

Heat-responsive microRNAs and phased small interfering RNAs in reproductive development of flax

Suresh Pokhrel^{1,2}  | Blake C. Meyers^{1,2} 

¹Division of Plant Science and Technology, University of Missouri, Columbia, Missouri, USA

²Donald Danforth Plant Science Center, St. Louis, Missouri, USA

Correspondence

Blake C. Meyers, Donald Danforth Plant Science Center, St. Louis, MO 63132, USA.
Email: bmeyers@danforthcenter.org

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Abstract

Plants will face increased heat stress due to rising global temperatures. Heat stress affects plant reproductive development and decreases productivity; however, the underlying molecular mechanisms of these processes are poorly characterized. Plant small RNAs (sRNAs) have important regulatory roles in plant reproductive development following abiotic stress responses. We generated sRNA transcriptomes of reproductive bud stages at three different time points to identify sRNA-mediated pathways responsive to heat stress in flax (*Linum usitatissimum*). With added sRNA transcriptomes of vegetative tissues, we comprehensively annotated miRNA and phasiRNA-encoding genes (*PHAS*) in flax. We identified 173 miRNA genes, of which 42 are newly annotated. Our analysis revealed that 141 miRNA genes were differentially accumulated between tissue types, while 18 miRNA genes were differentially accumulated in reproductive tissues following heat stress, including members of miR482/2118 and miR2275 families, known triggers of reproductive phasiRNAs. Furthermore, we identified 68 21-*PHAS* flax loci from protein-coding and noncoding regions, four 24-*PHAS* loci triggered by miR2275, and 658 24-*PHAS*-like loci with unknown triggers, derived mostly from noncoding regions. The reproductive phasiRNAs are mostly downregulated in response to heat stress. Overall, we found that several previously unreported miRNAs and phasiRNAs are responsive to heat stress in flax reproductive tissues.

KEYWORDS

flax, heat stress, microRNA, phasiRNA

1 | INTRODUCTION

Agricultural production is negatively impacted by climate change, especially because of higher temperatures. The global yield of three main staple crops is estimated to be reduced by 3.2%–6.0% per 1°C increase in global mean temperature (Zhao et al., 2017). The effect of higher temperature is greater on reproductive development, especially through impacts on pollen development, pollination, and embryo formation (Hedhly et al., 2009). Although there are studies that assess

the impacts of higher temperature on growth, physiological processes, and yields on crops such as wheat, maize, rice, canola, and flax (Cross et al., 2003; Pokharel et al., 2020; Wang et al., 2016; Zhao et al., 2017), limited knowledge is available on the underlying molecular mechanisms that play roles in heat stress; such knowledge would enable the development of thermo-tolerant crops.

Heat shock proteins (HSPs) are major players in heat tolerance (Hasanuzzaman et al., 2013). HSPs are produced in response to heat stress after activation by heat stress transcription factors (HSFs) such

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as MULTIPROTEIN BRIDGING FACTOR 1C, WRKYGQK motif-containing proteins, and basic leucine zipper proteins (He et al., 2019; Zhang et al., 2017). Multiple thermosensors initiate HSF activation after sensing heat stress. The conserved heat tolerance process in plants involves regulatory cascades of heat-responsive genes as well as other regulators such as noncoding RNAs that play a role in modulation of gene expression, and these noncoding molecules are yet to be identified.

Plant sRNAs are implicated in modulating expression of genes related to heat stress (He et al., 2019). These sRNAs are regulatory elements that modulate gene expression, genome-wide, by transcriptional and post-transcriptional gene silencing. Typically, sRNAs are 20–24 nucleotides (nt) in length and are diced by DICER-LIKE proteins (DCLs) from hairpin or double stranded RNA precursors formed by RNA-DEPENDENT RNA POLYMERASEs (RDRs). The sRNAs form a silencing complex with Argonaute (AGO) proteins to modulate the expression of genes and transposable elements (TEs). The major classes of plant sRNAs are as follows: microRNAs (miRNAs), phased secondary small interfering RNAs (phasiRNAs), and secondary small interfering RNA (siRNAs) (Axtell, 2013; Axtell et al., 2006; Fei et al., 2013; Reinhart et al., 2002; Zhai et al., 2011).

The miRNAs are the key regulators in the attenuation of gene expression, by transcript cleavage and degradation or translation inhibition in a homology-dependent manner (Zhai et al., 2011). There have been reports of heat-responsive miRNAs such as miR398, miR156, miR159, miR160, miR167, miR396, and miR408 during vegetative and reproductive stages of plants like Arabidopsis, sunflower, maize, and tomato (Giacomelli et al., 2012; Guan et al., 2013; He et al., 2019; Keller et al., 2020; Lin et al., 2018; Stief et al., 2014). PhasiRNAs are generated from mRNA transcripts of coding and noncoding regions of the genome mainly after the cleavage of trigger miRNAs (Fei et al., 2013; Johnson et al., 2009; Zhai et al., 2011). A special class of phasiRNAs that are produced from *trans*-acting siRNA loci (TAS) is called tasiRNAs (Fei et al., 2013; Howell et al., 2007). In Arabidopsis, tasiRNAs from the TAS1 locus are found to target transcripts of HEAT-INDUCED TAS1 TARGET 1 (HTT1) and HTT2 proteins that accumulate in response to heat stress (Li et al., 2014). Complete loss of reproductive 24-nt phasiRNAs after knock out of DCL5, a reproductive phasiRNA pathway gene, in maize, produces male sterility at high temperatures (Teng et al., 2020), while mutation in reproductive 21-nt phasiRNAs-generating loci produces photoperiod- and thermo-sensitive male sterility in rice (Fan et al., 2016; Zhou et al., 2012).

Flax is a widely cultivated crop species from the family Linaceae known for oil, fiber, and medicinally important compounds. Heat stress in flax reduces boll formation and seed set, and it may be due to the failure of pollen tubes to reach ovules (Cross et al., 2003). In this study, flax plants were subjected to 7- and 14-day heat treatments after flower initiation in a growth chamber to simulate heat stress conditions in the field. We harvested three reproductive bud stage tissues at three time points and generated sRNA transcriptomes to identify sRNA-mediated pathways responsive to heat stress. With added publicly available sRNA transcriptomes of leaves, stem, and root, we comprehensively annotated miRNA- and phasiRNA-

spawning genes (PHAS) in flax. We found that several miRNAs and both types of reproductive phasiRNAs (21 and 24 nt) are mostly downregulated in response to heat stress. Overall, we found that several previously unreported flax miRNAs and phasiRNAs are responsive to heat stress in reproductive tissues.

