181 metabolic novelty: A trichome-expressed invertase creates specialized metabolic
1182 diversity in wild tomato. Sci Adv 5: eaaw3754

1183 Letunic I, Bork P (2019) Interactive Tree Of Life (iTOL) v4: Recent updates and
1184 new developments. Nucleic Acids Res 47: W256–W259

1185 Liao Z, Hodén KP, Singh RK, Dixelius C (2020) Genome-wide identification of
1186 Argonautes in Solanaceae with emphasis on potato. Scientific Reports. doi:
1187 10.1038/s41598-020-77593-y

1188 Li H, Deng Y, Wu T, Subramanian S, Yu O (2010) Misexpression of miR482,
1189 miR1512, and miR1515 increases soybean nodulation. Plant Physiol 153: 1759–
1190 1770

1191 Liu Y, Teng C, Xia R, Meyers BC (2020) Phased secondary small interfering RNAs
1192 (phasiRNAs) in plants: their biogenesis, genic sources, and roles in stress
1193 responses, development, and reproduction. Plant Cell

1194 Lunardon A, Johnson NR, Hagerott E, Phifer T (2020) Integrated annotations and
1195 analyses of small RNA–producing loci from 47 diverse plants. Genome

1196 Martinez CC, Li S, Woodhouse MR, Sugimoto K, Sinha NR (2020) Spatial
1197 transcriptional signatures define margin morphogenesis along the proximal-distal
1198 and medio-lateral axes in tomato (*Solanum lycopersicum*) leaves. Plant Cell. doi:
1199 10.1093/plcell/koaa012

1200 Ma Z, Zhang X (2018) Actions of plant Argonautes: predictable or unpredictable?
1201 Curr Opin Plant Biol 45: 59–67

1202 Minh BQ, Nguyen MA, von Haeseler A (2013). Ultrafast approximation for
1203 phylogenetic bootstrap. Molecular biology and evolution. 30:1188-95.

1204 Mohorianu I, Schwach F, Jing R, Lopez-Gomollon S, Moxon S, Szittya G, Sorefan
1205 K, Moulton V, Dalmay T (2011) Profiling of short RNAs during fleshy fruit
1206 development reveals stage-specific sRNAome expression patterns: Time course
1207 study of short RNAs during fruit development. Plant J 67: 232–246

1208 Mosher RA, Melnyk CW (2010) siRNAs and DNA methylation: seedy epigenetics.
1209 Trends in Plant Science 15: 204–210

1210 Mueller LA, Solow TH, Taylor N, Skwarecki B, Buels R, Binns J, Lin C, Wright MH,

1211 Ahrens R, Wang Y, et al (2005) The SOL Genomics Network: a comparative
1212 resource for Solanaceae biology and beyond. *Plant Physiol* 138: 1310–1317

1213 Nakano M, McCormick K, Demirci C, Demirci F, Gurazada SGR, Ramachandruni
1214 D, Dusia A, Rothhaupt JA, Meyers BC (2020) Next-Generation Sequence
1215 Databases: RNA and Genomic Informatics Resources for Plants. *Plant Physiol* 182:
1216 136–146

1217 Nguyen LT, Schmidt HA, Von Haeseler A, Minh BQ (2015). IQ-TREE: a fast and
1218 effective stochastic algorithm for estimating maximum-likelihood phylogenies.
1219 *Molecular biology and evolution*. 32:268-74.

1220 Niederhuth CE, Bewick AJ, Ji L, Alabady MS, Kim KD, Li Q, Rohr NA, Rambani A,
1221 Burke JM, Udall JA, et al (2016) Widespread natural variation of DNA methylation
1222 within angiosperms. *Genome Biol* 17: 194

1223 Park M, Park J, Kim S, Kwon J-K, Park HM, Bae IH, Yang T-J, Lee Y-H, Kang B-C,
1224 Choi D (2012) Evolution of the large genome in *Capsicum annuum* occurred
1225 through accumulation of single-type long terminal repeat retrotransposons and their
1226 derivatives. *Plant J* 69: 1018–1029

1227 Pokhrel, S., Huang, K., & Meyers, B. C. (2021). Conserved and non-conserved
1228 triggers of 24-nt reproductive phasiRNAs in eudicots. *BioRxiv*,
1229 <https://doi.org/10.1101/2021.01.20.427321>

1230
1231 Pokhrel, S., Huang, K., Bélanger, S., Caplan, J. L., Kramer, E. M., & Meyers, B. C.
1232 (2020). Pre-meiotic, 21-nucleotide Reproductive PhasiRNAs Emerged in Seed
1233 Plants and Diversified in Flowering Plants. *BioRxiv*.
1234 <https://doi.org/10.1101/2020.10.16.341925>

