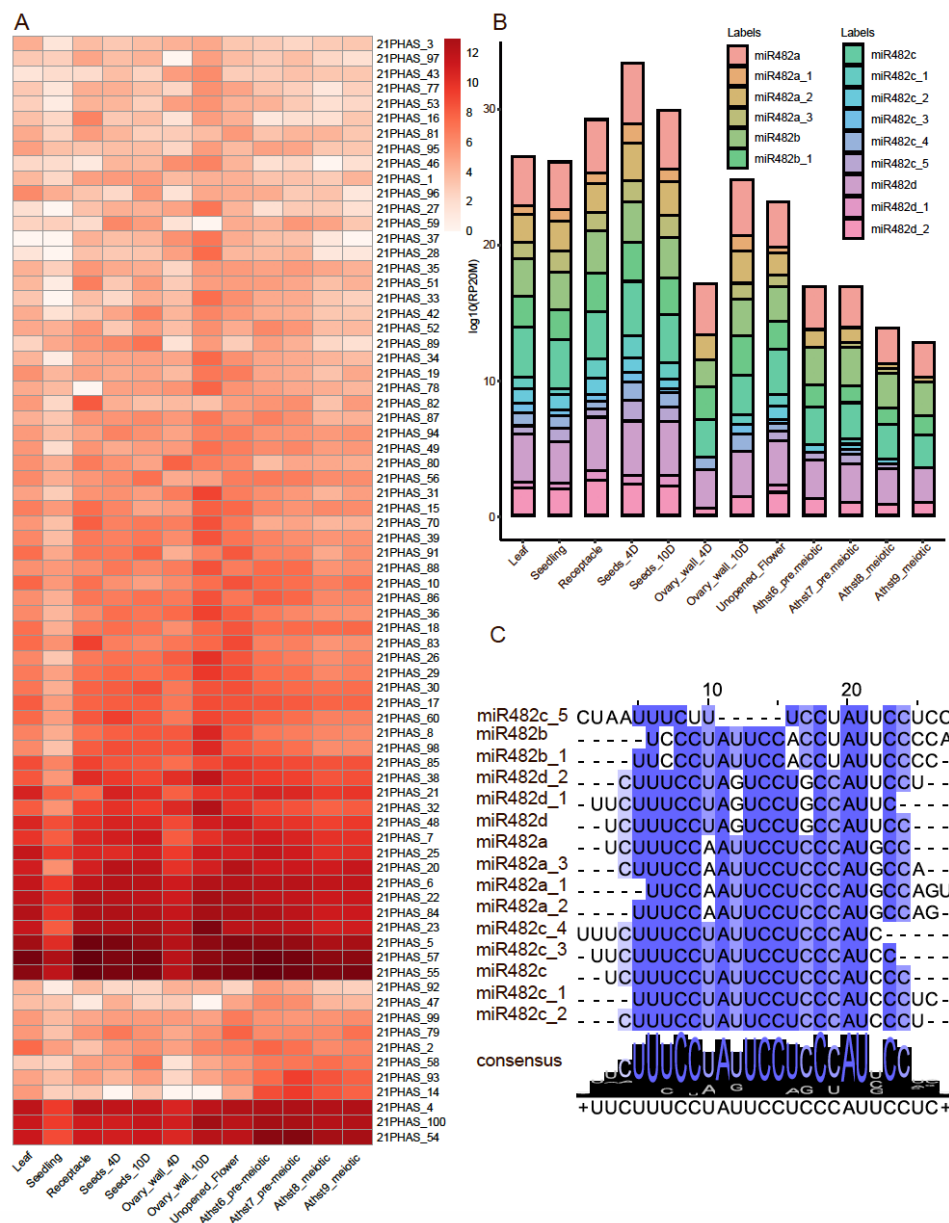


Pre-meiotic 21-nucleotide reproductive phasiRNAs emerged in seed plants and diversified in flowering plants

Pokhrel *et al.*



Supplementary Figure 2. miR2118/482 family and 21-nt phasiRNA pathway in wild strawberry.

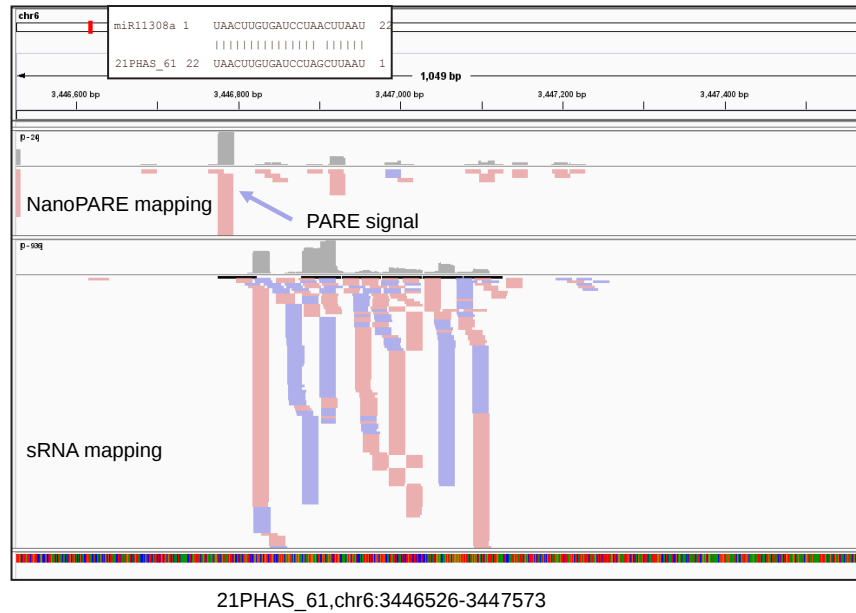
A. The abundance of miR2118/482-triggered 21-nt phasiRNAs across different tissues in wild strawberry. The key at right shows the abundance in unit of log₂(RP20M). The lowermost 11 loci are reproductive-enriched (fold change >1.5) while the lowermost six loci are non-coding.