2 | RESULTS

2.1 | Comprehensive annotation of miRNA in flax

In the latest release of miRBase version 22 (<http://www.mirbase.org>), there are 124 miRNA precursors deposited for flax, mainly identified from computational approaches (Barvkar et al., 2013). There are studies that annotated flax miRNAs based on sRNA sequencing in control, nutrient, saline, and alkaline stress conditions (Melnikova et al., 2016; Yu et al., 2016), but their miRNA lists are not available from miRBase; these studies predicted a high number of novel candidate miRNAs (233–475), in addition to observing known miRNAs, suggesting that most of them may be false positives. Because the highly studied model plant Arabidopsis only has a few hundred miRNAs, caution may be warranted for reports of more than 100 novel miRNAs in a species (Axtell & Meyers, 2018). Further, all these flax miRNA identifications were carried out using an older scaffold-based genome (Wang et al., 2012). Recently, a chromosome-scale genome assembly has been released for flax (You et al., 2018).

To comprehensively annotate miRNA precursors in the latest flax genome assembly version 2 (ASM22429v2) (You et al., 2018), sRNA libraries were sequenced from leaves, stem, and root tissues. In addition, to identify heat-responsive miRNAs in flax, sRNA transcriptomes from reproductive bud stages (Figure S1a) at three time points were sequenced (see Section 4 for details). In total, we generated 24 sRNA libraries, and clean reads (after removing rRNA/tRNA sequences) were used for miRNA prediction (Table S1). To accurately identify miRNA precursors, we used ShortStack and miR-PREFeR, some of the best-performing miRNA annotation tools in terms of precision and accuracy (Johnson et al., 2016; Lei & Sun, 2014). We identified 173 candidate miRNA precursors yielding 106 nonredundant mature miRNAs (Figure 1, outer circle; Table S2). Among them, 131 precursors produced known miRNAs, while 42 precursors generated novel miRNAs which contained at least five variants (mismatches plus unaligned nucleotides) with Viridiplantae miRNAs derived from miRBase version 22. We identified miRNAs from 31 known miRNA families; among them, the families having the greatest numbers of precursors encoded into the genome were miR167 (14), miR482/2118 (12), miR156 (11), miR166 (9), and miR160 (9) (Figure S1b, Table S2).

2.2 | Differentially accumulating miRNA precursors in flax tissues and heat treatments

miRNA species (miRNA–miRNA* pairs) are produced from precise cleavage of stem-loop RNA precursors, called miRNA precursors. The

