

Supplementary Information for:

Recent allopolyploidy alters *Spartina* microRNA expression in response to xenobiotic-induced stress.

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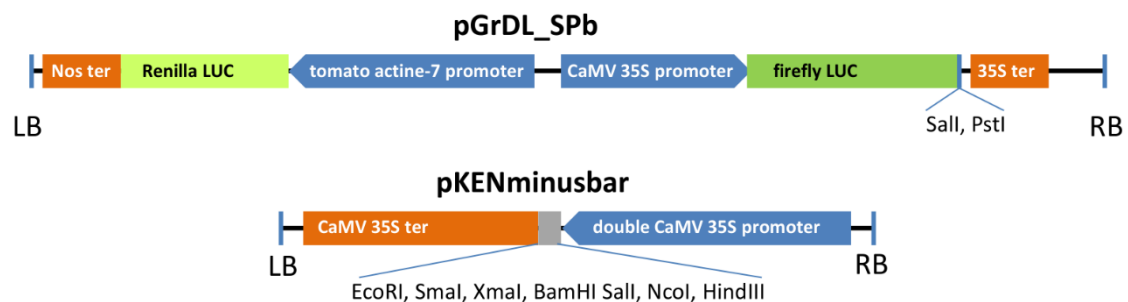


Figure S1. Plasmids used for transient assay systems to validate computationally predicted targets of miRNAs. The plasmid pGrDL_SPb contains the *Renilla* LUC expression cassette which acts as an internal control to standardize expression between replicates, while the *Firefly* LUC expression cassette containing the predicted target sequence cloned between *Sall* and *PstI* is used to report miRNA interaction. Expression plasmid pKENminusbar with multiple cloning sites is used for miRNA precursor insertion.

