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

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24 **Abstract:** Environmental contamination by xenobiotics represents a major threat for natural
25 ecosystems and public health. In response, xenobiotic detoxification is a fundamental trait of
26 organisms for developmental plasticity and stress tolerance, but the underlying molecular
27 mechanisms remain poorly understood in plants. To decipher this process, we explored the
28 consequences of allopolyploidy on xenobiotic tolerance in the genus *Spartina* Schreb.
29 Specifically, we focused on microRNAs (miRNAs) owing to their central function in the
30 regulation of gene expression patterns, including responses to stress.

31 Small RNA-Seq was conducted on the parents *S. alterniflora* and *S. maritima*, their F1 hybrid
32 *S. x townsendii* and the allopolyploid *S. anglica* under phenanthrene-induced stress (phe), a
33 model Polycyclic Aromatic Hydrocarbon (PAH) compound. Differentially expressed miRNAs in
34 response to phe were specifically identified within species. In complement, the respective
35 impacts of hybridization and genome doubling were detected, through changes in miRNA
36 expression patterns between *S. x townsendii*, *S. anglica* and the parents. The results support
37 the impact of allopolyploidy in miRNA-guided regulation of plant response to phe.

38 In total, we identified 17 phe-responsive miRNAs in *Spartina* among up-regulated *MIR156* and
39 down-regulated *MIR159*. We also describe novel phe-responsive miRNAs as putative *Spartina*-
40 specific gene expression regulators in response to stress. Functional validation using
41 *Arabidopsis* (L.) Heynh. T-DNA lines inserted in homologous *MIR* genes was performed, and
42 the divergence of phe-responsive miRNA regulatory networks between *Arabidopsis* and
43 *Spartina* was discussed.

44

45 **Keywords:** Allopolyploidy, hybridization, miRNA, phenanthrene, *Spartina*, xenome.

46 **1. Introduction**

47 Polyploidy represents a major event affecting the evolutionary history of plants (Comai 2005;
48 Van de Peer et al. 2017). Allopolyploidy, resulting in the coexistence of divergent
49 (homeologous) genomes in the same nucleus, is a substantial source of evolutionary novelties
50 (Wendel 2015; Wendel et al. 2018; Nieto Feliner et al. 2020). At the molecular level, it involves
51 genetic and epigenetic alterations (Chen, 2007), collectively considered consequences of the
52 “genomic shock” (McClintock 1984) induced by hybridization and genome redundancy
53 (Jackson and Chen 2010). Changes include novel gene expression patterns, and result in new
54 phenotypes that may impact the adaptive potential of allopolyploids (Doyle et al. 2008; te
55 Beest et al. 2012). In line with these reports, several examples of enhanced environmental
56 stress tolerance in polyploids compared to diploids are described (Del Pozo and Ramirez-Parra
57 2015; Fox et al. 2020).

58 In response to environmental pollutants foreign to biological systems (xenobiotics),
59 detoxification is an adaptive trait encountered by all living organisms, though it remains poorly
60 understood in plants. During the past decade, studying plant responses to organic xenobiotics
61 from Polycyclic Aromatic Hydrocarbons (PAHs) became a critical issue due to their
62 environmental persistence and toxicity (Harvey et al. 2002; Greenberg 2003). The absorption
63 of PAHs by plant roots occurs through passive or active transport mechanisms (Pilon-Smits
64 2005; Zhan et al. 2012). Once in plant tissues, xenobiotics may be accumulated, degraded,
65 and/or compartmented (Gao and Zhu 2004; Pilon-Smits 2005). To date, the physiological
66 impact of PAHs on plants is mostly documented in *Arabidopsis* (L.) Heynh. (Alkio et al. 2005;
67 Liu et al. 2009; Weisman et al. 2010; Shiri et al. 2015; Dumas et al. 2016) in response to
68 phenanthrene (phe), one of major PAHs found in crude oil (Liu et al., 2012). In plants, PAHs
69 reduce growth and development, for example by disrupting major metabolic functions such

70 as photosynthesis, or through the oxidative stress induced by reactive oxygen species (ROS)
71 (Alkio et al. 2005; Shiri et al. 2015; Dumas et al. 2016).

72 At the molecular level, the concept of “xenome” emerged to characterize genes involved in
73 xenobiotic detoxification pathways (Edwards et al. 2005; Edwards et al. 2011). It includes
74 transport, signaling and sensing genes, and several gene families involved in xenobiotic
75 conjugation and biotransformation such as α/β hydrolases, cytochromes P450 (CYPs),
76 glutathione-S transferases (GSTs), glycosyltransferases (GTs), peroxidases and
77 malonyltransferases. In addition, ATP-binding cassette transporters (ABC) may be responsible
78 for the transport of xenobiotic conjugates to the vacuole for storage (Weisman et al. 2010). In
79 *Arabidopsis*, transcriptomic analyses under phe-induced stress revealed significant up and
80 down-regulations of xenome genes (Weisman et al. 2010; Dumas et al. 2016), which
81 highlighted candidates involved in primary responses to xenobiotics and putative
82 metabolization process.

83

84 In this study, we explore the evolution of PAH-induced stress in the genus *Spartina* Schreb.
85 (cordgrasses), following recent allopolyploid speciation. Most *Spartina* species are salt marsh
86 plants, characterized by recurrent hybridization and genome doubling events (Ainouche et al.,
87 2009, 2012). In *Spartina*, a recent allopolyploidy event offers the opportunity to study the
88 respective impacts of hybridization and genome doubling on the structural and functional
89 genome dynamics (Ainouche et al. 2004; Salmon et al. 2005; Parisod et al. 2009; Chelaifa et
90 al. 2010b; Ferreira de Carvalho et al. 2017). The allopolyploid model system includes the
91 parental species *S. maritima* Curtis ($2n = 6x = 60$, paternal parent) and *S. alterniflora* Loisel ($2n$
92 $= 6x = 62$, maternal parent), which hybridized during the end of the 19th century and led to the
93 formation of the F1 sterile hybrid *S. x townsendii* ($2n = 6x = 62$; Groves & Groves, 1880). Whole

94 genome doubling of *S. x townsendii* resulted in the allododecaploid *S. anglica* ($2n = 12x = 120-$
95 124; Hubbard, 1968). In studied *Spartina* populations, very low intra- and inter-individual
96 genetic diversity was reported due to a strong genetic bottleneck during *S. anglica* speciation
97 (Ayres and Strong 2001; Baumel et al. 2001). To date, the formation of *S. anglica* is considered
98 as a unique event involving one genotype of each parental species (Ainouche et al., 2004).
99 In *Spartina*, a high degree of tolerance to stresses, salinity, anoxia and heavy-metals, have
100 been reported (Anderson and Treshow 1980; Thompson 1991; Castillo et al. 2000; Hacker et
101 al. 2001; Wang et al. 2006). *Spartina* species are also frequently exposed to xenobiotics in their
102 natural habitats, including PAHs resulting from oil spills (Lin and Mendelssohn 2012; Lin et al.
103 2016; Robertson et al. 2017). Recently, comparative physiological analyses between *S. anglica*
104 and its parents revealed enhanced tolerance to the following allopolyploidy (Cavé-Radet et
105 al. 2019). Moreover, the high resilience to crude oil reported in *S. alterniflora* also suggests
106 efficient tolerance to PAHs in this genus (Wu et al. 2012; Alvarez et al. 2018).