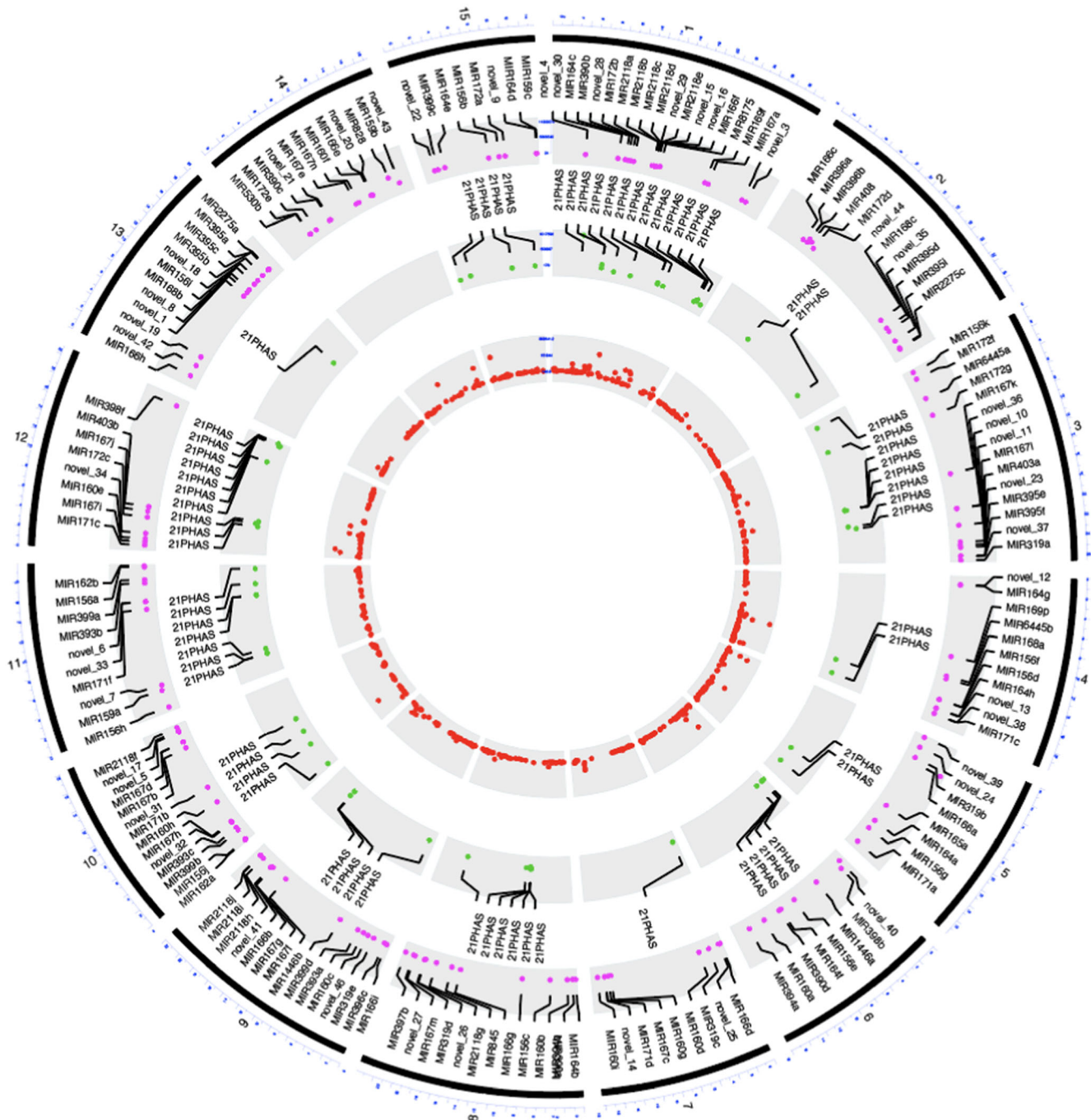


FIGURE 1 Genome-wide distribution of MIR genes and PHAS loci in flax. Chromosomal position of 173 MIR genes (outer circle, pink dots), 68 21-PHAS loci (middle circle, green dots), and 662 24-PHAS/24-PHAS-like loci (inner cycle, red dots). The MIR genes and 21-PHAS loci were annotated in each chromosome. The outermost cycle represents chromosomes from 1 to 15 as numbered

miRNA species accumulate differentially to perform their roles in developmental processes in vegetative and reproductive tissues. To assess miRNA precursor abundance, we summed the abundance of sRNAs that mapped to the entire region of the hairpin, and this was used also for subsequent differential expression (DE) analyses. We used this approach for DE of miRNA loci because it accounts for the abundance of the miRNA mature sequence, its variants, and the star

sequence, instead of using the abundance value of the single mature miRNA. Here, we characterized the abundance of miRNA loci in different tissue stages and summarized the results (Table 1). In total, we found 140 miRNA loci varying in abundance between vegetative and reproductive tissues; among them, 89 were enriched in vegetative tissues (Figure 2a, Table S3), while 51 were enriched in reproductive tissues (Figure 2b, Table S3). Vegetative tissue-enriched miRNA loci

TABLE 1 Differentially accumulated miRNA precursors in different tissues of flax

	Leaves	Stem	Root	B_2_4	B_5_7	B_8_10
Leaves		30D, 18U	35D, 46U	28D, 40U	33D, 31U	28D, 27U
Stem			11D, 32U	16D, 36U	16D, 25U	21D, 25U
Root				45D, 42U	51D, 39U	53D, 31U
B_2_4					5 D, 0U	14D, 4U
B_5_7						2D, 2U

Note: miRNAs that were significantly accumulated in different tissues were denoted as downregulated (D) and upregulated (U) ($p < .05$, FDR $< .05$, Table S3). B_2_4 indicates bud stage 1 with buds 2–4 mm in length, B_5_7 indicates bud stage 2 with buds 5–7 mm in length, and B_8_10 indicates bud stage 3 with buds 8–10 mm in length.

were divided into four sets based on their enrichment into different vegetative tissues. Set I represents miRNA loci more abundant in both leaves and root and includes members of miRNA families miR160, miR156, miR166, miR393, miR395, miR845, miR482, and several novel miRNAs (22, 26, and 46). Set II represents miRNA loci enriched mostly in root tissues only and composed of members of miR164, miR156, miR395, miR159, miR399, miR394, and several novel miRNAs (20, 38, and 13). Set III, mostly enriched in both stem and root tissues, is composed of members of miR8175, miR396, miR395, miR169, miR172, miR171, miR159, and novel miRNAs (15, 16, 25, 17, 10, 14, 11, 36, and 3). Set IV-related loci were enriched mostly in leaves only and composed of members related to miR167, miR171, miR166, miR162, miR172, miR396, miR530, miR396, miR6445, miR168, miR397, miR408, miR398, and several novel miRNAs (9, 31, 39, and 12). We divided the group of miRNA loci that were enriched more in reproductive tissues into three sets (Set V, VI, and VII). Set V, mostly enriched in all three reproductive tissues along with stem and root, includes members of miR156, miR319, and miR482/2118. Set VI, mostly enriched in reproductive tissues, includes members of miR171, miR390, miR1446, miR482/2118, miR2275, miR169, and novel miRNAs (30, 24, and 7). The last, Set VII, includes miRNA loci that were enriched in reproductive tissues and leaves, and consists of members of miR160, miR393, miR164, miR403, miR171, miR167, and novel miRNAs (5, 18, 33, 23, and 43).