B. Abundance of miR2118/482 variants in different tissues in wild strawberry.

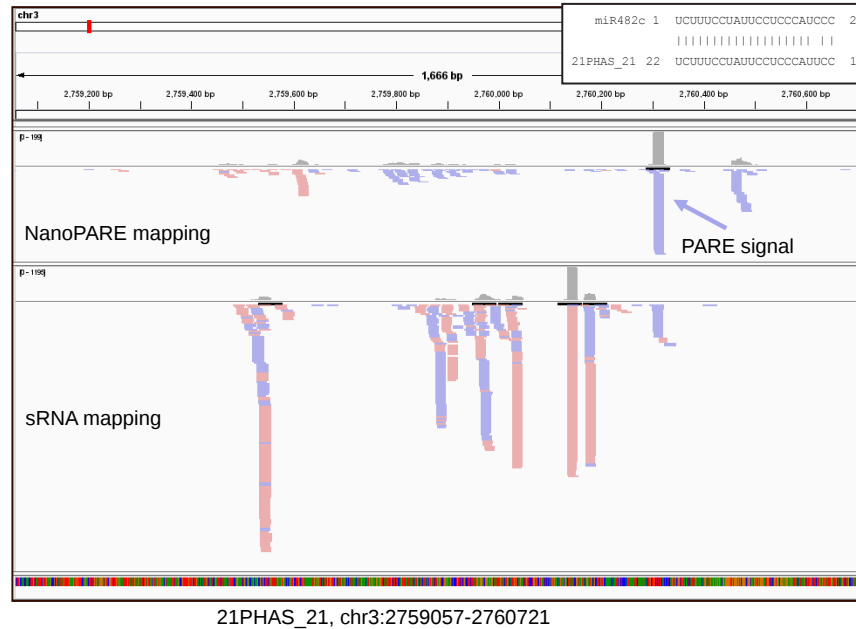
C. Alignment of members of the miR2118/482 family in wild strawberry. The degree of conservation is represented by intensity of blue color, and the consensus sequence of the alignment is shown with a sequence logo.

Source data underlying Supplementary Figure 2A and 2B are provided as Source Data file.

A



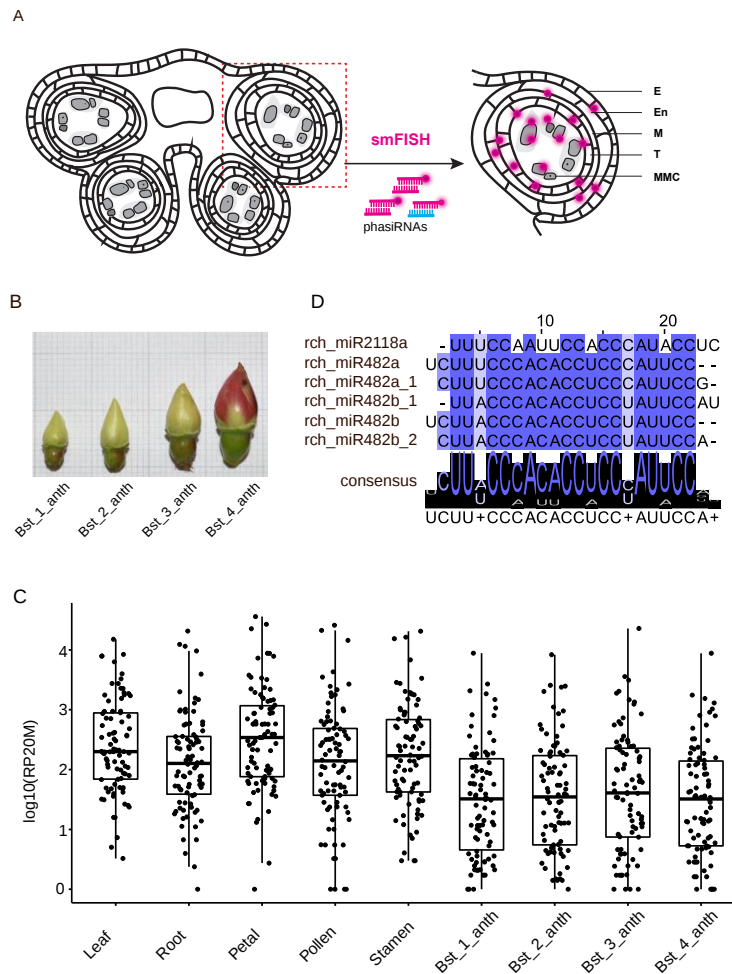
B



Supplementary Figure 3. PARE validation of miR11308 and miR482/2118 derived 21-nt phasiRNAs in wild strawberry.