107

108 To decipher the molecular mechanisms underlying PAH tolerance in *Spartina*, we focused on
109 microRNAs (miRNAs) owing to their central function in the regulation of gene expression
110 (Khraiweh et al. 2012; Sunkar et al. 2012; Zhang 2015; Shriram et al. 2016). MicroRNAs are
111 small RNAs (sRNAs) from approximately 21 to 24 bp, derived from single-strand hairpin
112 precursor sequence transcribed from intergenic regions referred as *MIR* genes (Axtell 2013;
113 Rogers and Chen 2013). Most of miRNAs are excised from the precursors by DICER-LIKE
114 proteins, and mature miRNAs will bind to reverse complementary mRNAs with ARGONAUTE
115 (AGO) proteins into an RNA-induced silencing complex for post-transcriptionally target gene
116 silencing (*i.e.* cleavage or translational inhibition of transcripts). The impact of miRNAs on gene
117 expression patterns has been widely reported within the literature, such as in response to

118 stress where up and down-regulated miRNAs will respectively target negative and positive
119 stress tolerance regulators (Khraiwesh et al. 2012). For example, the impact of miRNAs in
120 response to drought or salinity affecting plant growth and productivity are well reported in
121 crop species such as maize (Li et al. 2013; Fu et al. 2017), wheat (Feng et al. 2017; Bakhshi et
122 al. 2017) and tomato (Liu et al., 2017). However, studies depicting plant miRNA-mediated
123 responses to xenobiotics were mostly focused on heavy metals (Xu et al. 2013; Gupta et al.
124 2014; He et al. 2014; Noman and Aqeel 2017) and remain only explored in wheat for PAHs (Li
125 et al., 2017, 2020).

126 Several studies emphasized new expression patterns of sRNAs and their significant impact in
127 the regulation of genes, transposable elements (TEs), or in DNA methylations as a result of
128 interspecific hybridization and/or genome doubling. To date, most of the literature focused
129 on synthetic hybrids and/or polyploids such as in *Arabidopsis* (Ha et al., 2009), allotetraploid
130 wheat (Kenan-Eichler et al., 2011; Li et al., 2014), maize (Crisp et al. 2020) or *Brassica* (Fu et
131 al., 2016; Martinez Palacios et al., 2019). More recently, the impact of hybridization and/or
132 genome doubling was also reported in non-model species such as *Solidago* (Xu et al. 2019; Wu
133 et al. 2021) or *Cucumis* (Yu et al. 2020). Overall, sRNA-mediated epigenetic modifications in
134 newly formed hybrids and polyploids represent a significant process to recover from genome
135 instability, through changes of sRNAs expression patterns involved in heterosis and
136 adaptation. In this perspective, we hypothesized that miRNAs expression patterns in response
137 to phe and following allopolyploidy in *Spartina* may impact species tolerance abilities.

138

139 In this study, we relied on *Spartina* sRNA annotations recently published (Cavé-Radet et al.,
140 2020) and performed small RNA-Seq in *S. alterniflora*, *S. maritima*, *S. x townsendii* and *S.*
141 *anglica* under control and phe-induced stress conditions. Differential expression analysis was

142 conducted to study the impact of allopolyploidy under phe-induced stress on miRNA
143 expression patterns, and more generally to characterize phe-responsive miRNAs in *Spartina*.
144 Functional validation of phe-responsive miRNAs using *Arabidopsis* T-DNA mutant lines
145 inserted in homologous *MIR* genes was included. In addition, *in planta* interactions between
146 homologous phe-responsive miRNAs and their target genes in *Arabidopsis* were validated
147 using the dual luciferase (LUC) assay strategy (Moyle et al. 2017). To our knowledge, this work
148 is the first study focused on miRNA-guided regulation in response to organic xenobiotics,
149 integrating the consequences of allopolyploidy to distinguish the relative impacts of
150 hybridization and genome doubling in such processes.

151

152 **2. Material and methods**

153 **2.1 Plant material and experimental design**

154 Plants were collected in their natural habitats, where low intra- and inter-population genetic
155 diversity within species was reported (Baumel et al. 2001; Baumel et al. 2003; Ainouche et al.
156 2003; Yannic et al. 2004). Here, we sampled rhizomes as seed germination is erratic in
157 *Spartina*. The parental species were sampled at Le Faou (Finistère, France) for *S. alterniflora*,
158 and in Brillac-Sarzeau and Le Hezo (Morbihan, France) for *S. maritima*. *S. x townsendii* was
159 collected in Hythe (Kent, England), and *S. anglica* was sampled at La Guimorais (Ille-et-Vilaine,
160 France). After rhizomes regrowth, plants (one genotype per species) were maintained three
161 weeks in the greenhouse under controlled conditions (natural sunlight for 13-14h
162 photoperiod, an average ambient temperature of 18-20°C, and 50-60% hygrometry). On the
163 sampled populations, chromosome number and ploidy level were previously analyzed by flow
164 cytometry, and confirmed hexaploid cytotypes for *S. maritima* ($2n = 60$), *S. alterniflora* ($2n =$

165 62) and *S. x townsendii* ($2n = 62$), while *S. anglica* is allododecaploid with a chromosome
166 number of $2n = 124$.

167 Mature plants were then transferred to phe-treated and control substrates following the
168 method described in Hong et al. (2015). Briefly, bulk sediments were collected from the
169 sampling site of *S. anglica*, and air-dried subsamples of sediments were supplemented with
170 phe diluted in absolute ethanol (100 mM phe, or with an identical volume of absolute ethanol
171 to serve as the control). These samples of sediments were then mixed with the remaining wet
172 sediments to reach a final concentration of 150 mg phe.kg⁻¹ (dry weight) in phe-treated
173 substrate, which represents a moderate exposure compared to concentrations reported in
174 polluted environments (*e.g.* see Shi et al. 2005; Watts et al. 2006; Wang et al. 2010). Plants
175 were potted with treated and control substrates supplemented with sterile vermiculite
176 (volume 1 : 3), and placed in a phytotron chamber with a light : dark regime of 16 : 8h and
177 an average ambient temperature of 20°C. Plants were watered each day with 150 mL of half-
178 strength Hoagland's nutrient solution (Arnon and Hoagland 1950). After 5 days, leaves at a
179 mature developmental stage were harvested, immediately frozen in liquid nitrogen, and kept
180 at -80°C. We performed RNA extraction with TRIzol® Reagent (Thermo Fisher) according to
181 the method described by Chelaifa et al. (2010b,a). In total, 24 RNA samples were extracted,
182 corresponding to three biological replicates per species (different ramets from a same
183 genotype) and treatments (phe-treated and control).