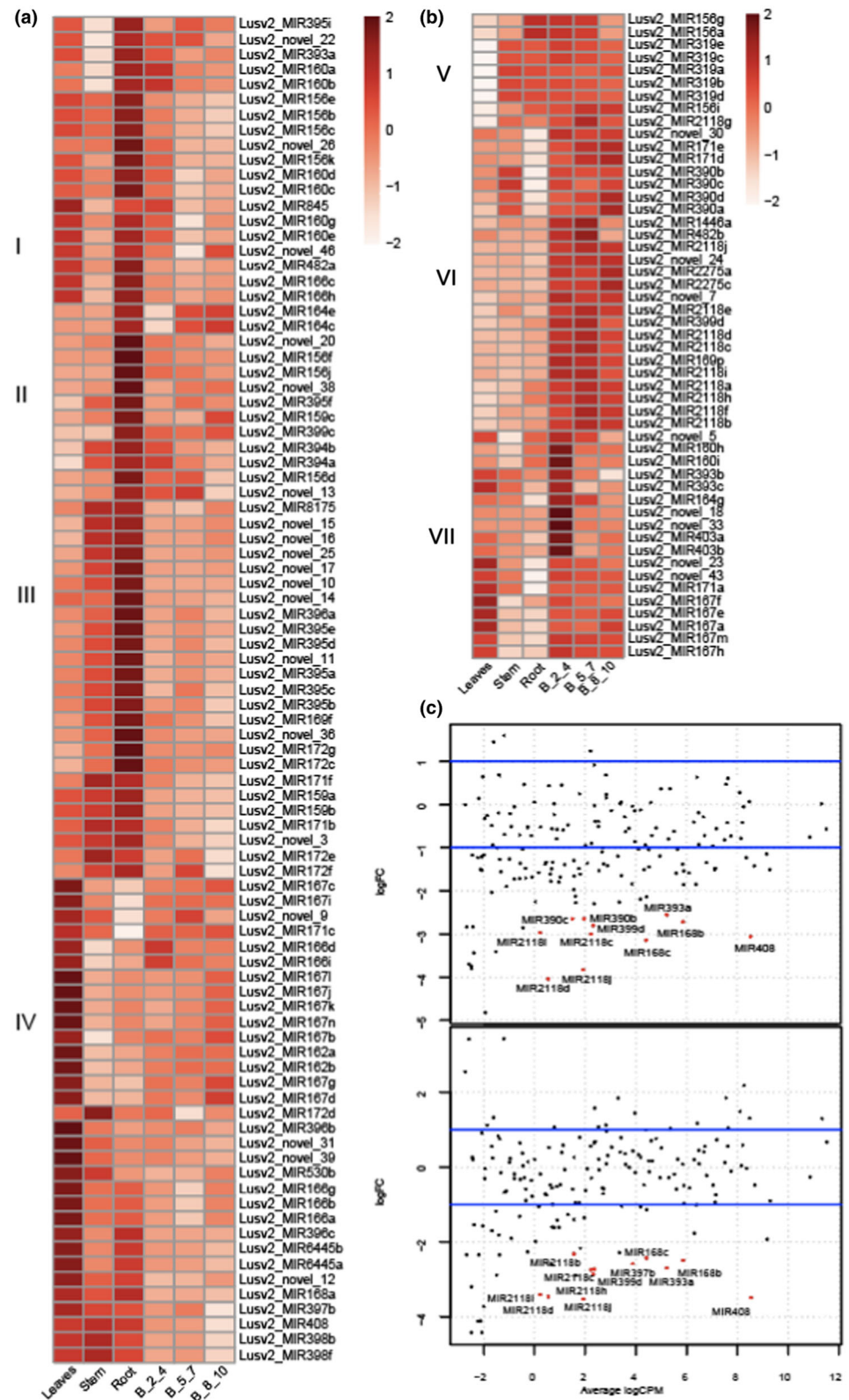
In this study, we subjected flax to two heat treatments during flowering as reported in (Cross et al., 2003) (more in Section 4 for details); and these heat treatments resulted in differential abundances of a total of 18 miRNA loci (Figure 2c, Figure S2a, Table S4). These were mostly downregulated due to heat stress, with one exception: miR8175 (Figure S2b). The downregulated miRNA loci under heat stress are members of the families of miR482/2118, miR390, miR168, and miR393a, miR399d, miR397b, miR408, and novel_7. Compared with other bud stages, bud stage 2 is the most sensitive to heat stress, and 11 miRNA loci were differentially accumulated after 7 days of heat treatment. There were no differentially accumulated miRNA loci identified between the two different treatments in all stages, but with an increased number of heat stress days, from 7 to 14, more miRNA loci were differentially expressed (Table 2). Overall, members of the miR482/2118 family were enriched in reproductive tissues and they initiate the production of 21-nt reproductive phasiRNAs (Pokhrel, Huang, Bélanger, et al., 2021).

2.3 | Genome-wide identification of phasiRNAs in flax

Plant genomes encode loci that produce secondary siRNAs that are generated from a miRNA cleavage site within a polyadenylated RNA; these are generated in a phased pattern and are called *PHAS* loci. These loci can be derived from both protein-coding (mRNA) and non-coding (long, noncoding RNA or lncRNA) transcripts. We used small RNA libraries from both vegetative and reproductive tissues to identify *PHAS* loci in flax. We identified 68 21-*PHAS* loci (i.e., loci-generating 21-nt phasiRNAs) from both coding and noncoding regions (Figure 1, middle circle; Table 3; Table S5). Out of 68 loci, 51 were enriched in reproductive tissues (Figure 3a), while 17 loci were enriched in vegetative tissues (Figure S3a). We identified three loci of miR390-*TAS3*, the most conserved phasiRNA pathway in land plants (Xia et al., 2015) (example locus in Figure 3b). We found two noncoding *TAS-like* (*TASL*) loci enriched in leaves and reproductive tissues triggered by miR167h (example locus in Figure 3c, miR167 variants in Figure 3d) and another 12 noncoding loci enriched in reproductive tissues with unknown triggers (Figure 3a). We found triggers of at least 30 21-*PHAS* loci, including miR167 as a trigger for a locus encoding a metal tolerance protein, miR168 for *AGO1*, miR393 for *AFB2*, miR6445 for *NAC*, miR408 for a laccase-encoding gene, and miR482/2118 for noncoding reproductive 21-*PHAS* loci that were recently described (Pokhrel, Huang, Bélanger, et al., 2021). The other gene families for which triggers were not identified included genes encoding BHLH, SCD, RING-type, Zf-C3H4, and BZIP domain-containing proteins, a eukaryotic translation initiation factor, and an E3 ubiquitin protein ligase (Table 3).

Recently, 24-nt phasiRNAs were reported in several eudicots (Pokhrel, Huang, & Meyers, 2021; Xia et al., 2019); thus, we looked for the conservation of this pathway in flax. We identified four 24-*PHAS* loci triggered by miR2275 and an additional 658 24-*PHAS*-like loci with unknown triggers (Figure 1, inner circle; Figure S3; Table S6). These 24-*PHAS*-like loci passed the software's filtering criteria for *PHAS* loci, and the loci were mostly derived from nonrepetitive regions of the genome. However, when we examined a subset in our genome browser (Nakano et al., 2020), we found these loci display poor phasing, with phasing scores ranging from 15 to 20. Due to their poor phasing and unknown triggering mechanism, it is more likely that these 24-nt sRNAs could be hc-siRNAs that are abundant

FIGURE 2 Differentially accumulated *MIR* genes in different tissues type and heat treatments. (a) Heatmap showing differentially accumulated *MIR* genes enriched in vegetative tissues with four sets of *MIR* genes: Set I, enriched in leaves and root; Set II, enriched in roots; Set III, enriched in stem and roots; and Set IV, enriched in leaves. (b) Similar to (a) except enriched in reproductive tissues, and with three sets of *MIR* genes: Set V, enriched in all reproductive tissues and stem/roots; Set VI, enriched in reproductive tissues; and Set VII, enriched in reproductive tissues and leaves. The key at right shows z-score. B_2_4 indicates bud stage 1 with 2–4 mm in length, B_5_7 indicates bud stage 2 with 5–7 mm in length, and B_8_10 indicates bud stage 3 with 8–10 mm in length. (c) Smear plot showing differentially accumulated microRNAs (miRNAs) in reproductive bud stages: Upper track, bud stage 1 before (B_2_4) and after 14 days (B_2_4_F) of heat treatment; and in lower track, bud stage 2 before (B_5_7) and after 14 days (B_5_7_F) of heat treatment



at specific stages of reproductive development, misidentified as 24-nt phasiRNAs as previously described (Polydore et al., 2018). However, a subset of these loci may be derived from a 12-nt phasing mechanism similar to the 24-nt phasiRNAs of Solanaceous species (Xia et al., 2019).