1235
1236 Pombo MA, Rosli HG, Fernandez-Pozo N, Bombarely A (2020) *Nicotiana*
1237 *benthamiana*, A Popular Model for Genome Evolution and Plant–Pathogen
1238 Interactions. *The Tobacco Plant Genome* 231–247

1239 Potato Genome Sequencing Consortium, Xu X, Pan S, Cheng S, Zhang B, Mu D,
1240 Ni P, Zhang G, Yang S, Li R, et al (2011) Genome sequence and analysis of the
1241 tuber crop potato. *Nature* 475: 189–195

1242 Qin C, Yu C, Shen Y, Fang X, Chen L, Min J, Cheng J, Zhao S, Xu M, Luo Y, et al
1243 (2014) Whole-genome sequencing of cultivated and wild peppers provides insights
1244 into *Capsicum* domestication and specialization. *Proc Natl Acad Sci U S A* 111:
1245 5135–5140

1246 Särkinen T, Bohs L, Olmstead RG, Knapp S (2013) A phylogenetic framework for
1247 evolutionary study of the nightshades (*Solanaceae*): a dated 1000-tip tree. *BMC*
1248 *Evol Biol* 13: 214

1249 Schoft VK, Chumak N, Mosiolek M, Slusarz L, Komnenovic V, Brownfield L, Twell

1250 D, Kakutani T, Tamaru H (2009) Induction of RNA-directed DNA methylation upon
1251 decondensation of constitutive heterochromatin. *EMBO Rep* 10: 1015–1021

1252 Seo E, Kim T, Park JH, Yeom S-I, Kim S, Seo M-K, Shin C, Choi D (2018)
1253 Genome-wide comparative analysis in Solanaceous species reveals evolution of
1254 microRNAs targeting defense genes in *Capsicum* spp. *DNA Res* 25: 561–575

1255 Shinozaki Y, Nicolas P, Fernandez-Pozo N, Ma Q, Evanich DJ, Shi Y, Xu Y, Zheng
1256 Y, Snyder SI, Martin LBB, et al (2018) High-resolution spatiotemporal transcriptome
1257 mapping of tomato fruit development and ripening. *Nature Communications*. doi:
1258 10.1038/s41467-017-02782-9

1259 Shivaprasad PV, Chen H-M, Patel K, Bond DM, Bruno A C, Baulcombe DC (2012)
1260 A MicroRNA Superfamily Regulates Nucleotide Binding Site–Leucine-Rich Repeats
1261 and Other mRNAs. *Plant Cell* 24: 859–874

1262 Slotkin RK, Vaughn M, Borges F, Tanurdzić M, Becker JD, Feijó JA, Martienssen
1263 RA (2009) Epigenetic reprogramming and small RNA silencing of transposable
1264 elements in pollen. *Cell* 136: 461–472

1265 Song Q-X, Liu Y-F, Hu X-Y, Zhang W-K, Ma B, Chen S-Y, Zhang J-S (2011)
1266 Identification of miRNAs and their target genes in developing soybean seeds by
1267 deep sequencing. *BMC Plant Biol* 11: 5

1268 Sun W, Xiang X, Zhai L, Zhang D, Cao Z, Liu L, Zhang Z (2018) AGO18b
1269 negatively regulates determinacy of spikelet meristems on the tassel central spike
1270 in maize. *J Integr Plant Biol* 60: 65–78

1271 Sun X, Zhang J, Li A, Yuan X (2013) mirPD: A pattern-based approach for
1272 identifying microRNAs from deep sequencing data. *Digital Signal Processing* 23:
1273 1887–1896

1274 Taller D, Bálint J, Gyula P, Nagy T, Barta E, Baksa I, Szittya G, Taller J, Havelda Z
1275 (2018) Correction: Expansion of *Capsicum annum* fruit is linked to dynamic tissue-
1276 specific differential expression of miRNA and siRNA profiles. *PLoS One* 13:
1277 e0203582

1278 Tomato Genome Consortium (2012) The tomato genome sequence provides
1279 insights into fleshy fruit evolution. *Nature* 485: 635–641

1280 Turner SD (2014) qqman: an R package for visualizing GWAS results using Q-Q
1281 and manhattan plots. *bioRxiv* 005165

1282 Vaucheret H, Vazquez F, Crété P, Bartel DP (2004) The action of ARGONAUTE1
1283 in the miRNA pathway and its regulation by the miRNA pathway are crucial for
1284 plant development. *Genes Dev* 18: 1187–1197