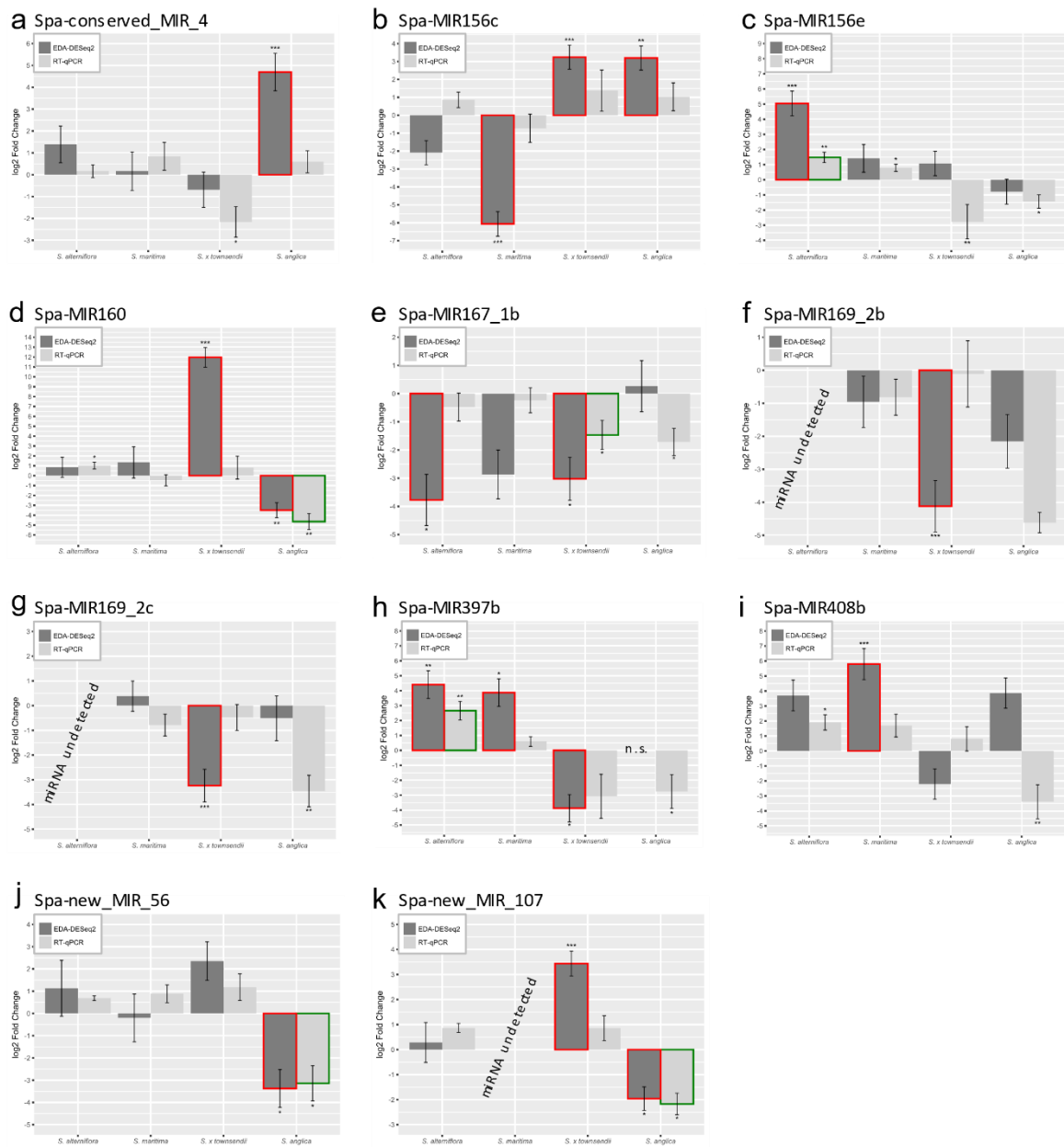


Figure S2. Expression patterns obtained for 11 miRNAs in response to phe within *Spartina* species. On this subset of 11 miRNAs, we compared miRNA expression patterns (log₂ Fold Changes ± SE) from intraspecific comparisons of phe-treated versus control libraries within *Spartina* species, as estimated by the *in-silico* (EDAseq-DESeq2; dark grey) and the RT-qPCR approaches (light grey). Significant differences in miRNA expression patterns were reported for 18 comparisons using EDAseq-DESeq2 (bars surrounded in red). Using RT-qPCR, we confirmed significant differences in miRNA expression patterns for 6 comparisons (bars surrounded in green). MicroRNA expressions patterns measured *in silico* are significantly DE according to DESeq2 Bonferroni adjusted p.value, while miRNA expressions patterns measured by RT-qPCR are significantly DE according to Wilcoxon signed rank tests (*: p.value < 0.05; **: p.value < 0.01; ***: p.value < 0.001).