2.4 | Differentially expressed phasiRNAs

Because the two classes of phasiRNAs enriched in anthers of angiosperms demonstrate short periods of peak abundance (Pokhrel, Huang, Bélanger, et al., 2021; Pokhrel, Huang, & Meyers, 2021;

TABLE 2 Differentially accumulated microRNA (miRNA) precursors in flax due to heat treatments

	B_2_4	B_5_7	B_8_10	B_2_4_S	B_5_7_S	B_8_10_S	B_2_4_F	B_5_7_F	B_8_10_F
B_2_4				1D, 0U			11D, 0U		
B_5_7					10D, 1U			12D, 0U	
B_8_10						1D, 0U			0D, 0U
B_2_4_S							0D, 0U		
B_5_7_S								0D, 0U	
B_8_10_S									0D, 0U

Note: miRNAs that were significantly accumulated in different tissues were denoted as downregulated (D) and upregulated (U) ($p < .05$, FDR $< .05$, Table S4). B_2_4 indicates bud stage 1 with buds 2–4 mm in length, B_5_7 indicates bud stage 2 with buds 5–7 mm in length, and B_8_10 indicates bud stage 3 with buds 8–10 mm in length. "_S" and "_F" in the codes indicate 7 and 14 days of heat treatment.

TABLE 3 Summary of 21-PHAS loci and their triggers identified in flax

Trigger miRNA	Locus type, name, or encoded protein	Number of loci
Lusv2_MIR167h/j	Metal tolerance protein	3
Lusv2_MIR167h	Noncoding	2
Lusv2_MIR168a	AGO1	2
Lusv2_MIR482/2118	Noncoding	13
Lusv2_MIR390	TAS3	3
Lusv2_MIR393b	AFB2	2
Lusv2_MIR408	Laccase	2
Lusv2_MIR6445a	NAC	3
N.D.	Noncoding	13
N.D.	Other protein-coding genes	25

Note: Detailed information for these loci can be found in Table S5. Abbreviations: AFB2, AUXIN SIGNALING F-BOX 2; AGO, Argonaute protein; microRNA, miRNA; NAC, NAC domain-containing protein; N.D., not determined.

Xia et al., 2019), we examined the differential accumulation of these reproductive phasiRNAs. We found among 68 21-PHAS loci present in flax that 40 loci were differentially expressed, with most (37) being downregulated in heat-treated samples (Table S7). Among the differentially expressed loci, 37 loci were enriched in reproductive tissues, 12 of which are 21-PHAS loci targeted by miR482/2118, three encode metal tolerance proteins (triggered by miR167h/f), nine are noncoding loci with unknown triggers, and the remaining 13 loci are from other protein-coding genes (Figure 4a, Table S7). Both Stages 1 and 2 had similar numbers of 21-PHAS differentially expressed during 7 and 14 days of heat treatments; however, for Stage 3, the 14-day heat treatment increased the number of differentially accumulated 21-PHAS loci (Table 4).

In the case of 24-PHAS loci, for bud stages 1 and 2, 14 days of heat treatment resulted in more downregulated 24-PHAS loci compared with 7 days of heat treatment, although the abundance of 24-PHAS loci in bud stage 3 did not change due to either of the heat treatments (Table 5). Each of the four reproductive 24-PHAS loci

triggered by miR2275 was downregulated in heat-treated samples, while 450 24-PHAS-like loci were also downregulated by heat, out of 658 total loci (Figure 4b, Table S8).