A. miR11308a-triggered cleavage produces a strong PARE signal at the predicted cleavage site, validating the cleavage by this miRNA. NanoPARE and sRNA data were loaded into IGV for visualization.

B. miR482c-triggered cleavage of 21-PHAS loci, similar to A.



Supplementary Figure 4. Schematic diagram of smFISH of phasiRNAs plus the miR2118/482 family and 21-nt phasiRNA pathway in rose.

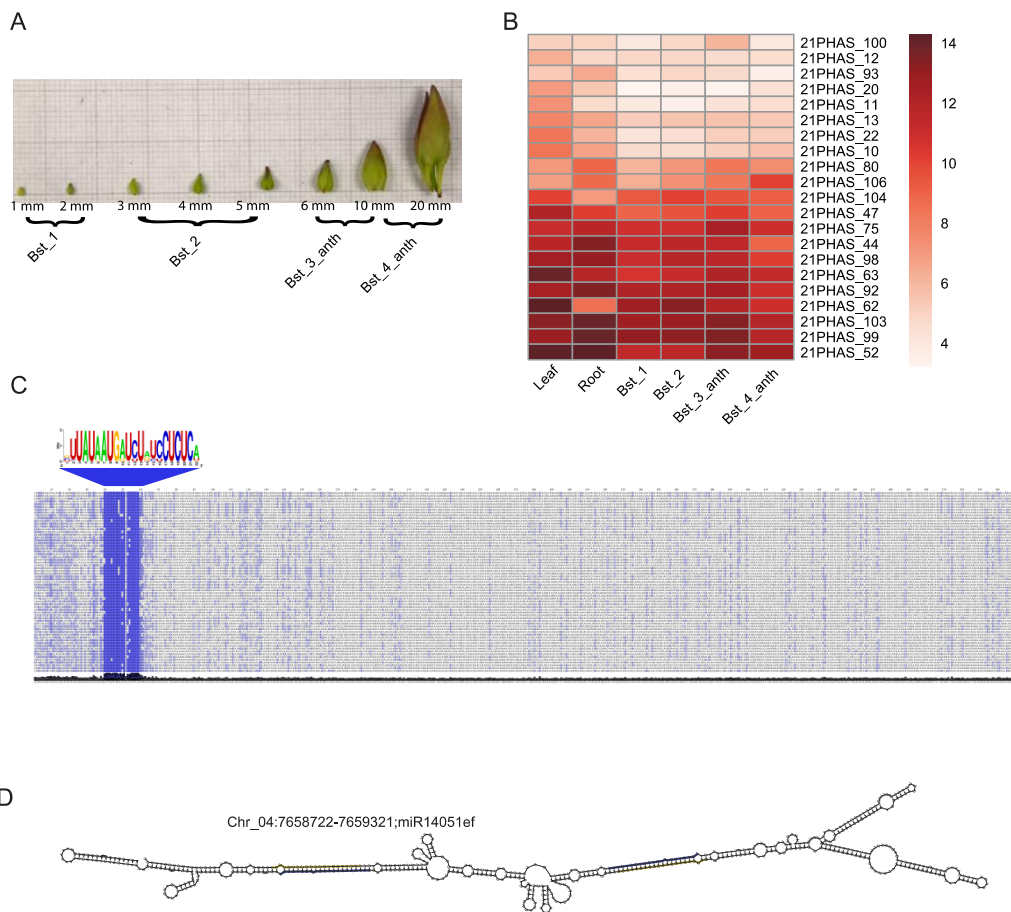
A. Schematic diagram of smFISH using a pool of 50 abundant phasiRNAs in wild strawberry, yielding the data shown in Figure 3. E: epidermis, En: endodermis, M: middle layer, T: tapetum, MMC: microspore mother cell.

B. Bud stages of rose from which anthers were harvested. Bst indicates bud stage, anth indicates anther, stage 1: 1 cm buds, stage 2: 1.25 cm buds, stage 3: 1.5 cm buds, Stage 4: 2 cm buds.

C. The abundance of miR2118/482-triggered 21-nt phasiRNAs abundance among different tissues in rose. In the boxplot, the center line represents the median, box limits are the upper and lower quartiles; whiskers are the 1.5x interquartile range of upper or lower quartiles; points show the scatter of data points for eighty-six (n=86) 21-PHAS loci.

D. Alignment of members of the miR2118/482 family in rose. The degree of conservation is represented by the intensity of the blue color and consensus sequence of the alignment is shown with a sequence logo.