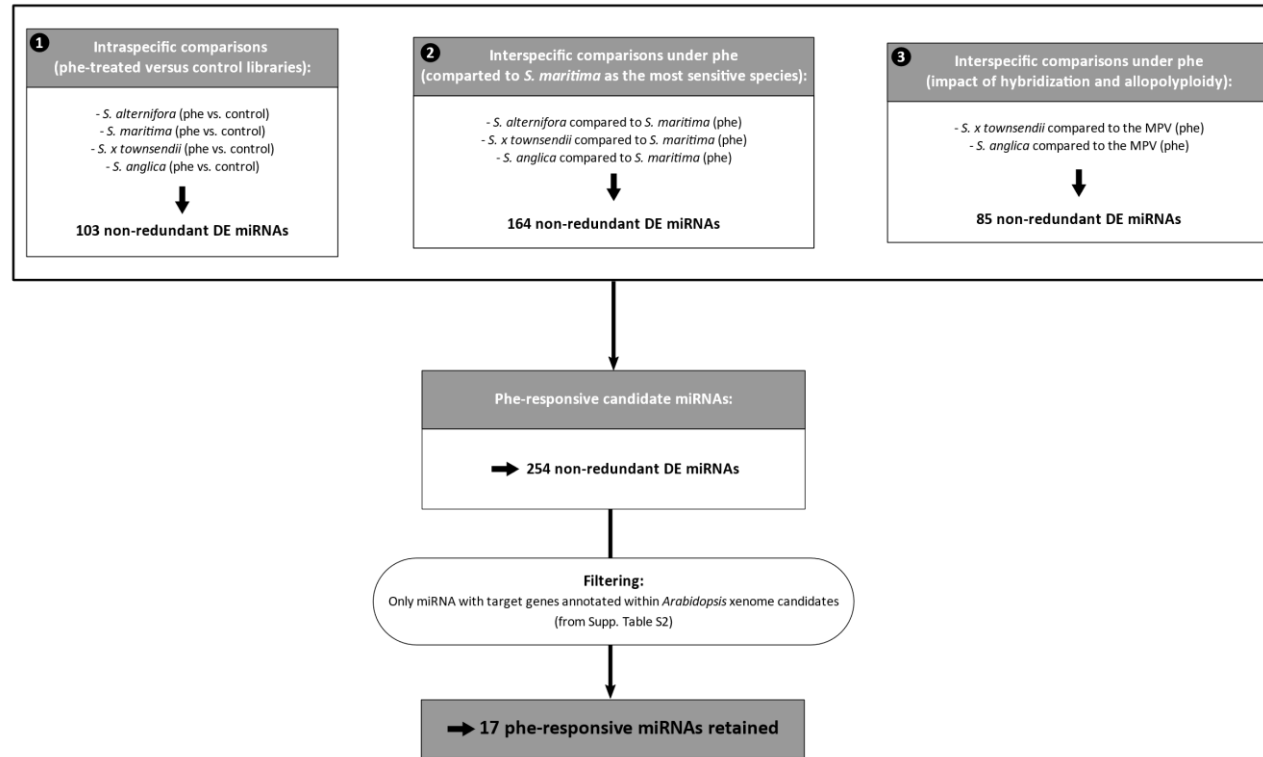


Figure S3. Identification process of phe-responsive miRNAs in *Spartina*. From the *in silico* differential expression analysis, (1) 103 non-redundant phe-responsive candidate miRNAs were identified from intraspecific comparisons (phe-treated versus control). In complement, we also analysed (2) DE miRNAs from pairwise interspecific comparisons under phe (*S. alterniflora* compared to *S. maritima*, *S. x townsendii* compared to *S. maritima*, and *S. anglica* compared to *S. maritima*) to detect additional phe-responsive candidate miRNAs, representing 164 non-redundant miRNAs. Finally, we also included (3) DE miRNAs by comparing *S. x townsendii* and *S. anglica* to the MPV under phe as additional phe-responsive candidate miRNAs, representing in total 85 non-redundant miRNAs. When combining the differential expression analyses, phe-responsive candidate miRNAs represent 254 non-redundant miRNAs. Finally, only miRNAs with target genes annotated within *Arabidopsis* xenome candidates were retained, which represent 17 *Spartina* phe-responsive miRNAs.