184

185 **2.2 Small RNA sequencing and data preprocessing**

186 Small RNA-Seq was performed in single end, on a NextSeq500 (Illumina®) sequencer (1×50 bp,
187 20 million reads) on 16 libraries (two of the three biological replicates). Preprocessing of small
188 RNA-Seq data was conducted as described in Cavé-Radet et al. (2020). Briefly, raw reads were

189 trimmed by Cutadapt (Martin 2011) to remove 3' sequencing adaptors, filtered by size (from
190 16 to 28 nucleotides), and subjected for quality check (Phred Q-score ≥ 30) by FASTQC
191 (Andrews 2010). Clean small RNA-Seq reads were further screened to identify previously
192 annotated *Spartina* sRNAs from Cavé-Radet et al. (2020), which include 594 miRNAs (113
193 conserved miRNAs and 481 novel miRNAs, that are those not annotated in other plant
194 species). *Spartina* miRNAs are associated with 3,552 putative target genes, identified from
195 previously assembled *Spartina* transcriptomes (Boutte et al., 2016; Ferreira de Carvalho et al.,
196 2013). Here, the numbers of small RNA-Seq reads corresponding to *Spartina* miRNAs (perfect
197 match: no gap, 100% identity and coverage) were calculated across libraries using custom
198 python scripts, and combined into a count matrix.

199

200 **2.3 *In silico* differential expression analysis in *Spartina***

201 The matrix of miRNA counts across libraries was normalized with EDAsq (Risso et al. 2011)
202 for within-lane effects (related to GC-content) and library size normalization (using the full-
203 quantile method). Normalized matrix was then subjected for differential expression analysis
204 with DESeq2 (Love et al. 2014). DE miRNAs were identified according to Wald tests with \log_2
205 Fold Changes (FC) significantly different from zero at Bonferroni adjusted p.value < 0.05 .

206 In this study, multiple pairwise comparisons between species and/or treatments were
207 performed for miRNA differential expression analysis. For a better overview, all comparisons
208 performed were summarized in Supplemental Table S1 to remind how miRNAs impacted by
209 allopolyploidy under phe exposure and phe-responsive candidate miRNAs were respectively
210 identified.

211

212 **2.3.1 Differentially expressed *Spartina* miRNAs following allopolyploidy under phe-**
213 **induced stress**

214 We first aimed to analyze the impact of allopolyploidy on miRNA expression patterns under
215 phe-induced stress, while the results under control condition for this experiment are already
216 published (Cavé-Radet et al. 2020). Here, we performed interspecific comparisons under phe
217 exposure, and determined the impact of hybridization by comparing miRNA expression
218 patterns between the MPV (Mid-Parent Value: the average of parental expression expected
219 under additive patterns) and *S. x townsendii*. Similarly, the impact of allopolyploidy (combining
220 hybridization and genome doubling) was investigated by comparing miRNA expression
221 patterns of *S. anglica* to the MPV, while the impact of genome doubling *per se* was determined
222 comparing *S. anglica* to the F1 hybrid (Supp. Table S1).

223

224 **2.3.2 Identification of phe-responsive miRNAs in *Spartina***

225 Beside studying the impact of allopolyploidy on miRNA expression patterns under phe-
226 induced stress, we more generally aimed to characterize phe-responsive miRNAs in *Spartina*.
227 In order to characterize miRNA-regulated gene networks potentially involved in phe tolerance,
228 we first identified DE miRNAs within *Spartina* species in response to stress (intraspecific
229 comparisons of phe-treated versus control libraries). In complement, because of previous
230 published data depicting PAH sensitivity in *S. maritima* compared to other investigated
231 *Spartina* species (Cavé-Radet et al. 2019), we also included as phe-responsive candidate
232 miRNAs DE miRNAs from interspecific pairwise comparisons under phe (*S. alterniflora*
233 compared to *S. maritima*, *S. x townsendii* compared to *S. maritima*, and *S. anglica* compared
234 to *S. maritima*). Finally, because of enhanced tolerance to phe in *S. anglica* following
235 allopolyploidy (Cavé-Radet et al. 2019), DE miRNAs between *S. x townsendii* and *S. anglica*

236 compared to the MPV were ultimately identified as phe-responsive candidate miRNAs whose
237 abundance was specifically affected by hybridization or allopolyploidy. A summary of pairwise
238 comparisons (intra- and interspecific) considered to identify phe-responsive candidate
239 miRNAs is provided in Supp. Table S1.

240 Afterwards, we crossed annotated targets of phe-responsive candidate miRNAs from *Spartina*
241 with *Arabidopsis* (TAIR10) gene families potentially involved in xenobiotic tolerance
242 mechanisms (<https://www.arabidopsis.org/browse/genefamily/index.jsp>), including 1151
243 loci. *Arabidopsis* xenome genes are distributed between 94 NAC transcription factors, 17
244 SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPLs), 191 α/β hydrolases, 130 ATP-binding
245 cassette (ABC) transporters, 245 CYPs, 319 GTs, 53 GSTs, 7 related malonyl-transferases, and
246 ROS scavenging enzymes including 3 catalases (CAT), 7 superoxide dismutases (CSDs, MSDs
247 and FSDs), 73 class III peroxidases, 8 ascorbate peroxidases (APX), and 4 ATP synthase proteins
248 (ATPS) (see Supplemental Table S2). These genes are xenome candidates and putative positive
249 or negative stress tolerance regulators (*e.g.* involved in oxidative stress responses; see Singh,
250 2002; Edwards et al., 2005, 2011; Alkio et al., 2005; Weisman et al., 2010; Dumas et al., 2016;
251 Li et al., 2017). Among phe-responsive candidate miRNAs, only miRNAs with target genes
252 annotated among these putative stress tolerance regulators were retained.