1285 Verhoeven KJF, Preite V (2014) Epigenetic variation in asexually reproducing

1286 organisms. *Evolution* 68: 644–655

1287 Vicient CM, Casacuberta JM (2017) Impact of transposable elements on polyploid
1288 plant genomes. *Ann Bot* 120: 195–207

1289 Vrbsky J, Akimcheva S, Watson JM, Turner TL, Daxinger L, Vyskot B, Aufsatz W,
1290 Riha K (2010) siRNA-mediated methylation of Arabidopsis telomeres. *PLoS Genet*
1291 6: e1000986

1292 de Vries S, Kloesges T, Rose LE (2015) Evolutionarily Dynamic, but Robust,
1293 Targeting of Resistance Genes by the miR482/2118 Gene Family in the
1294 Solanaceae. *Genome Biol Evol* 7: 3307–3321

1295 Waese J, Fan J, Pasha A, Yu H, Fucile G, Shi R, Cumming M, Kelley L, Sternberg
1296 M, Krishnakumar V, Ferlanti E, Miller J, Town C, Stuerzlinger W, Provart NJ
1297 (2017). ePlant: Visualizing and Exploring Multiple Levels of Data for Hypothesis
1298 Generation in Plant Biology. *Plant Cell* 29:1806-1821.

1299 Wang X, Weigel D, Smith LM (2013) Transposon variants and their effects on gene
1300 expression in Arabidopsis. *PLoS Genet* 9: e1003255

1301 Wang Z, Hardcastle TJ, Pastor AC, Yip WH, Tang S, Baulcombe DC (2018) A
1302 novel DCL2-dependent miRNA pathway in tomato affects susceptibility to RNA
1303 viruses. *Genes & Development* 32: 1155–1160

1304 Won SY, Yumul RE, Chen X (2014) Small RNAs in Plants. In SH Howell, ed,
1305 *Molecular Biology*. Springer New York, New York, NY, pp 95–127

1306 Wu, H., Li, B., Iwakawa, H. oki, Pan, Y., Tang, X., Ling-hu, Q., Liu, Y., Sheng, S.,
1307 Feng, L., Zhang, H., Zhang, X., Tang, Z., Xia, X., Zhai, J., & Guo, H. (2020). Plant
1308 22-nt siRNAs mediate translational repression and stress adaptation. *Nature* 581:
1309 81-93

1310 Wu L, Zhang Q, Zhou H, Ni F, Wu X, Qi Y (2009) Rice MicroRNA Effector
1311 Complexes and Targets. *The Plant Cell* 21: 3421–3435

1312 Xia R, Meyers BC, Liu Z, Beers EP, Ye S, Liu Z (2013) MicroRNA superfamilies
1313 descended from miR390 and their roles in secondary small interfering RNA
1314 Biogenesis in Eudicots. *Plant Cell* 25: 1555–1572

1315 Xia R, Xu J, Arikait S, Meyers BC (2015) Extensive Families of miRNAs and PHAS
1316 Loci in Norway Spruce Demonstrate the Origins of Complex phasiRNA Networks in
1317 Seed Plants. *Mol Biol Evol* 32: 2905–2918

1318 Xie Z, Kasschau KD, Carrington JC (2003) Negative feedback regulation of Dicer-
1319 Like1 in Arabidopsis by microRNA-guided mRNA degradation. *Curr Biol* 13: 784–
1320 789

1321 Zhai J, Jeong D-H, De Paoli E, Park S, Rosen BD, Li Y, González AJ, Yan Z, Kitto
1322 SL, Grusak MA, et al (2011) MicroRNAs as master regulators of the plant NB-LRR
1323 defense gene family via the production of phased, trans-acting siRNAs. *Genes Dev*
1324 25: 2540–2553

1325 Zhang H, Xia R, Meyers BC, Walbot V (2015) Evolution, functions, and mysteries of
1326 plant ARGONAUTE proteins. *Curr Opin Plant Biol* 27: 84–90

1327 Zhang R, Marshall D, Bryan GJ, Hornyik C (2013) Identification and
1328 characterization of miRNA transcriptome in potato by high-throughput sequencing.
1329 *PLoS One* 8: e57233

1330 Zuo J, Grierson D, Courtney LT, Wang Y, Gao L, Zhao X, Zhu B, Luo Y, Wang Q,
1331 Giovannoni JJ (2020) Relationships between genome methylation, levels of non-
1332 coding RNAs, mRNAs and metabolites in ripening tomato fruit. *Plant J* 103: 980–
1333 994

1334

FIGURES

Figure 1: Solanaceae-specific diversification of the AGO family highlights expansion and subfunctionalization of subfamily members. Phylogenetic tree of proteins encoded by the AGO gene family in the Solanaceae (A). Clades of AGO subfamily members are drawn with distinct colors. Yellow boxes highlight groups of AGO genes that have expanded in Solanaceae. Red circles indicate AGO homologs in tomatoes. Heatmaps show the relative expression of AGO1 and AGO2 from public data (B). Orthologous protein groups were identified using OrthoFinder and SonicParanoid. Protein alignments and phylogeny analysis were completed using MUSCLE and IQ-TREE. Phylogenetic tree was illustrated using iTOL.