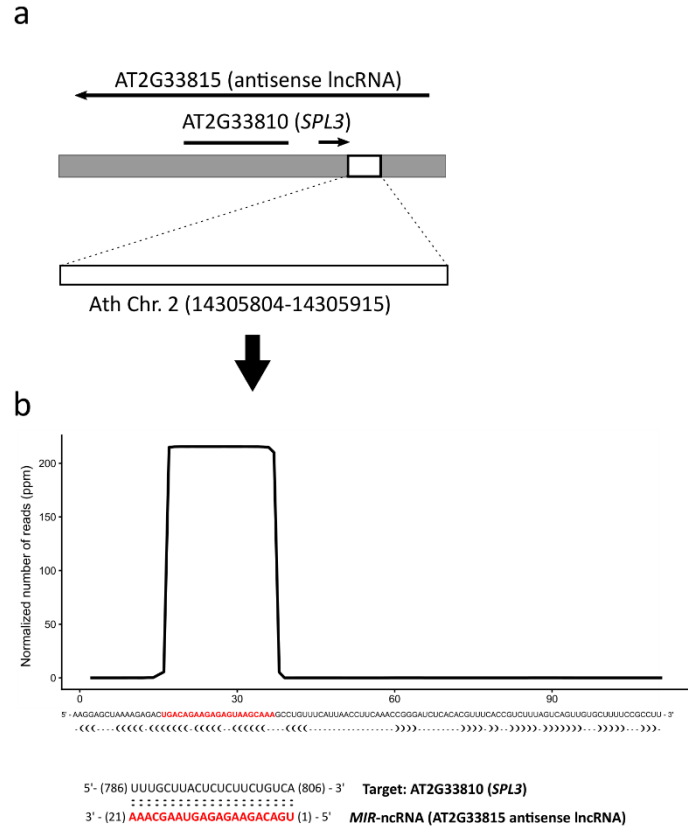


Figure S4. *De novo* prediction of a putative primary *MIR156* transcript in *Arabidopsis* (*MIR*-ncRNA) homologous to *Spa-MIR156e* and *Spa-MIR156j*. This putative *MIR156* primary transcript (a) was identified following mapping of *Spartina* miRNAs on the *Arabidopsis* genome in the lncRNA AT2G33815 (Chr. 2 positions 14305804 to 14305915). This putative miRNA precursor presents a stem-loop design (b), producing a *MIR156* candidate (in red) predicted to target *SPL3* by perfect matching with the opposite complementary strand.