253

254 **2.4 Quantitative analysis of miRNA expression by RT-qPCR**

255 Validation of miRNA abundance was conducted for a subset of 11 miRNAs (see below), by RT-
256 qPCR using the poly(T) adaptor method (Shi et al. 2012) on the 24 RNA samples. After DNase
257 treatments of RNA extracts using the Turbo DNA-free kit (Thermo Fisher), cDNAs were
258 produced by poly(A)-tailing (Poly(A) tailing kit, Thermo Fisher) and reverse transcription was
259 performed using a universal poly(T) adaptor primer (SuperScript™ III Reverse Transcriptase

260 kit, Thermo Fisher). PCR amplifications were monitored with a reverse primer annealing with
261 the universal poly(T) adaptor sequence of cDNA. Reactions were carried out with 10 μ L of
262 SYBR Green Master Mix (Thermo Fisher), 5 ng of cDNA ($3 \text{ ng} \cdot \mu\text{L}^{-1}$), forward and universal
263 reverse primers ($0.8 \mu\text{M}$), and completed with sterile water for a final volume of 20 μ L.
264 Forward primers used for specific miRNA amplifications were designed manually
265 (Supplemental Table S3).

266 We performed a two-steps PCR protocol on a LifeCycler[®] 480 (Roche Life Science), using the
267 following program: 3 min. of pre-incubation at 95°C , followed by 45 cycles of 15 sec.
268 denaturation (95°C), 20 sec. annealing and elongation (60°C), and optical reading. Normalized
269 expression levels of miRNAs were compared to the 5S rRNA as a housekeeping gene, which
270 remains stable under experimental conditions (amplified with forward primer sequence
271 AAGTCCTCGTGTTCATCCCT). We estimated \log_2 FC corrected by PCR efficiency (E) of
272 amplicons (calculated with standard curves: $E = 10^{(-1/\text{slope})}$) with the following formula : $R =$
273 $(E_{\text{miRNA}}) \Delta\text{Cp}_{\text{miRNA}}^{(\text{control-treatment})} / (E_{5\text{S rRNA}}) \Delta\text{Cp}_{5\text{S rRNA}}^{(\text{control-treatment})}$ (Pfaffl, 2001). We performed
274 statistical analyses by Wilcoxon signed rank tests and considered \log_2 FC significantly different
275 from zero at $p.\text{value} < 0.05$.

276

277 **2.5 Functional validation of *Spartina* phe-responsive miRNAs in *A. thaliana***

278 **2.5.1 Selection of *Arabidopsis* T-DNA insertional *MIR* mutants**

279 For functional validation of phe-responsive miRNAs, we first identified homologous *Spartina*
280 miRNAs in *Arabidopsis*. Homologous miRNAs were identified by mapping *Spartina* miRNAs
281 onto the *Arabidopsis* TAIR10 genome (Bowtie2 end-to-end alignment, 2 accepted
282 mismatches; Langmead & Salzberg, 2012), prior to *MIR* gene prediction of aligned flanking
283 regions by miR-PREFeR (default parameters; Lei & Sun, 2014). The target genes of homologous

284 *Spartina* miRNAs identified in *Arabidopsis* were predicted by psRNATarget
285 (<http://plantgrn.noble.org/psRNATarget/>) based on the *Arabidopsis* TAIR10 genome
286 (expectation score ≤ 5.0 ; Dai et al., 2018).

287 Hence, related *MIR* genes in *Arabidopsis* were retained and we selected corresponding T-DNA
288 mutants. *Arabidopsis* T-DNA mutants were obtained from the SALKseq and SAIL collections
289 (Colombia control grown counterpart: Col-0), and from the FLAG collection (Wassilewskija
290 control grown counterpart: WS). Homozygous lines of T-DNA mutants were selected as
291 described by O'Malley et al. (2015) in two-step genotyping assays, using genomic DNA
292 extracted as described by Edwards et al. (1991). Primers used to amplify flanking regions of
293 the insertion site are available online (<http://signal.salk.edu/tdnaprimers.2.html>).

294

295 **2.5.2 T-DNA mutants: *in vitro* culture and phenotyping**

296 *Arabidopsis* seeds were surface-sterilized and sown in Petri dishes on half-strength Murashige
297 and Skoog (MS/2) solid medium containing 0.8 % (w : v) agar-agar type E (Sigma Aldrich) and
298 1.0 % sucrose. We germinated seeds vertically in a growth chamber (16 : 8h light : dark cycle,
299 8,500 lux, 22 °C, 70 % hygrometry) after cold treatment for 48h at 4°C. Within Petri dishes, 10
300 homozygous seeds per T-DNA mutant line and its control grown counterpart were cultivated
301 in three technical replicates under control (0 μ M) and phe-induced stress. Phe was diluted in
302 ethanol and mixed in substrates for moderate (50 μ M), and high phe-induced stress
303 concentrations (200 μ M as sub-lethal concentration, see Dumas et al. 2016). In controls, the
304 same volumes of ethanol used for phe solubilization were added.

305 Plants were photographed after 7, 14 and 20 days of growth. We measured primary root
306 growth as plantlet tolerance trait to xenobiotic during early *Arabidopsis* developmental stages
307 using ImageJ (v1.51j8). We performed pairwise comparisons of primary root lengths between

308 treatments on T-DNA mutants compared to their control grown counterparts using non-
309 parametric Kruskal-Wallis tests (Bonferroni adjusted p.value < 0.05) in R 3.5 (R Core Team
310 2015).

311

312 **2.6 Experimental validation of miRNAs and target genes interactions from *A. thaliana* by** 313 **dual LUC assays**

314 In order to confirm the impact of homologous phe-responsive *Spartina* miRNAs identified in
315 *Arabidopsis*, experimental validation quantifying miRNA repression of targeted genes was
316 performed using the dual LUC assay strategy (Moyle et al. 2017). Detailed method about the
317 dual LUC assay procedure followed is provided as Supplemental Note S1. Briefly, the strategy
318 consists in cloning both the predicted target sequences and the miRNA precursors within
319 expression plasmids (Supplemental Fig. S1). Plasmid containing the miRNA target sequences
320 (pGrDL_SPb) includes two expression cassettes for a quantitative dual luciferase reporter
321 system. One cassette contains the *Renilla* LUC acting as internal control to standardize
322 expression between replicates, while the other cassette contains the *Firefly* LUC and the
323 target sequence used to report miRNA interaction. The other plasmid (pKENminusbar) is used
324 for miRNA precursor insertion. Agroinfiltration of expression plasmids was carried out in
325 *Nicotiana benthamiana*, and agroinfiltrated leaves were harvested prior to measurement by
326 dual LUC assay (*Firefly* LUC / *Renilla* LUC ratios). If the miRNA can bind to the target gene and
327 regulate its expression, changes in *Firefly* LUC level will affect the dual LUC ratio, while controls
328 consist of assays where the miRNA precursor culture was replaced with a precursor construct
329 culture that does not match with the target sequence of interest. A significant reduction of
330 dual LUC ratios compared with the controls is used to report and validate miRNA-target
331 interactions. Statistical analysis was performed using GraphPad Prism 6 software.