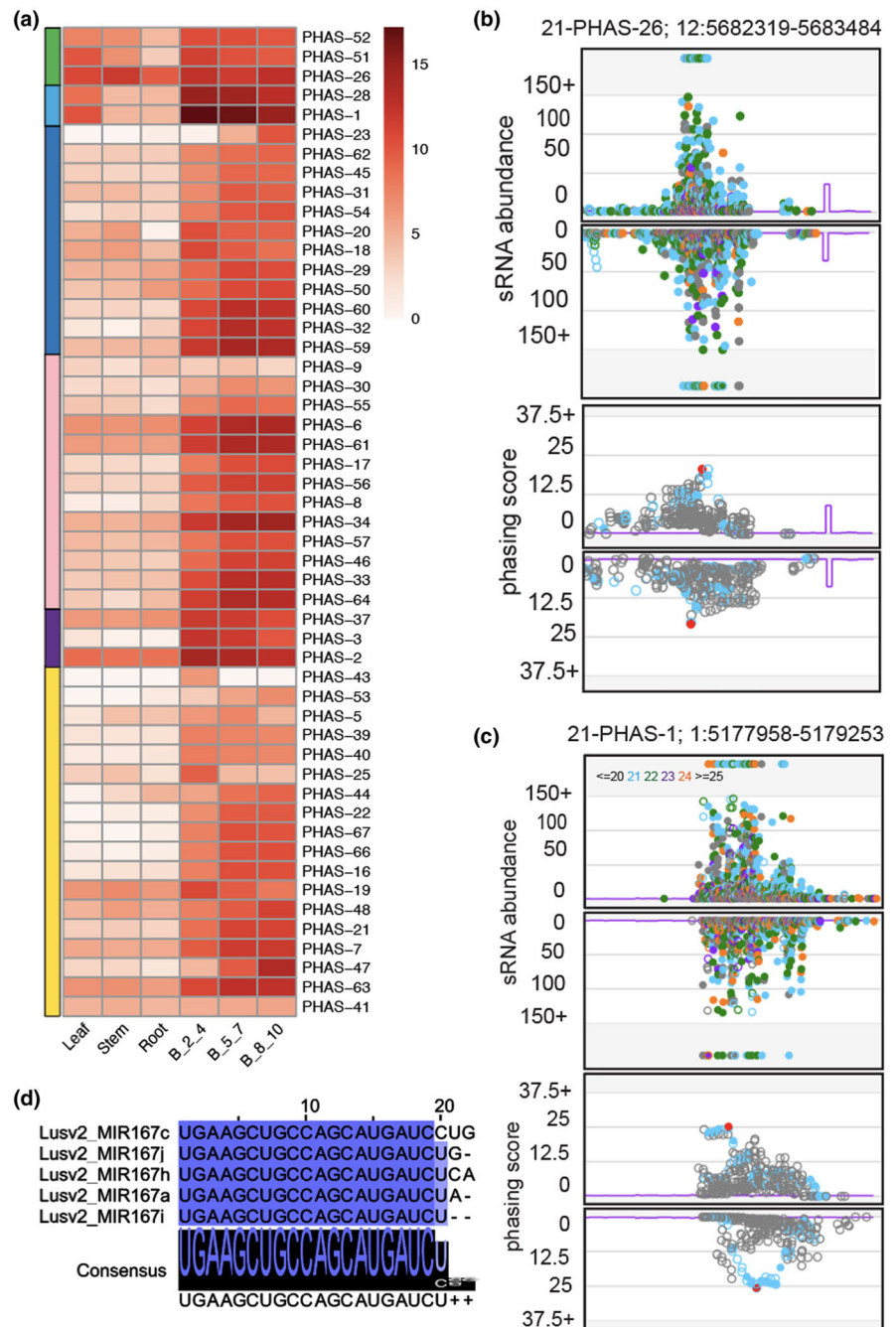
3 | DISCUSSION

We conducted a genome-wide annotation of miRNAs and phasiRNAs in flax. On the basis of the updated criteria of miRNA annotation (Axtell & Meyers, 2018), we comprehensively identified 173 *MIR* genes that generated miRNAs from 31 known families and 42 novel miRNAs. As miRNAs play important regulatory roles in plant growth and reproductive development, we found that most of the miRNA loci (141 out of 173) were preferentially accumulated in a tissue-specific manner. Further, we categorized differentially accumulated miRNAs loci based on their enrichment in vegetative and reproductive tissues. Because miRBase (v22) contains only 124 *MIR* genes for flax, most of which were identified using computational prediction based on the flax genome sequence, our current analysis would substantially improve the miRBase data for flax.

Furthermore, we identified 68 21-PHAS loci that include three *TAS3* loci, two *TASL* loci, and additional loci from coding and noncoding regions. We found no *PHAS* loci related to *PPR* genes or *NB-LRR* defense genes, which is surprising as these are conserved and numerous *PHAS* loci in many eudicots (Zhai et al., 2011). There are cases of the near absence or absence of *PHAS* loci at *PPR* genes in all Solanaceous species, although some of the Solanaceous species (petunia and *Nicotiana*) harbor few *PHAS* loci related to *NB-LRR* defense genes (Baldrich et al., 2021). Instead of protein-coding *PHAS* loci triggered by miR482/2118 members, we found that these miRNAs initiate the production of reproductive tissue-enriched 21-nt phasiRNAs from noncoding loci in flax, a eudicot (Pokhrel, Huang, Bélanger, et al., 2021; and this study). We identified several miRNAs and phasiRNA pathways in flax that are responsive to heat stress. The two members of miR390, the conserved trigger of *TAS3*, were downregulated under heat stress, although the abundance of *TAS3* derived phasiRNAs did not change significantly. We found no miR167 members to be differentially accumulated under heat stress, but we observed a reduction in phasiRNAs from miR167-triggered metal

FIGURE 3 Characterization of reproductive 21-nt phased secondary small interfering RNAs (phasiRNAs) in flax.

(a) Expression of reproductive-enriched multi-microRNA (miRNA) triggered 21-nt reproductive phasiRNAs in different tissue types. Left color-coded panel indicates different trigger miRNA and their phasiRNA: green, miR390 triggered *TAS3* loci; light blue, miR167 triggered *TASL* loci; dark blue, noncoding loci with unknown trigger, noncoding loci with miR482/2118 trigger; purple, miR167 derived metal protein-related loci; and yellow, all other protein-coding loci with unknown triggers. The key at right indicates the abundance in unit of $\log_2(\text{RP20M})$. B_2_4, B_5_7, and B_8_10 indicate bud stages 1, 2, and 3, respectively. (b) Upper track: Abundance (RP10M) of small RNAs (sRNAs) in both strands of an example *TAS3* locus padded with 250 base pairs, each side. Name and coordinates of the *PHAS* locus are indicated in top. sRNA sizes are denoted by colors, as indicated at top. Below track: Phasing score of the same locus; the red dot indicates the highest phased sRNA position. (c) Similar to (b) for an example *TASL* locus as indicated in the top. (d) Alignment of members of miR167 in flax. The intensity of blue color denotes the conservation in nucleotide level. The consensus sequence of the alignment is shown with a sequence logo



tolerance proteins phasing pathways that were enriched in reproductive tissues.

In recent studies, conservation of the miR2275-24-*PHAS* pathway in angiosperms was reported, including several eudicots (Pokhrel, Huang, & Meyers, 2021; Xia et al., 2019); the pathway is also conserved in flax, but with few (only four) 24-*PHAS* loci. Here, we identified a larger number (658) of 24-*PHAS*-like loci that are reproductive tissue-enriched but that lack obvious trigger miRNAs, possibly similar to the Solanaceous 24-nt phasiRNAs (Xia et al., 2019) or possibly some sort of loci of reproductive-tissue-enriched hc-siRNAs. We found that all 24-*PHAS* and 450 24-*PHAS*-like loci were heat responsive and all are downregulated due to heat stress.