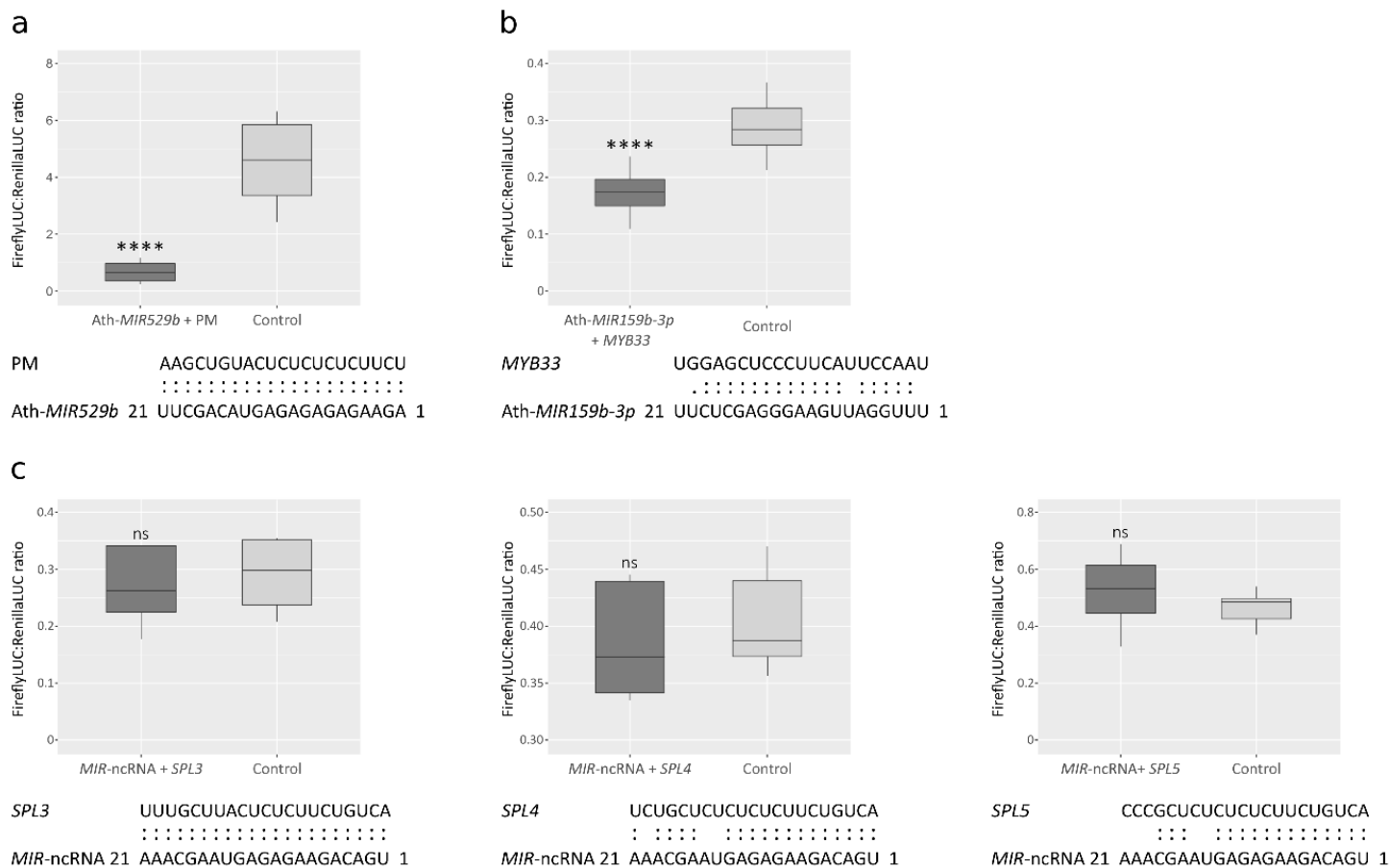


Figure S5. Additional experimental validation by dual LUC assay of computational interactions of *Arabidopsis* miRNAs (homologous to phen-responsive miRNAs in *Spartina*) and their predicted targets. We used the same a positive control as presented in Fig. 5 (a). Interactions tested are Ath-MIR159-3p with target gene MYB33 domain protein (AT5G06100) (b) and MIR-ncRNA with target genes SPL3: SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (AT2G33810), SPL4 (AT1G53160) and SPL5 (AT3G15270) (c). Data were depicted as box and whisker plots with interquartile range boxes and min/max whiskers (n=6). Asterisks represent statistically significant differences using a simple two-tailed unpaired *t*-test with ****p.value < 0.0001.

Table S1. Pairwise comparisons between treatments and species performed for miRNA differential expression analysis. This table classifies intraspecific and interspecific comparisons performed, and reminds how phe-responsive candidate miRNAs and miRNAs impacted by allopolyploidy under phe exposure were respectively identified.

		Phe-responsive candidate miRNAs	Impact of allopolyploidy on miRNAs under phe
Intraspecific comparisons	<i>S. alterniflora</i> (phe vs. control)	x	
	<i>S. maritima</i> (phe vs. control)	x	
	<i>S. x townsendii</i> (phe vs. control)	x	
	<i>S. anglica</i> (phe vs. control)	x	
Interspecific comparison	<i>S. alterniflora</i> compared to <i>S. maritima</i> (phe)	x	
	<i>S. x townsendii</i> compared to <i>S. maritima</i> (phe)	x	
	<i>S. anglica</i> compared to <i>S. maritima</i> (phe)	x	
	<i>S. x townsendii</i> compared to the MPV (phe)	x	x (impact of hybridization)
	<i>S. anglica</i> compared to the MPV (phe)	x	x (impact of allopolyploidy)
	<i>S. anglica</i> compared to <i>S. x townsendii</i> (phe)		x (impact of genome doubling <i>per se</i>)

Table S2. TAIR10 gene families potentially involved in xenobiotic detoxification. It includes xenome genes (Edwards *et al.*, 2011), or genes annotated as antioxidants enzymes and transcription factors (TFs) (<https://www.arabidopsis.org/browse/genefamily/index.jsp>).

[Excel file submitted separately]

Table S3. Primers used for RT-qPCR of Spa-miRNAs. The poly(T) adaptor used in reverse transcription of polyadenylated RNA is provided, as well as the universal poly(T) reverse primer sequence.

	Primer sequence
poly(T)_adaptor	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTTVN
poly(T)_adaptor_RP	GCGAGCACAGAATTAATACGAC
Spa-new_MIR_25 (rRNA 5s)	AAGTCCTCGTGTGCATCCCT
Spa-MIR156c	TTGACAGAAGATAGAGAGCAC
Spa-MIR156e	TGACAGAAGAGAGTGAGCACA
Spa-MIR397b	TTGAGTGCAGCGTTGATGAAA
Spa-MIR408b	CTGCACTGCCTCTTCCCT
Spa-MIR169_2b	ATCGGCAAGTTGTCCTTG
Spa-MIR169_2c	TGCAGCCAAGGATGACTTGCC
Spa-MIR167_1b	TGAAGCTGCCAGCATGATCTA
Spa-MIR160	GTGCAAGGGGCCAAGCAT
Spa-conserved_MIR_4	AAACCTGGCTCTGATACCA
Spa-new_MIR_56	GCTCTGAAATTGTTGTCATTAGG
Spa-new_MIR_107	ATTGGTATCGGCTGATGTAAG

Table S4. Primers used to construct target site adaptors in the plasmid pGrDL_SPb for dual LUC assays. Plasmid pGrDL_SPb with *Sall* and *PstI* restriction sites is used for target sequence insertion.