332

333 **3. Results**

334 **3.1 Preprocessing and analysis of small RNA-Seq libraries**

335 From all libraries, we obtained 460,502,187 sRNAs reads (deposited on NCBI under accession
336 PRJNA552944). After preprocessing, we retained 31,611,711 non-redundant putative sRNA
337 sequences (Supplemental Table S5).

338 Most miRNAs previously annotated in *Spartina* (Cavé-Radet et al., 2020) were expressed
339 under phe-induced stress in *S. alterniflora*, *S. maritima*, *S. x townsendii* and *S. anglica* (Fig.
340 **1a,b**). Both the hybrid and *S. anglica* expressed all parental conserved miRNAs (Fig. **1a**), except
341 for a single miRNA (Spa-MIR393c) undetected in the parental species under phe. Concerning
342 novel miRNAs annotated in *Spartina* (Fig. **1b**), the hybrid harbored between 97.72% and
343 98.48% of the parental miRNAs (214/219 from *S. alterniflora* and 389/395 from *S. maritima*).
344 The same pattern was observed in *S. anglica* (expressing ca. 95% of parental novel miRNAs :
345 209/219 from *S. alterniflora* and 377/395 from *S. maritima*). Interestingly in *S. x townsendii*
346 and *S. anglica*, each species harbored 30 novel miRNAs undetected in the parental species
347 under phe (Figure **1b**).

348

349 **3.2 Differentially expressed miRNAs in response to phe within *Spartina* species**

350 Expression levels between phe-treated and control libraries were analysed in each species. In
351 total, we identified 103 non-redundant DE miRNAs (Fig. **2a**), distributed between 20 miRNAs
352 in *S. alterniflora* (9 up-regulated and 11 down-regulated), 14 miRNAs in *S. maritima* (9 up-
353 regulated and 5 down-regulated), 54 miRNAs in *S. x townsendii* (26 up-regulated and 28 down-
354 regulated), and 33 miRNAs in *S. anglica* (19 up-regulated and 14 down-regulated). Based on
355 hierarchical clustering of expression profiles, we found that most of DE miRNAs appeared

356 species-specific (*i.e.* only differentially expressed in response to phe in one species: 13 in *S.*
357 *alterniflora*, 11 in *S. maritima*, 39 in *S. x townsendii* and 23 in *S. anglica*). In contrast, DE
358 miRNAs specific to *S. x townsendii* and *S. anglica* are scarce, with only three miRNAs (Spa-
359 *MIR395d*, Spa-new-*MIR_384* and Spa-*MIR156c*) significantly up-regulated in response to phe
360 in both species. Nonetheless, few miRNAs exhibited opposite expression patterns between
361 species, for example Spa-new-*MIR_154* or Spa-*MIR160* which were up-regulated in *S. x*
362 *townsendii* but down-regulated in the allopolyploid.

363 Experimental validation of DE miRNAs was performed by RT-qPCR for 11 miRNAs for which we
364 successfully designed miRNA specific primers. Here, we compared miRNA expression patterns
365 within *Spartina* species in response to stress (intraspecific comparisons of phe-treated versus
366 control libraries), as estimated by the *in-silico* (EDAseq-DESeq2) and the RT-qPCR approaches
367 (Supplemental Fig. S2). On this subset of 11 miRNAs, we detected significant differences in
368 miRNA expression patterns for 18 comparisons using EDAseq-DESeq2 (corresponding to 3
369 miRNAs DE in *S. alterniflora*, 3 miRNAs DE in *S. maritima*, 7 miRNAs DE in *S. x townsendii*, and
370 5 miRNAs DE in *S. anglica*; bars surrounded in red in Supp. Fig. S2). From RT-qPCR, we
371 confirmed significant differences in miRNA expression patterns for 6 comparisons (bars
372 surrounded in green in Supp. Fig. S2). For the other 12 comparisons with significantly DE
373 miRNAs reported by EDAseq-DESeq2, we observed similar regulation profiles (up- or down-
374 regulated miRNAs using both methods) but log₂ FC were not statistically significant using the
375 RT-qPCR approach. Overall, experimental validation by RT-qPCR is consistent with *in-silico*
376 miRNA differential expression analysis.

377

378 **3.3 Impact of allopolyploidy on *Spartina* miRNA expression patterns under phe-induced**
379 **stress**

380 Interspecific comparisons of miRNA expression patterns under phe were performed between
381 species in order to characterize the relative impact of hybridization and allopolyploidy under
382 stress exposure (for the results under control condition, see Cavé-Radet et al. 2020). When
383 comparing *S. x townsendii* expression levels to the MPV (Fig. **2b**), non-additive expression
384 profiles were detected for 73 miRNAs following hybridization (18 up-regulated and 55 down-
385 regulated). Among them, 14 and 31 miRNAs were respectively expressed under paternal and
386 maternal dominance, whereas 22 miRNAs exhibited transgressive expression profiles. By
387 comparing *S. anglica* to the MPV and to *S. x townsendii* (Fig. **2b**), we respectively identified 32
388 DE miRNAs (9 up-regulated and 23 down-regulated) and 28 DE miRNAs (12 up-regulated and
389 16 down-regulated). Concerning the 32 DE miRNAs identified following allopolyploidy, 10 and
390 8 miRNAs were respectively expressed under paternal and maternal dominance, whereas 14
391 exhibited transgressive expression profiles. Annotated target genes of DE miRNAs in response
392 to hybridization and allopolyploidy are provided in Supplemental Table S6. Here, target genes
393 of DE miRNAs following hybridization or allopolyploidy are mostly annotated with GO terms
394 related with developmental and stress responsive processes such as ‘cell growth’, ‘cell
395 differentiation’ or ‘response to stress’. Interestingly, 20 miRNAs were found DE following
396 hybridization (comparing *S. x townsendii* to the MPV) and allopolyploidy (comparing *S. anglica*
397 to the MPV) under phe-induced stress. For example, we observed down-regulated Spa-
398 *MIR159b* and Spa-*MIR159f* targeting MYB domain transcription factors (AT5G06100), among
399 other DE novel miRNAs.

400 Changes in miRNA expression patterns following allopolyploidy under phe-induced stress are
401 summarized in Fig. **3**. Here, changes were mostly detected between *S. alterniflora* and *S.*
402 *maritima* (19.19%). While comparing *S. x townsendii* to the MPV, we found more changes in
403 miRNA expression patterns following hybridization (12.87%) compared with allopolyploidy

404 (5.62%, comparing *S. anglica* to the MPV) or genome doubling *per se* (4.84%, comparing *S.*
405 *anglica* to *S. x townsendii*). In contrast, changes measured under control condition previously
406 published in Cavé-Radet et al. (2020) are provided (obtained from the same control samples
407 we used in this study), where most changes were detected between the MPV and *S. anglica*
408 (16.87%) instead of following hybridization (6.52%) or genome doubling (14.33%). For further
409 details, we crossed DE miRNAs following allopolyploidy (considering up and down-regulated
410 expression patterns) between control condition and phe-induced stress (for each of the
411 interspecific comparisons performed: *S. alterniflora* compared to *S. maritima*, *S. x townsendii*
412 compared to the MPV, *S. anglica* compared to the MPV, and *S. anglica* compared to *S. x*
413 *townsendii*). As presented on the Venn diagrams from Fig. 3, DE miRNAs following
414 allopolyploidy in *Spartina* are mostly different between treatments.