Figure 2: Phylogenetic analysis of DCL family members encoded in the Solanaceae highlights a *Solanum/Capsicum*-specific expansion of DCL2. Phylogenetic tree of protein sequences indicating *DCL* subfamily members in the Solanaceae (A). Subclades of the *DCL* family are drawn with distinct colors and yellow boxes highlight groups of *DCL2* that expanded in *Capsicum* and *Solanum* genera. Red circles indicate tomato paralogs for *DCL2*. Heatmaps show the relative abundance of these tomato *DCL2* paralogs in diverse samples (B). Protein orthologous groups were identified using OrthoFinder and SonicParanoid. Protein alignments and phylogeny analysis were done using MUSCLE and IQ- TREE. Phylogenetic tree was visualized using iTOL.

Figure 3: MicroRNA predictions across Solanaceae support a lineage-specific expansion of miRNAs. Venn diagrams of total miRNAs predicted by miRPrefer (red), Shortstack (yellow), and both programs (orange) for each species (A). Upset plots showing novel (B) and known (C) miRNAs that are shared across Solanaceae species. For each Upset plot: the bottom left panel shows the entire size of each set as a horizontal histogram, the bottom right panel shows the intersection matrix, and the upper right panel shows the size of each combination as a vertical histogram. The red line marks miRNAs common to all species, the purple line marks miRNAs common to both *Petunia* species, the orange line marks miRNAs common to both *Solanum* species, the green line marks miRNAs common to both *Nicotiana* species, and the blue and yellow lines mark benthi- and pepper-specific miRNAs, respectively.

Figure 4: miR5303 aligns to MITE terminal inverted repeat (TIR) sequences. The previously-annotated Solanaceae-specific miRNA family, miR5303, exhibits many features that disqualify it as a bonafide miRNA. The majority of the miR5303 miRBase entries include mature sequences that are 24-nt in length, indicating that these sRNAs are likely derived from siRNA producing loci (A). miR5303 miRBase entries are highly variable in their sequence; we identified a consensus sequence to isolate true miR5303 family members (highlighted in the yellow box) (A). We filtered out miRNAs > 22-nt in length; however, our predicted miR5303 family members that are 21 or 22 nt follow the same 5' consensus sequence (UUUUG) as the miRBase entries (B). The predicted precursors of miR5303 family members have near perfect hairpin folding, indicating that these loci are likely inverted repeats, rather than real miRNA precursors (C), and BLAST alignment of miR5303 family members to the plant MITE database shows perfect/near perfect alignment of the precursors to MITE entries, with the mature miRNA sequences aligning to the TIR sequences (D).

Figure 7: hc-siRNA expression across chromosomes in Solanaceae. Normalized read counts for 24-nt TE-associated hc-siRNAs are plotted across the assembled chromosomes for tobacco, tomato, potato, and pepper. The x-axis represents the chromosome number, alternating between grey and black, and the y-axis represents the average number of reads at each chromosome position. The average read number was calculated based on the number of available libraries for each species. As expected, the majority of hc-siRNAs map to telomeric ends. Unexpectedly, potato accumulates a low proportion of 24-nt hc-siRNAs relative to total sRNAs.

Figure 8: Tomato genome produces 22-nt sRNA clusters in response to geminivirus infection.

sRNA reads mapped to two genomic regions in an infected-control sample pair are shown. The central red and blue boxes represent gene models from the 3.0 version of *S. lycopersicum* genome, and shaded boxes represent repetitive sequences. Dots represent sRNA reads, color-coded by length, mapped to the genomic region, with abundance represented by the position along the Y axis. Hollow dots are multi-mappers, and solid dots are unique-mappers. (A) and (C) are from a control, mock-infected sample (Sly_leaves_t1) and (B) and (D) are from the corresponding virus-infected sample (Sly_leaves_t2). (A) and (B) show the genomic region Ch05:268043..272842. (C) and (D) show the genomic region Ch05:2979082..2987879.