Primer Name	Primer Sequence
Ath-MIR529b_PM_F	<u>TCGACGCCCAAAGCTGTACTCTCTCTTCTCCATTCTGCA</u>
Ath-MIR529b_PM_R	GAATGGAGAAGAGAGAGAGTACAGCTTTGGGCG
AT5G06100, MYB33_domain_protein_F	<u>TCGACTGGAGCTCCCTTCATTCCAATCTGCA</u>
AT5G06100, MYB33_domain_protein_R	GATTGGAATGAAGGGAGCTCCAG
AT5G43270, SPL2_F	<u>TCGACGTGCTCTCTCTTCTGTCACTGCA</u>
AT5G43270, SPL2_R	GTGACAGAAGAGAGAGAGCACG
AT2G33810, SPL3_F	<u>TCGACTTTGCTTACTCTCTTCTGTCACTGCA</u>
AT2G33810, SPL3_R	GTGACAGAAGAGAGTAAGCAAAG
AT1G53160, SPL4_F	<u>TCGACTCTGCTCTCTCTTCTGTCACTGCA</u>
AT1G53160, SPL4_R	GTGACAGAAGAGAGAGAGCAGAG
AT3G15270, SPL5_F	<u>TCGACCCCGCTCTCTCTTCTGTCACTGCA</u>
AT3G15270, SPL5_R	GTGACAGAAGAGAGAGAGCGGGG
AT5G50670, SPL13B_F	<u>TCGACCTTCTCTTTCTTTTTTTCACTGCA</u>
AT5G50670, SPL13B_R	GTGAAAAAAAAAGAAAGAGAAGG

Table S5. Summary of small RNA-seq data processing in *Spartina*. Number of reads retained on each *Spartina* species for control and phe-treated libraries are presented.

Species	<i>S. alterniflora</i>		<i>S. maritima</i>		<i>S. x townsendii</i>		<i>S. anglica</i>	
	Control	PHE	Control	PHE	Control	PHE	Control	PHE
Raw reads	37,551,835	60,444,313	17,828,306	32,274,148	100,752,127	125,380,090	46,650,696	39,620,672
Clean reads	22,231,291	31,443,611	11,042,707	21,336,208	73,548,538	97,497,156	35,270,284	25,825,213
Putative sRNA reads	5,718,925	7,367,871	3,389,799	4,676,878	25,809,487	52,366,512	20,524,897	9,612,503
Putative sRNA reads (non-redundant)	2,252,788	2,284,748	1,613,905	1,853,687	7,238,893	9,208,854	4,434,910	2,723,926

Table S6. *Spartina* annotated target genes of DE miRNAs in response to hybridization and allopolyploidy.

[Excel file submitted separately]

Table S7. *Spartina* miRNAs and homologous *MIR* genes identified in *Arabidopsis*. In total, 40 homologous *MIR* genes were retained (39 annotated *MIR* genes and 1 *de novo* predicted *MIR* gene from prediction: *MIR*-ncRNA Chr2. 14305804-14305915). Lines in bold are *Arabidopsis* homologous *MIR* genes related to phe-responsive miRNAs retained in *Spartina*, *MIR* genes in grey were tested by functional validation using T-DNA mutant lines.