415

416 **3.4 Phe-responsive miRNAs and regulatory modules potentially involved in xenobiotic** 417 **tolerance**

418 In differential expression analysis, 103 non-redundant phe-responsive candidate miRNAs were
419 identified from intraspecific comparisons (phe-treated versus control, Fig. 2a). In complement,
420 we also analysed DE miRNAs from pairwise interspecific comparisons under phe (*S. alterniflora*
421 compared to *S. maritima*, *S. x townsendii* compared to *S. maritima*, and *S. anglica* compared
422 to *S. maritima*) to detect additional phe-responsive candidate miRNAs. We found 57, 115 and
423 80 DE miRNAs when comparing *S. alterniflora*, *S. x townsendii* and *S. anglica* with *S. maritima*
424 under phe-induced stress respectively, representing 164 non-redundant miRNAs (46
425 conserved and 118 novel miRNAs). Finally, we also included DE miRNAs by comparing *S. x*
426 *townsendii* and *S. anglica* to the MPV under phe as additional phe-responsive candidate
427 miRNAs, representing in total 85 non-redundant miRNAs (18 conserved and 67 novel miRNAs).

428 When combining the results from differential expression analysis, phe-responsive candidate
429 miRNAs represent 254 non-redundant miRNAs (69 conserved and 185 novel miRNAs). Among
430 them, we only retrieved miRNAs with annotated *Spartina* target genes potentially involved in
431 tolerance to xenobiotics based on *Arabidopsis* annotations (Cavé-Radet et al., 2020). For a
432 better overview into phe-responsive miRNAs identification, the process and results were
433 summarized in Supplemental Fig. S3.

434 In total, we identified 17 phe-responsive miRNAs in *Spartina* (6 conserved and 11 novel
435 miRNAs; Table 1) which may affect the functionality of negative and positive tolerance
436 regulators. Among them, miRNAs from the *MIR156* family (Spa-*MIR156c*, Spa-*MIR156d*, Spa-
437 *MIR156e* and Spa-*MIR156h*) and Spa-new-*MIR_3* are targeting SPLs annotated *Spartina*
438 contigs. We also found down-regulated miRNAs from the *MIR159* family (Spa-*MIR159d* and
439 Spa-*MIR159j*), and other novel miRNAs (Spa-new-*MIR_1*, Spa-new-*MIR_426*, Spa-new-
440 *MIR_207*, Spa-new-*MIR_458* and Spa-new-*MIR_151*) targeting GTs and CYPs. Additional
441 down-regulated novel miRNAs (Spa-new-*MIR_56*, Spa-new-*MIR_72*, Spa-new-*MIR_181*, Spa-
442 new-*MIR_447* and Spa-new-*MIR_316*) targeting CYPs and α/β hydrolases were also detected.
443

444 **3.5 Functional validation of phe-responsive conserved miRNAs**

445 Following miR-PREFeR predictions (Lei and Sun 2014) of *Spartina* miRNAs in *Arabidopsis*, we
446 identified 40 homologous *MIR* genes (Supplemental Table S7). These *MIR* genes are all related
447 with conserved miRNAs, except one that was not annotated in *Arabidopsis* (homologous to
448 Spa-*MIR156e* and Spa-*MIR156j*, referred as *MIR-ncRNA*). In *MIR-ncRNA*, the precursor was
449 predicted between positions 14305804 and 14305915 on *Arabidopsis* Chr2 in an antisense
450 long non-coding RNA (lncRNA; AT2G33815), and overlaps with *SPL3* as one of its putative
451 target gene (AT2G33810). Some important criteria of miRNA precursor were reported on *MIR-*

452 ncRNA (Supplemental Fig. S4), but it most likely represents a false positive from the prediction
453 as no interaction with its putative target genes (*SPL3* or other SPLs) was observed by dual LUC
454 assays (Supplemental Fig. S5).

455

456 In total, 14 *MIR* genes homologous to *Spartina* phe-responsive miRNAs were identified in
457 *Arabidopsis*. Homologous *MIR* genes belong to the *MIR156* (with the closely related *MIR157*)
458 and the *MIR159* (with the closely related *MIR319*) families. Their target genes (predicted by
459 psRNATarget) from the *Arabidopsis* xenome are provided in Supplemental Table S8. For
460 functional validation, most T-DNA insertional mutants for the *MIR* genes selected were
461 available within stock centers, and we performed a primary phenotypic screening using the
462 same culture conditions as described in section 2.5.2. Once received, seeds were germinated
463 under 0 and 25 μM phe, but only T-DNA mutants with a significant impact of phe on the
464 primary root growth compared to their control grown counterparts were retrieved prior to
465 the selection of homozygous lines. Hence, we only considered *Ath-MIR156e* (homologous to
466 *Spa-MIR156e* and *Spa-MIR156j*) and *Ath-MIR159d* (homologous to *Spa-MIR159d*) using two
467 independent homozygous T-DNA lines for both *MIR* genes (Supplemental Table S9). For
468 functional validation, 10 homozygous seeds per T-DNA mutant line and its control grown
469 counterpart were cultivated in three technical replicates under control (0 μM) and phe-
470 induced stress (50 and 200 μM).

471 We obtained results from the SAIL_770_G05 and SALKseq_030399.2 lines, where T-DNA
472 insertions are found in the *Ath-MIR159b* precursor sequence (AT1G18075, Fig. 4a). Cultivated
473 under control and phe-induced stress (0 and 200 μM phe), SAIL_770_G05 and
474 SALKseq_030399.2 exhibit tolerant phenotypes with significantly longer root lengths under
475 phe-induced stress compared to its control grown counterpart (Fig. 4b,c). In response to

476 stress, primary root growth is inhibited between 32 to 56% in Col-0, and by 17% and 23%
477 respectively in SAIL_770_G05 and SALKseq_030399.2. Opposite patterns are observed in the
478 FLAG_090F02 and FLAG_104E09 lines, where T-DNA insertions are found in the *Ath-MIR156e*
479 precursor sequence (AT5G11977, Fig. **4d**). When cultivated under control and phe-induced
480 stress conditions (0 and 200 μ M phe), FLAG_090F02 did not present significant differences in
481 primary root growth in response to phe compared to its control grown counterpart (Fig. **4e**).
482 However, when cultivated under moderate xenobiotic concentration (50 μ M phe), this mutant
483 showed senescence phenotypes on aged leaves, which turned yellow and lost all the
484 chlorophyll content. This sensitivity was confirmed by the results obtained for FLAG_104E09
485 where primary root growth is significantly reduced compared to its control grown counterpart
486 in response to phe (Fig. **4f**). In response to stress, primary root growth is inhibited by 36% in
487 Ws, and decreased up to 59% in FLAG_104E09.