Notably, we observed downregulation of the reproductive phasiRNA triggers, miR482/2118 and miR2275 in response to heat treatments, as well as the reproductive 21- and 24-nt phasiRNAs triggered by these miRNAs, respectively. In rice, mutations in the precursors of two 21-*PHAS* loci resulted in temperature-sensitive genic male sterility (Fan et al., 2016; Zhou et al., 2012), although in maize, knock-down of *DCL5*, a gene responsible for the production of reproductive 24-nt phasiRNAs, greatly reduced the phasiRNAs and resulted in temperature-sensitive male sterile phenotype (Teng et al., 2020). Cross et al. reported that two heat treatments reduce boll formation and seed set in flax (Cross et al., 2003), the same conditions that we used; however, the molecular mechanisms underlying these results

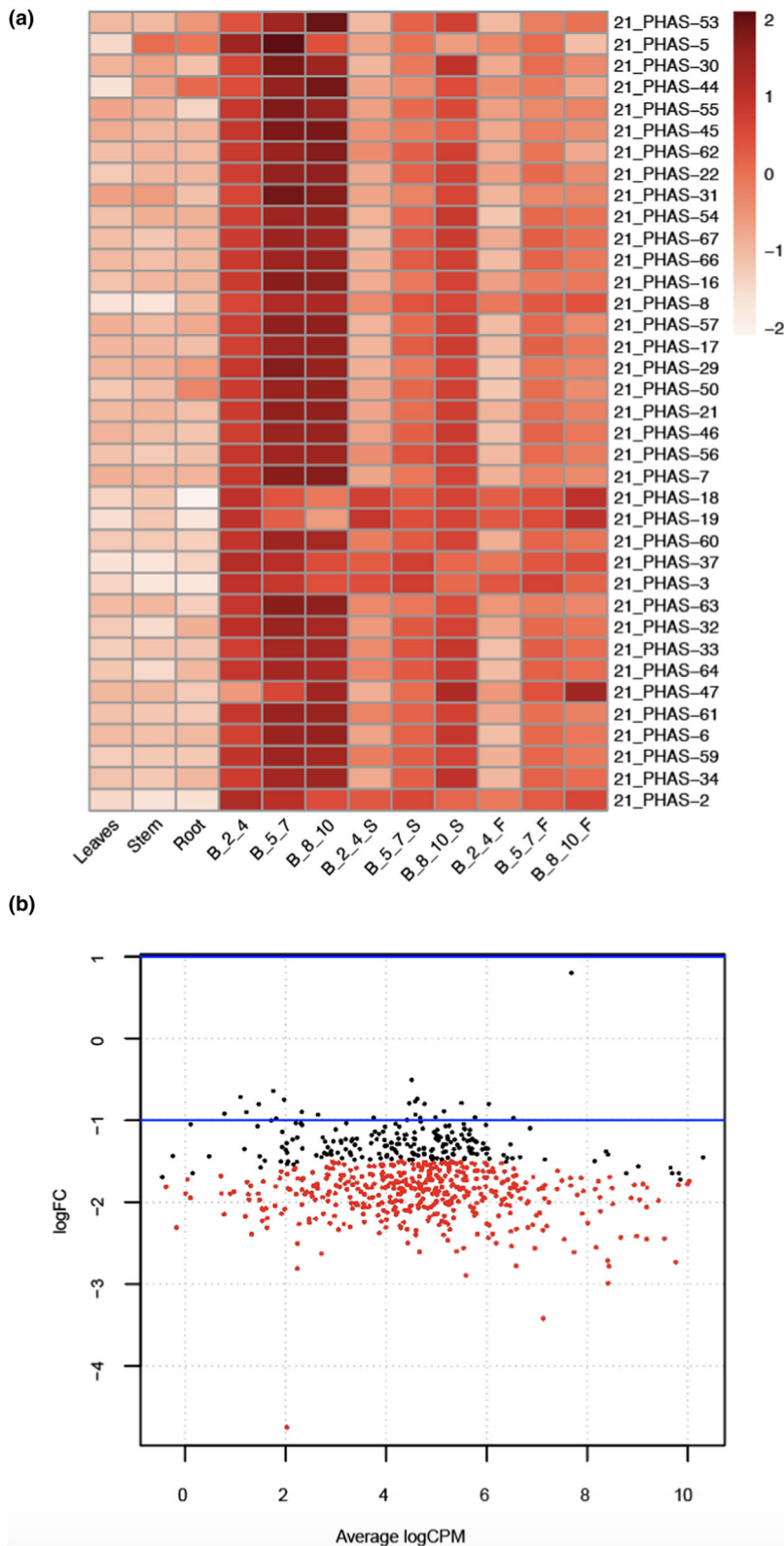


FIGURE 4 Profiling of differential accumulation of reproductive 21- and 24-nt phased secondary small interfering RNAs (phasiRNAs) in flax. (a) Differential accumulation of 21-nt reproductive phasiRNAs in reproductive stages after heat treatments. B_2_4, B_5_7, and B_8_10 indicate bud stage 1, 2 and 3 respectively. S and F indicates 7 and 14 days of heat treatment. The key at right indicates z-score. (b) Smear plot showing differentially accumulated 24-nt phasiRNAs in bud stage 1 before (B_2_4) and after 14 days (B_2_4_F) of heat treatment

are unknown. We hypothesize that a decrease in abundance of rate-limiting trigger miRNAs due to heat stress results in the down-regulation of reproductive phasiRNAs that ultimately produces sterility phenotypes in flax. Future studies are needed to elucidate a mechanistic understanding of the roles for miRNA-phasiRNAs in reproductive development under heat stress.