Spa_miRNAs	Ath_miRNAs	Chr	prec_start	prec_stop
Spa-MIR156c	Ath-MIR157b	Chr1	24921100	24921199
Spa-MIR156c	Ath-MIR157c	Chr3	6244500	6244716
Spa-MIR156c; Spa-MIR156j; Spa-MIR156e	Ath-MIR156h	Chr5	22597018	22597112
Spa-MIR156e	Ath-MIR156c	Chr4	15415426	15415513
Spa-MIR156e	Ath-MIR156d	Chr5	3456632	3456749
Spa-MIR156e	Ath-MIR156a	Chr2	10676470	10676556
Spa-MIR156e; Spa-MIR156c; Spa-MIR156j	Ath-MIR157a	Chr1	24913202	24913301
Spa-MIR156e; Spa-MIR156j	Ath-MIR156g	Chr2	8412523	8412612
Spa-MIR156e; Spa-MIR156j	Ath-MIR156f	Chr5	9136123	9136219
Spa-MIR156e; Spa-MIR156j	Ath-MIR156e	Chr5	3867210	3867312
Spa-MIR156j; Spa-MIR156e	MIR-ncRNA	Chr2	14305804	14305915
Spa-MIR159d	Ath-MIR159b	Chr1	6220646	6220841
Spa-MIR159d; Spa-MIR159i	Ath-MIR159c	Chr2	18994638	18994851
Spa-MIR159f; Spa-MIR159h	Ath-MIR319b	Chr5	16660468	16660642
Spa-MIR159f; Spa-MIR159h; Spa-MIR159d	Ath-MIR319c	Chr2	17029707	17029894
Spa-MIR159f; Spa-MIR159h; Spa-MIR159i	Ath-MIR319a	Chr4	12352955	12353133
Spa-MIR160c	Ath-MIR160c	Chr5	19009093	19009184
Spa-MIR166b	Ath-MIR166e	Chr5	16775519	16775664
Spa-MIR166b; Spa-MIR166h; Spa-MIR166k; Spa-MIR166n	Ath-MIR165b	Chr4	369849	369999
Spa-MIR166b; Spa-MIR166h; Spa-MIR166k; Spa-MIR166n	Ath-MIR165a	Chr1	78927	79037
Spa-MIR166h; Spa-MIR166k; Spa-MIR166l; Spa-MIR166n; Spa-MIR166b	Ath-MIR166f	Chr5	17516306	17516401
Spa-MIR167_1b	Ath-MIR167c	Chr3	1306624	1306779
Spa-MIR167_1b; Spa-MIR167_1g	Ath-MIR167a	Chr3	8108072	8108209
Spa-MIR167_1b; Spa-MIR167_1g	Ath-MIR167b	Chr3	23406168	23406277
Spa-MIR168f	Ath-MIR168a	Chr4	10578634	10578774
Spa-MIR168f	Ath-MIR168b	Chr5	18358793	18358906
Spa-MIR169_2b; Spa-MIR169_2c	Ath-MIR169d	Chr1	20039495	20039583
Spa-MIR169_2c	Ath-MIR169b	Chr5	8527486	8527637
Spa-MIR169_2c	Ath-MIR169g	Chr4	11483019	11483128
Spa-MIR169_2c; Spa-MIR169_2b	Ath-MIR169e	Chr1	20041580	20041667
Spa-MIR395d	Ath-MIR395f	Chr1	26273857	26273971
Spa-MIR395d	Ath-MIR395b	Chr1	9364476	9364566
Spa-MIR395d	Ath-MIR395c	Chr1	9367089	9367171
Spa-MIR395d	Ath-MIR395e	Chr1	26272785	26272862
Spa-MIR395d	Ath-MIR395a	Chr1	9363205	9363280
Spa-MIR395d	Ath-MIR395d	Chr1	26269988	26270070
Spa-MIR396	Ath-MIR396b	Chr5	13611798	13611932
Spa-MIR397b	Ath-MIR397b	Chr4	7878656	7878757
Spa-MIR399	Ath-MIR399c	Chr5	24962485	24962598
Spa-MIR408b; Spa-MIR408c	Ath-MIR408	Chr2	19319856	19319963

Table S8. Target prediction results of phe-responsive homologous miRNAs in *Arabidopsis*. Only target genes corresponding to putative xenobiotic stress tolerance regulators are included (psRNATarget maximum expectation score set to 5.0; Dai *et al.*, 2018).

[Excel file submitted separately]

Table S9. *Spartina* homologous *MIR* genes in *Arabidopsis* and their corresponding T-DNA mutant lines. *Arabidopsis thaliana* T-DNA insertional mutants were obtained from the SALK and SAIL collections (Colombia control grown counterpart Col-0), and from the FLAG collection (Wassilewskija control grown counterpart Ws). The primers used for genotyping are indicated as LP and RP.

[Excel file submitted separately]

Note S1. Detailed method followed for the experimental validation of miRNAs and target genes interactions from *A. thaliana* by dual LUC assays.

• Cloning strategy of miRNAs and target sites

For transient assay systems to validate computationally predicted targets of miRNAs, we used the plasmid pGrDL_SPb which contains the *Renilla* LUC expression cassette acting as internal control to standardize expression between replicates, while the *Firefly* LUC expression cassette containing the predicted target sequence cloned between *Sall* and *PstI* is used to report miRNA interaction (Supplemental Fig. S1).