488 From dual LUC assays, we experimentally validated the impact of *Ath-MIR156e* in the
489 regulation of SPLs (the significant repression when *Ath-MIR529b* was co-infiltrated with the
490 control perfect match target construct is used as a positive control, Fig. **5a**). Based on
491 significant differences in dual LUC assays compared with the controls, we validated that *Ath-*
492 *MIR156e* is targeted to regulate *SPL2* and *SPL4*, but no interaction was observed with *SPL13B*
493 (Fig. **5b**).

494

495 **4. Discussion**

496 In this study, we explored the impact of phe on miRNA expression patterns in *Spartina*, in the
497 context of recent allopolyploid speciation. Plants were exposed 5 days under 150 mg phe.kg⁻¹
498 and such concentration induced little observable phytotoxicity. Nonetheless, the phenotypic
499 effect of phe exposure has been previously reported by Hong et al. (2015) in *S. alterniflora*,

500 with a significant decrease of leaf dry biomass in plants exposed to lower phe concentrations
501 (70 days under 10 and 100 mg phe.kg⁻¹). In complement, differences in tolerance, ROS
502 production, and phe contents in leaf tissues were previously reported between the
503 investigated *Spartina* species under phe-induced stress (Cavé-Radet et al. 2019). Such reports
504 suggest intraspecific differences in phe accumulation and detoxification in *Spartina* following
505 allopolyploidy. While accumulation and detoxification both affect plant tolerance to
506 xenobiotics, we aimed to compare the related molecular pathways between species with a
507 focus on miRNAs as major regulators of gene expression, and particularly regarding gene
508 networks involved in developmental processes and response to stress.

509

510 **4.1 Hybridization and genome doubling reprogrammed miRNAs under phe-induced stress in** 511 ***Spartina***

512 We found the highest miRNA transcriptional differences (19.19%) between the parental
513 species *S. maritima* and *S. alterniflora*, as a result of species divergence sometimes 2-4 Mya
514 (Rousseau-Gueutin et al. 2015). This report is consistent with the transcriptional divergence
515 previously detected between the parental species (Chelaifa et al. 2010a; Ferreira de Carvalho
516 et al. 2013, 2017; Giraud et al. 2021). Thus, the recently formed hybrid and allopolyploid have
517 resulted from the merger of divergent regulatory networks, and miRNAs undetected in the
518 parental species represent candidates triggered by allopolyploidy.

519

520 Altogether, our data indicated that allopolyploidy under phe exposure (with differential
521 impacts of hybridization and genome doubling) affected miRNA expression patterns in
522 *Spartina* (Fig. 3). Such patterns are consistent with the role of genetic buffer of sRNAs against
523 the genomic 'shock' following genome merger and redundancy in the hybrid and *S. anglica*,

524 as reported in *Arabidopsis* by Ha et al. (2009). Although single genotypes were selected as
525 representatives of each species, the recent origin, a very low intra-and inter-individual genetic
526 diversity, and clonal propagation are limiting the effects of selection acting on different
527 genotypes in *Spartina*. Hence, results we obtained are mostly related to the immediate
528 evolutionary effects of allopolyploidy rather than population divergence, even if we cannot
529 strictly exclude that other mechanisms resulting from the history of species might interact.

530 In response to allopolyploidy, major changes in miRNA expression patterns under phe-induced
531 stress were detected following hybridization (12.87%, comparing *S. x townsendii* to the MPV)
532 compared with allopolyploidy (5.62%, comparing *S. anglica* to the MPV) or genome doubling
533 *per se* (4.84%, comparing *S. anglica* to *S. x townsendii*). Our results under phe-induced stress
534 are in contrast with recent reports obtained in control condition, where major changes
535 resulted from allopolyploidy (16.87%) compared to hybridization (6.52%, Fig. 3; Cavé-Radet et
536 al., 2020). Differences in DE miRNAs suggest that under phe exposure, miRNA-mediated
537 epigenetic modifications are mostly observed following hybridization rather than genome
538 doubling. In *S. x townsendii*, DE miRNAs under phe following hybridization may represent
539 important regulators related with heterosis, as reported in rice (He et al., 2010), tomato
540 (Shivaprasad et al. 2012), sugarcane (Zanca et al. 2010) or wheat (Kenan-Eichler et al. 2011).

541 Following hybridization, we found for example down-regulated miRNAs from the *MIR166*
542 family (*Spa-MIR166f*, *Spa-MIR166o* and *Spa-MIR166s*), targeting homeobox-leucine zipper
543 family protein and lipid-binding START domain-containing protein genes, which are
544 transcription factors involved in plant developmental processes (Chew et al. 2013). We also
545 found down-regulated *Spa-new_MIR_471*, which is targeting a double-stranded-RNA-binding
546 protein. Such proteins are reported to impact sRNA biosynthetic pathways (Curtin et al. 2008),
547 and might be related with enhanced miRNA transcriptional changes observed in *S. x*

548 *townsendii* compared to *S. anglica*. Another possible reason could be related to the impact of
549 TEs on miRNA expression patterns in response to stress (Hou et al. 2019). For example, a burst
550 of TEs specifically activated in response to stress following hybridization rather than
551 allopolyploidy might result in additional changes of miRNA expression patterns as observed in
552 the hybrid (e.g. TE-derived miRNAs or TEs adjacent to *MIR* genes).