Source data underlying Supplementary Figure 4C are provided as a Source Data file.



Supplementary Figure 5. The 21-nt phasiRNA pathway in columbine.

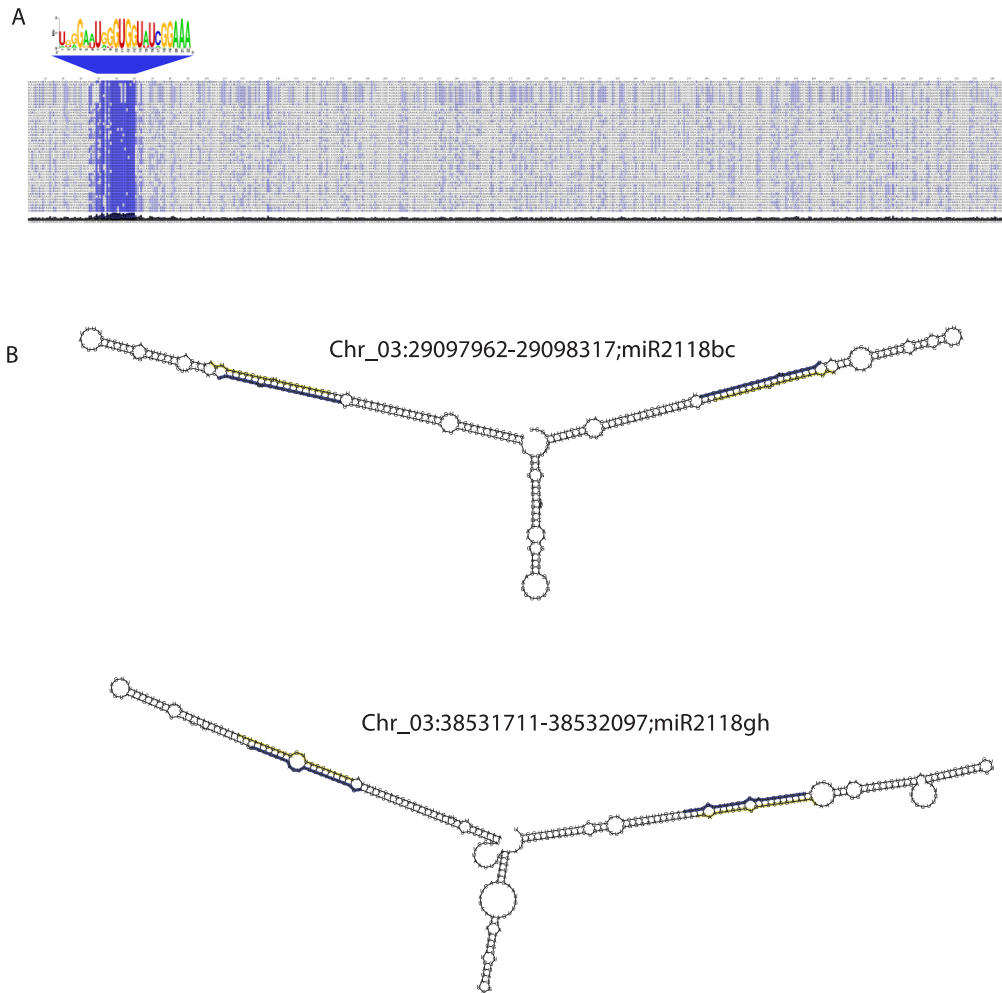
A. Bud stages in columbine, Bst indicates bud stage, anth indicates anther, stage 1: 1 to 2 mm buds, stage 2: 3 to 5 mm buds, stage 3: 6 to 10 mm buds, stage 4: 10 to 20 mm buds.

B. The abundance of miR2118/482- and miR14051-triggered 21-nt phasiRNAs in vegetative/reproductive tissues in columbine. The key at right displays the abundance in units of $\log_2(\text{RP20M})$.

C. Above: Sequence logo denoting conservation of the target site of miR14051, determined from 65 21-*PHAS* loci. Below: Nucleotide sequence alignment of 21-*PHAS* loci with sequence similarity denoted by the intensity of the blue color, showing that the miR14051 target site is the only conserved region for all the precursors.

D. A polycistronic precursor cluster of miR14051e and miR14051f in chromosome 4. The mature miR14051 and miR14051* sequences are marked in blue and yellow respectively.

Source data underlying Supplementary Figure 5B are provided as a Source Data file.



Supplementary Figure 6. Target site conservation of miR2118/482 derived 21-PHAS loci in columbine and polycistronic precursors of miR2118/482.