Target site adaptors were made by annealing two complementary primers (Supplemental Table S4), synthesized with 5'-phosphate additions and *Sall* and *PstI* overhangs (synthesized by Integrated DNA Technologies). A volume of 10 nmol of each primer were mixed together in 60 μ L of DNase-free water, heated to 90°C for 5 min. in a water bath and allowed to slowly cool down to room temperature (at a rate of 1 degree per minute). The resulting adaptors were diluted 100-fold prior to directional cloning into a *Sall* and *PstI* digested and Antarctic Phosphatase (New England Biolabs) treated pGrDL_SPb plasmid, and subsequently verified by Sanger sequencing (Australian Genome Research Facility).

The miRNA precursors were synthesized with flanking restriction enzyme sites (GenScript) and cloned between the double *Cauliflower mosaic virus (CaMV)* 35S promoter and 35S terminator of the expression plasmid pKENminusbar. A similar sized construct, derived from *Capsicum chlorosis* virus, was cloned into pKENminusbar and used as a control (see Moyle *et al.*, 2017). This control sequence is predicted to form a secondary structure similarly to a miRNA precursor but does not contain enough homology to produce small RNA capable of interacting with the reporter gene target sequence.

• Agroinfiltration of *Nicotiana benthamiana* leaves

Agroinfiltration of expression plasmids was carried out as described by Moyle *et al.* (2017) with minor modifications. Expression plasmids were transformed by electroporation into *A. tumefaciens* strain GV3101 (harboring pSOUP) and plated on LB agar media with rifampicin (25 mg.mL⁻¹), kanamycin (50 mg.mL⁻¹), and tetracycline (10 mg.mL⁻¹) selection. Starter cultures were prepared by inoculating a single colony in 2 mL of LB with rifampicin, kanamycin and tetracycline selection, and grown overnight at 28°C in a shaking incubator. Starter culture was used to inoculate the working culture (20–50 mL), which was grown overnight under the same media, selection and incubation conditions. Working cultures were harvested by centrifugation at room temperature and 2,400g for 15 min. Cell pellets were resuspended in 10 mM MgCl₂ and the OD600 adjusted to 0.5 prior to the addition of acetosyringone (200 mM). The resulting cell cultures were stored in the dark at room temperature for 4h prior to infiltration. *Nicotiana benthamiana* plants were grown at room temperature with 16h light per day, under LumiBar LED strip lighting (LumiGrow). Seedlings were grown for approximately 3–4 weeks prior to agroinfiltration. For each miRNA and target gene module tested, three expanded leaves per plant were infiltrated by applying pressure on the abaxial surface of the leaf with a disposable 5 mL syringe containing the *Agrobacterium* suspension. Each leaf was treated as a biological replicate. Agroinfiltrated plants were incubated for a specified period (typically 72h) in a growth chamber set to 22°C with 16h light per day. Agroinfiltrated leaves were harvested individually, snap-frozen in liquid nitrogen, powdered using a ball mill tissue-lyser (Retsch), and stored at -80°C prior to measurement by dual LUC assays.

• Dual LUC assays

Dual LUC assays (*Firefly* LUC / *Renilla* LUC ratios) extracts were prepared using the Dual Luciferase Reporter Assay System kit (Promega). Five mg of powdered tissue was mixed with 100 μ L of the passive lysis buffer (PLB), and the cellular debris pelleted by centrifugation at 7,500g for 1 min. The supernatant was diluted 20-fold in PLB and 15 μ L loaded into a well of a white flat bottom Costar 96 well plate (Corning). The assay was performed using a GloMax 96 microplate luminometer (Promega). The dual injectors were used to introduce 75 μ L of LUC assay reagent and Stop & Glo reagent, respectively, per well.

If the miRNA can bind to the target gene and regulate its expression, changes in *Firefly* LUC level will affect the dual LUC ratio, while controls consist of assays where the miRNA precursor culture was replaced with a precursor construct culture that does not match with the target sequence of interest. A significant reduction of dual LUC ratios compared with the controls is used to report and validate miRNA-target interactions. Statistical analysis was performed using GraphPad Prism 6 software.