4 | METHODS

4.1 | Heat stress treatment and plant materials

Flax cultivar “Stormont cirrus” was grown in a walk-in Conviron growth chamber with 16-/8-h light/dark cycle with 25/20°C

**TABLE 4** Differentially accumulated 21-PHAS loci in flax due to heat treatments

	B_2_4	B_5_7	B_8_10	B_2_4_S	B_5_7_S	B_8_10_S	B_2_4_F	B_5_7_F	B_8_10_F
B_2_4		2D, 8U	7D, 21U	32D, 0U			33D, 0U		
B_5_7			0D, 2U		34D, 1U			31D, 0U	
B_8_10						16D, 1U			30D, 2U
B_2_4_S							0D, 0U		
B_5_7_S								0D, 0U	
B_8_10_S									19D, 0U

Note: 21-PHAS loci that were significantly accumulated in different tissues and heat treatment were denoted as downregulated (D) and upregulated (U) ($p < .05$, FDR $< .05$, Table S7). B_2_4 indicates bud stage 1 with buds 2–4 mm in length, B_5_7 indicates bud stage 2 with buds 5–7 mm in length, and B_8_10 indicates bud stage 3 with buds 8–10 mm in length. "_S" and "_F" in the codes indicate 7 and 14 days of heat treatment.

TABLE 5 Differentially accumulated 24-PHAS loci in flax due to heat treatments

	B_4_2	B_5_7	B_8_10	B_2_4_S	B_5_7_S	B_8_10_S	B_2_4_F	B_5_7_F	B_8_10_F
B_2_4				62D, 0 U			449D, 0 U		
B_5_7					68D, 0 U			143D, 0 U	
B_8_10						1D, 0 U			0D, 0 U
B_2_4_S							0D, 0 U		
B_5_7_S								0D, 0 U	
B_8_10_S									0D, 0 U

Note: 24-PHAS loci that were significantly accumulated in different tissues were denoted as downregulated (D) and upregulated (U) ($p < .05$, FDR $< .05$, Table S8). B_2_4 indicates bud stage 1 with buds 2–4 mm in length, B_5_7 indicates bud stage 2 with buds 5–7 mm in length, and B_8_10 indicates bud stage 3 with buds 8–10 mm in length. "_S" and "_F" in the codes indicate 7 and 14 days of heat treatment.

temperatures, respectively. From 1-month-old seedlings, leaves, stem, and root tissues were harvested and immediately frozen in liquid nitrogen. After 2 weeks following the initiation of flowering, two groups of seedlings, each containing at least nine plants, were transferred to a new growth chamber where during the day (16 h) cycle, temperatures would increase at $\sim 3^\circ\text{C}/\text{h}$ from 20°C to 40°C , over a 7-h period, held at 40°C for 1 h, and decreased at the same rate for 7 h. The heat treatment was continued for 14 days, and samples of three bud stages (Figure S1a) were harvested before initiation of heat stress, and after 7 and 14 days during peak heat stress (40°C), from two groups as two biological replicates, and frozen immediately in liquid nitrogen. We sampled two replicates for three different bud stages at three different time points (before heat stress, 7 and 14 days after heat treatment), resulting in 18 total libraries; six more libraries from other tissue types, for a total of 24, were subsequently used.

4.2 | RNA extraction, libraries preparation, and sequencing

Total RNA from all flax vegetative and reproductive tissues was extracted using the PureLink Plant RNA Reagent (ThermoFisher Scientific, cat. #12322012) following the manufacturer's instructions with minor modifications. RNA quality was determined by running denaturing gels, and quantity was assessed using Qubit. First,

sRNAs (20–30 nt) were size selected in a 15% polyacrylamide/urea gel and used for sRNA library preparation. An input amount $5 \mu\text{g}$ of total RNA was used for library construction. The sRNA libraries were constructed following manufacturer's instruction using the NEBNext Small RNA Library Preparation set for Illumina (NEB, cat # E7300).

4.3 | Data analysis and visualization

Preprocessing and filtering of sRNA libraries were conducted as previously described (Pokhrel, Huang, Bélanger, et al., 2021). Two miRNA identification tools: ShortStack and miR-PREFeR were used (Johnson et al., 2016; Lei & Sun, 2014) for genome-wide MIR gene identification. To increase the sensitivity of miRNA detection (a result of increased supporting data), in ShortStack, two libraries were used at a time using default parameters except $-\text{mismatch } 0$ and $-\text{mincov } 10$. As ShortStack has relatively high precision and accuracy, miRNA loci predicted using miR-PREFeR were again re-evaluated with ShortStack, and only the loci with passed the criteria were included in the final set. The miRNA loci from two tools/runs were merged in BEDTools using the "merge" command with default parameters with $-s -c 4,6 -o \text{distinct}$ (Quinlan & Hall, 2010).

PHAS loci were also identified using two tools, PHASIS (Kakrana et al., 2017) with a p -value cut-off of .001 and number of phase cycle

(k) = 7 and ShortStack (Johnson et al., 2016) with default parameters except –mismatch 0 and –mincov 10. BEDTools was used to merge PHAS loci from two different softwares (Quinlan & Hall, 2010). 24-PHAS loci with unknown triggers were annotated as 24-PHAS-like loci. Target prediction was carried out by sPARTA (Kakrana et al., 2014) with a target cut off score of ≤ 5 . The sRNA abundance and phasing score were viewed at a customized browser (Nakano et al., 2020). The 250 base pairs were padded in both upstream and downstream of predicted PHAS loci and subsequently used as the de novo annotated set for analysis by BLASTX (Altschul et al., 1997) against UniRef90. The summed abundance of sRNAs from given locus was calculated by ShortStack using –locfile parameter. Differential analysis ($p < .05$, false discovery rate [FDR] $< .05$) of sRNA loci was conducted in R using the edgeR package (Robinson et al., 2010). Circular plots were constructed using OmicCircos (Hu et al., 2014) for the chromosomal distributions of miRNAs and phasiRNAs generating loci. pheatmap (<https://rdrr.io/cran/pheatmap/>) was used to draw heatmaps in R.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHOR CONTRIBUTIONS

B.C.M. and S.P. designed the research; S.P. performed the experiments and analyzed the data; S.P. and B.C.M wrote and revised the manuscript.

DATA AVAILABILITY STATEMENT

All the sRNA data generated during this study were deposited in NCBI's SRA (Sequence Read Archive) under the accession numbers PRJNA669702 and PRJNA768110.

ORCID

Suresh Pokhrel  <https://orcid.org/0000-0002-5323-1940>

Blake C. Meyers  <https://orcid.org/0000-0003-3436-6097>

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SUPPORTING INFORMATION

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