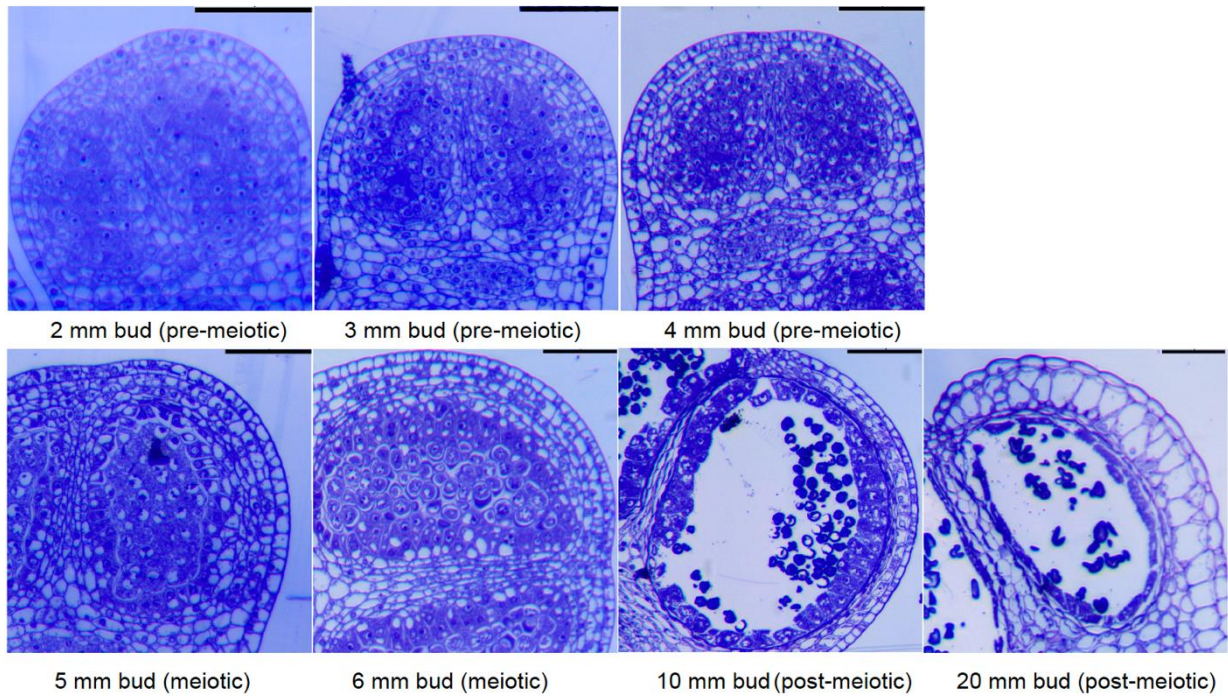
A. Above: Sequence logo denoting conservation of the target site of miR2118/482 for 47 21-PHAS loci. Below: Nucleotide sequence alignment of 21-PHAS loci with sequence similarity denoted by the intensity of blue color showing that miR2118/482 target site is the only conserved region for all the precursors.

B. Two polycistronic precursor clusters of miR2118/482 four variants in chromosome 3. The mature miR2118/482 and miR2118/482* sequences are marked in blue and yellow respectively.

The figure displays a circular genome map of *Z. mays* (Zea mays) showing the distribution of 21PHAS and miRNA loci across six chromosomes (Chr_01 to Chr_06). The map is organized into concentric rings, with the outermost ring representing the chromosome and the inner rings representing the 21PHAS and miRNA loci. The 21PHAS loci are labeled with names such as 21PHAS_1, 21PHAS_2, 21PHAS_3, 21PHAS_4, 21PHAS_5, 21PHAS_6, 21PHAS_7, 21PHAS_8, 21PHAS_9, 21PHAS_10, 21PHAS_11, 21PHAS_12, 21PHAS_13, 21PHAS_14, 21PHAS_15, 21PHAS_16, 21PHAS_17, 21PHAS_18, 21PHAS_19, 21PHAS_20, 21PHAS_21, 21PHAS_22, 21PHAS_23, 21PHAS_24, 21PHAS_25, 21PHAS_26, 21PHAS_27, 21PHAS_28, 21PHAS_29, 21PHAS_30, 21PHAS_31, 21PHAS_32, 21PHAS_33, 21PHAS_34, 21PHAS_35, 21PHAS_36, 21PHAS_37, 21PHAS_38, 21PHAS_39, 21PHAS_40, 21PHAS_41, 21PHAS_42, 21PHAS_43, 21PHAS_44, 21PHAS_45, 21PHAS_46, 21PHAS_47, 21PHAS_48, 21PHAS_49, 21PHAS_50, 21PHAS_51, 21PHAS_52, 21PHAS_53, 21PHAS_54, 21PHAS_55, 21PHAS_56, 21PHAS_57, 21PHAS_58, 21PHAS_59, 21PHAS_60, 21PHAS_61, 21PHAS_62, 21PHAS_63, 21PHAS_64, 21PHAS_65, 21PHAS_66, 21PHAS_67, 21PHAS_68, 21PHAS_69, 21PHAS_70, 21PHAS_71, 21PHAS_72, 21PHAS_73, 21PHAS_74, 21PHAS_75, 21PHAS_76, 21PHAS_77, 21PHAS_78, 21PHAS_79, 21PHAS_80, 21PHAS_81, 21PHAS_82, 21PHAS_83, 21PHAS_84, 21PHAS_85, 21PHAS_86, 21PHAS_87, 21PHAS_88, 21PHAS_89, 21PHAS_90, 21PHAS_91, 21PHAS_92, 21PHAS_93, 21PHAS_94, 21PHAS_95, 21PHAS_96, 21PHAS_97, 21PHAS_98, 21PHAS_99, 21PHAS_100, 21PHAS_101, 21PHAS_102, 21PHAS_103, 21PHAS_104, 21PHAS_105, 21PHAS_106, 21PHAS_107, 21PHAS_108, 21PHAS_109, 21PHAS_110, 21PHAS_111, 21PHAS_112, 21PHAS_113, 21PHAS_114, 21PHAS_115, 21PHAS_116, 21PHAS_117, 21PHAS_118, 21PHAS_119, 21PHAS_120, 21PHAS_121, 21PHAS_122, 21PHAS_123, 21PHAS_124, 21PHAS_125, 21PHAS_126, 21PHAS_127, 21PHAS_128, 21PHAS_129, 21PHAS_130, 21PHAS_131, 21PHAS_132, 21PHAS_133, 21PHAS_134, 21PHAS_135, 21PHAS_136, 21PHAS_137, 21PHAS_138, 21PHAS_139, 21PHAS_140, 21PHAS_141, 21PHAS_142, 21PHAS_143, 21PHAS_144, 21PHAS_145, 21PHAS_146, 21PHAS_147, 21PHAS_148, 21PHAS_149, 21PHAS_150, 21PHAS_151, 21PHAS_152, 21PHAS_153, 21PHAS_154, 21PHAS_155, 21PHAS_156, 21PHAS_157, 21PHAS_158, 21PHAS_159, 21PHAS_160, 21PHAS_161, 21PHAS_162, 21PHAS_163, 21PHAS_164, 21PHAS_165, 21PHAS_166, 21PHAS_167, 21PHAS_168, 21PHAS_169, 21PHAS_170, 21PHAS_171, 21PHAS_172, 21PHAS_173, 21PHAS_174, 21PHAS_175, 21PHAS_176, 21PHAS_177, 21PHAS_178, 21PHAS_179, 21PHAS_180, 21PHAS_181, 21PHAS_182, 21PHAS_183, 21PHAS_184, 21PHAS_185, 21PHAS_186, 21PHAS_187, 21PHAS_188, 21PHAS_189, 21PHAS_190, 21PHAS_191, 21PHAS_192, 21PHAS_193, 21PHAS_194, 21PHAS_195, 21PHAS_196, 21PHAS_197, 21PHAS_198, 21PHAS_199, 21PHAS_200, 21PHAS_201, 21PHAS_202, 21PHAS_203, 21PHAS_204, 21PHAS_205, 21PHAS_206, 21PHAS_207, 21PHAS_208, 21PHAS_209, 21PHAS_210, 21PHAS_211, 21PHAS_212, 21PHAS_213, 21PHAS_214, 21PHAS_215, 21PHAS_216, 21PHAS_217, 21PHAS_218, 21PHAS_219, 21PHAS_220, 21PHAS_221, 21PHAS_222, 21PHAS_223, 21PHAS_224, 21PHAS_225, 21PHAS_226, 21PHAS_227, 21PHAS_228, 21PHAS_229, 21PHAS_230, 21PHAS_231, 21PHAS_232, 21PHAS_233, 21PHAS_234, 21PHAS_235, 21PHAS_236, 21PHAS_237, 21PHAS_238, 21PHAS_239, 21PHAS_240, 21PHAS_241, 21PHAS_242, 21PHAS_243, 21PHAS_244, 21PHAS_245, 21PHAS_246, 21PHAS_247, 21PHAS_248, 21PHAS_249, 21PHAS_250, 21PHAS_251, 21PHAS_252, 21PHAS_253, 21PHAS_254, 21PHAS_255, 21PHAS_256, 21PHAS_257, 21PHAS_258, 21PHAS_259, 21PHAS_260, 21PHAS_261, 21PHAS_262, 21PHAS_263, 21PHAS_264, 21PHAS_265, 21PHAS_266, 21PHAS_267, 21PHAS_268, 21PHAS_269, 21PHAS_270, 21PHAS_271, 21PHAS_272, 21PHAS_273, 21PHAS_274, 21PHAS_275, 21PHAS_276, 21PHAS_277, 21PHAS_278, 21PHAS_279, 21PHAS_280, 21PHAS_281, 21PHAS_282, 21PHAS_283, 21PHAS_284, 21PHAS_285, 21PHAS_286, 21PHAS_287, 21PHAS_288, 21PHAS_289, 21PHAS_290, 21PHAS_291, 21PHAS_292, 21PHAS_293, 21PHAS_294, 21PHAS_295, 21PHAS_296, 21PHAS_297, 21PHAS_298, 21PHAS_299, 21PHAS_300, 21PHAS_301, 21PHAS_302, 21PHAS_303, 21PHAS_304, 21PHAS_305, 21PHAS_306, 21PHAS_307, 21PHAS_308, 21PHAS_309, 21PHAS_310, 21PHAS_311, 21PHAS_312, 21PHAS_313, 21PHAS_314, 21PHAS_315, 21PHAS_316, 21PHAS_317, 21PHAS_318, 21PHAS_319, 21PHAS_320, 21PHAS_321, 21PHAS_322, 21PHAS_323, 21PHAS_324, 21PHAS_325, 21PHAS_326, 21PHAS_327, 21PHAS_328, 21PHAS_329, 21PHAS_330, 21PHAS_331, 21PHAS_332, 21PHAS_333, 21PHAS_334, 21PHAS_335, 21PHAS_336, 21PHAS_337, 21PHAS_338, 21PHAS_339, 21PHAS_340, 21PHAS_341, 21PHAS_342, 21PHAS_343, 21PHAS_344, 21PHAS_345, 21PHAS_346, 21PHAS_347, 21PHAS_348, 21PHAS_349, 21PHAS_350, 21PHAS_351, 21PHAS_352, 21PHAS_353, 21PHAS_354, 21PHAS_355, 21PHAS_356, 21PHAS_357, 21PHAS_358, 21PHAS_359, 21PHAS_360, 21PHAS_361, 21PHAS_362, 21PHAS_363, 21PHAS_364, 21PHAS_365, 21PHAS_366, 21PHAS_367, 21PHAS_368, 21PHAS_369, 21PHAS_370, 21PHAS_371, 21PHAS_372, 21PHAS_373, 21PHAS_374, 21PHAS_375, 21PHAS_376, 21PHAS_377, 21PHAS_378, 21PHAS_379, 21PHAS_380, 21PHAS_381, 21PHAS_382, 21PHAS_383, 21PHAS_384, 21PHAS_385, 21PHAS_386, 21PHAS_387, 21PHAS_388, 21PHAS_389, 21PHAS_390, 21PHAS_391, 21PHAS_392, 21PHAS_393, 21PHAS_394, 21PHAS_395, 21PHAS_396, 21PHAS_397, 21PHAS_398, 21PHAS_399, 21PHAS_400, 21PHAS_401, 21PHAS_402, 21PHAS_403, 21PHAS_404

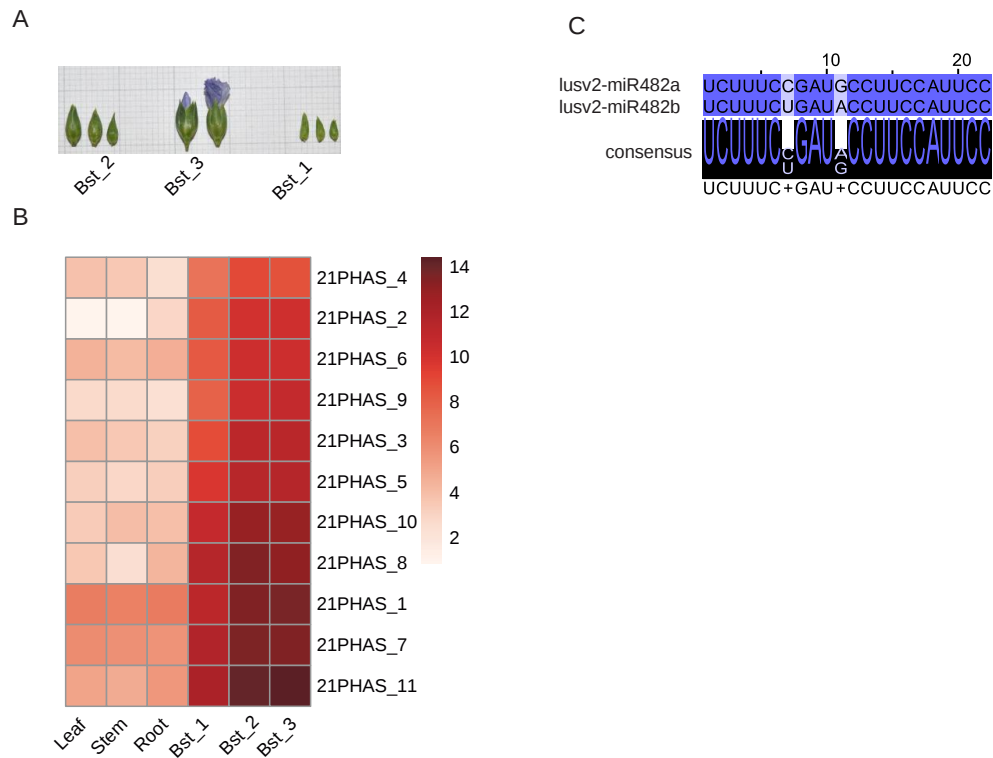
Supplementary Figure 7. Genome-wide distribution of triggers and 21-*PHAS* Loci in columbine.

Genome-wide distribution of miR14051 and miR2118/482 family members and their 21-*PHAS* loci represented by inner (red) and outer (green) cycles respectively. Source data are provided as a Source Data file.



Supplementary Figure 8. Transverse sections of anthers from 2-20 mm buds in size covering four different stages in columbine.

Anthers were fixed in a FAA solution and embedded using the Quetol epoxy resin, sectioning at $0.5\ \mu\text{m}$ and stained using 0.05% toluidine blue O. Black scale bars correspond to $50\ \mu\text{m}$. The transverse sections were taken from two replicates with similar results.



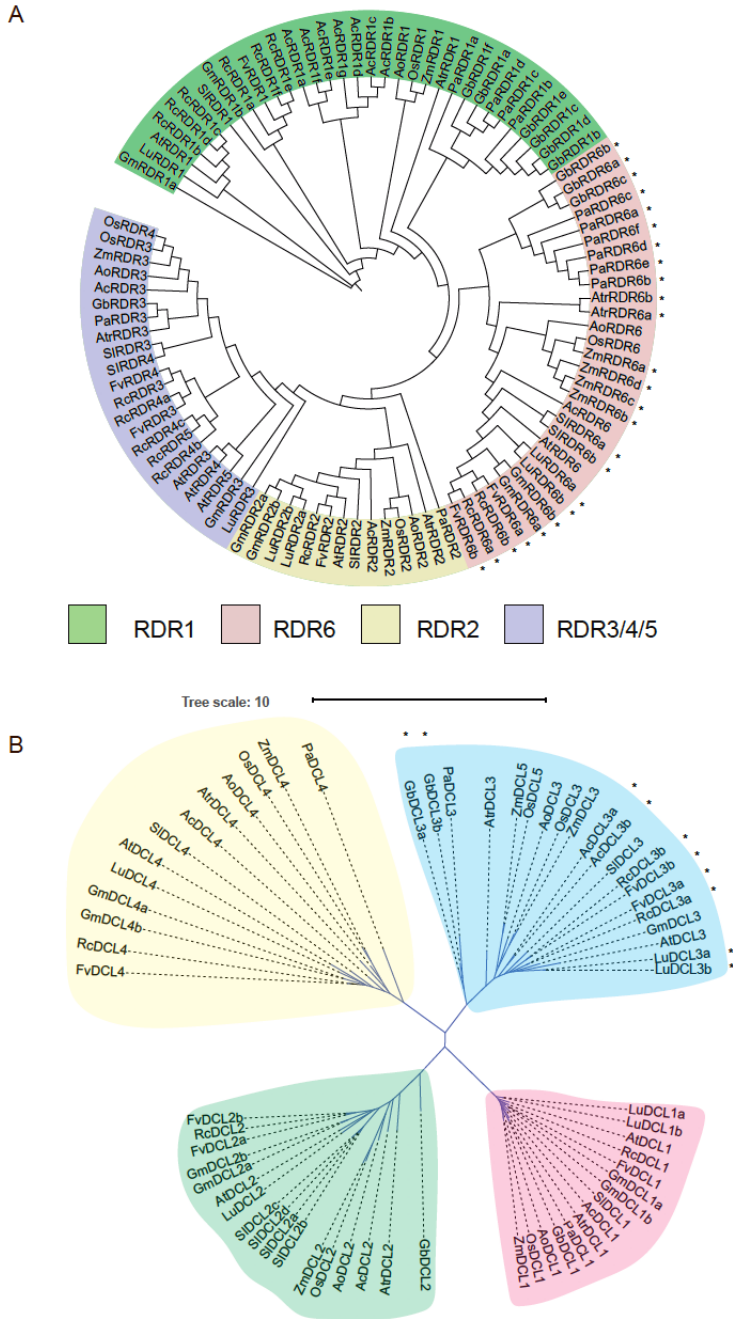
Supplementary Figure 9. Reproductive 21-nt phasiRNAs in flax and their trigger miR2118/482.

A. Bud stages in flax, Bst indicates the bud stage, stage 1: 3 to 5 mm buds, stage 2: 5 to 8 mm buds, stage 3: 8 to 10 mm buds.

B. Accumulation of 21-nt phasiRNAs in different tissues in flax. The key at right indicates the abundance in unit of $\log_2(\text{RP20M})$.

C. Alignment of variants of miR2118/482 family in flax.

Source data underlying Supplementary Figure 9B are provided as a Source Data file.



Supplementary Figure 10. Protein diversity of RNA-dependent RNA polymerase (RDR) and Dicer-like (DCL) family members encoded in genomes of wild strawberry, rose, columbine, flax, other representative species from gymnosperms, eudicots, and monocots.

A. Phylogenetic tree of RDR family members of wild strawberry (Fv), rose (Rc), columbine (Ac), flax (Lu), norway spruce (Pa), ginkgo (Gb), *Amborella* (Atr), soybean (Gm), tomato (Sl), *Arabidopsis* (At), *Asparagus* (Ao), maize (Zm), and rice (Os). The asterisk indicates the species with duplicated copies mentioned in the main text.

B. Phylogenetic tree of DCL family members of same species as A. The asterisk indicates the species with duplicated copies mentioned in the main text.