553

554 More globally, changes in miRNA expression patterns in *S. x townsendii* and *S. anglica*
555 identified are most likely related with enhanced tolerance abilities to PAH following
556 allopolyploidy, including different responses from regulatory networks of stress-responsive
557 miRNAs. However, among phe-responsive miRNAs we reported only two miRNAs directly
558 affected by hybridization (*Spa-new_MIR_207* and *Spa-new_MIR_458*) when comparing *S. x*
559 *townsendii* to the MPV, while no phe-responsive miRNA affected by allopolyploidy has been
560 identified when comparing *S. anglica* to the MPV. Nevertheless, we found that most of target
561 genes associated with DE miRNAs following hybridization and/or allopolyploidy are related to
562 developmental and response to stress processes (Supplemental Table S6), such as recently
563 reported by Martinez Palacios et al. (2019) in *Brassica* allopolyploids. However, given the lack
564 of a reference genome in *Spartina* and target gene annotations for many DE miRNAs, it is
565 difficult to report additional phe-responsive miRNA regulatory modules in *Spartina*.
566 Considering the evolutionary divergence between *Spartina* and *Arabidopsis*, the molecular
567 response to xenobiotics most likely involves species-specific detoxification metabolic
568 pathways (Alvarez et al., 2018; Cavé-Radet et al., 2019), potentially including miRNA
569 regulatory modules and target genes we did not consider within the *Arabidopsis* xenome
570 genes. For example, among the 20 miRNAs DE following both hybridization and allopolyploidy
571 under phe-induced stress (comparing both *S. x townsendii* and *S. anglica* to the MPV), we

572 identified downregulated *MIR159* targeting MYB domain transcription factors (AT5G06100
573 annotated *Spartina* contig). The impact of MIR159-MYB regulatory modules was previously
574 reported in many stress responses (Roy 2016; Li et al. 2016; Chen et al. 2018), and might be
575 involved in phe tolerance mechanisms following allopolyploidy in *Spartina*. Other DE miRNAs
576 with transgressive expression patterns following hybridization and allopolyploidy
577 (Supplemental Table S6) also represent candidates of interest for further prospects, which
578 potentially ensure the genome stability in response to genomic ‘shock’ from allopolyploidy
579 under phe-induced stress.

580

581 **4.2 Phe-responsive miRNAs and regulatory modules retained in *Spartina***

582 In complement to report miRNAs whose abundance was affected by hybridization and
583 allopolyploidy under phe exposure in *Spartina*, we more generally aimed to detect phe-
584 responsive candidate miRNAs by comparing expression patterns within and among *Spartina*
585 species. From intraspecific comparisons, 103 DE miRNAs in response to phe were identified
586 and validation by RT-qPCR is consistent with *in-silico* miRNA differential expression analysis
587 performed by EDAseq-DESeq2 (Supplemental Fig. S2). Interestingly, we rarely observed
588 opposite miRNA regulations between *Spartina* species (*e.g.* up- versus down-regulated
589 miRNAs). Such patterns are mainly contrasting *S. x townsendii* to *S. anglica* and confirm that
590 phe impacts differently miRNA expression patterns among species.

591 Based on interspecific comparisons under phe-induced stress, we retained 254 non-redundant
592 phe-responsive candidate miRNAs (69 conserved and 185 novel miRNAs). In total, we
593 retrieved 17 phe-responsive miRNAs (Table 1), distributed between 6 conserved and 11 novel
594 miRNAs. Among them, we identified phe-responsive miRNAs targeting SPLs annotated
595 *Spartina* contigs, and down-regulations of these genes are correlated with enhanced growth,

596 development, and tolerance to several stresses (Li et al., 2017). We also found phe-responsive
597 miRNAs targeting GTs, CYPs and α/β hydrolases, for which up-regulations of these genes may
598 impact tolerance abilities to xenobiotic and oxidative stress.

599

600 So far, stress-responsive miRNAs in *Spartina* were only explored in *S. alterniflora* under salinity
601 (Qin et al. 2015; Zandkarimi et al. 2015), while few studies have explored the role of plant
602 miRNAs in response to phe. Recently, Li et al. (2017) reported 11 phe-responsive miRNAs from
603 wheat. Here, the authors focused on roots to perform differential expression analysis of
604 miRNAs in early xenobiotic exposure (0, 12, 24 and 48h), while we exposed plants for 5 days
605 under phe to specifically focus on detoxification responses once xenobiotics accumulate in
606 leaf tissues. We crossed DE miRNAs from *Spartina* and wheat, but we observed little overlap
607 between the two taxa. We found only Spa-*MIR156e* (up-regulated in both taxa) and Spa-
608 *MIR398* (up-regulated in *Spartina* leaves but down-regulated in wheat roots). *MIR156* and
609 *MIR398* are shared *MIR* families among spermaphytes, and their impact in stress responses
610 are also well described. It includes for example the *MIR156*-SPL regulatory system through
611 enhanced plant development and growth under stress conditions (Aung et al., 2015), or
612 *MIR398* regulating superoxide dismutases involved in ROS scavenging (Ding et al. 2010; Li et
613 al. 2020). In *Spartina*, similar functions may be expected in response to PAHs, but the
614 differences we observed could also result from spatiotemporal specificities (e.g. organs, time
615 exposure) between experiments and/or from species divergence.

616

617 **4.3 *MIR156* and *MIR159* regulatory modules are targeted to adapt the xenome expression**
618 **and developmental plasticity in *Arabidopsis* in response to phe**

619 For functional validation of phe-responsive *Spartina* miRNAs, homologous *MIR* genes were
620 identified in *Arabidopsis* prior to T-DNA mutant phenotyping. Unfortunately, only homologous
621 *MIR* genes related with conserved *Spartina* miRNAs were identified in *Arabidopsis*
622 (Supplemental Table S7), most likely because of the evolutionary distance between species.
623 For a broader functional validation, homologous *MIR* genes were similarly identified in
624 *Brachypodium* and *Oryza* (phylogenetically related species among Poaceae), but only few
625 mutants were available in T-DNA collections, which was unsatisfactory in our approach. Hence
626 we only focused on miRNAs from conserved *MIR* gene families within embryophytes (Taylor
627 et al. 2014) such as Ath-*MIR156e* and Ath-*MIR159d*, and we were unable to perform functional
628 validation on novel phe-responsive *Spartina* miRNAs.

629

630 Concerning Ath-*MIR159d* (*Spa-MIR159d* homolog), tolerant phenotypes were observed in
631 SAIL_770_G05 and SALKseq_030399.2 under phe-induced stress. As described in *Spartina*,
632 *Arabidopsis MIR159* target genes include CYPs, in complement with other xenome genes such
633 as GTs and ABC transporters (Supplemental Table S8). In *Arabidopsis*, down-regulation of Ath-
634 *MIR159d* most likely induces up-regulation of positive stress tolerance regulators to phe.
635 Among *Arabidopsis MIR159* target genes, we detected genes involved in xenobiotic
636 detoxification, or in responses to oxidative stress such as ROS scavenging (Dumas et al., 2016;
637 Edwards et al., 2005, 2011; Li et al., 2017; Taguchi et al., 2010; Weisman et al., 2010).
638 *Arabidopsis MIR159* target genes include α/β hydrolases (AT5G18630, AT5G18640), CYPs
639 (AT1G67110, AT2G34500, AT5G35715), transferases (AT1G22360, AT4G36770), and
640 peroxidases (AT3G49960) involved in xenobiotic conjugation and biotransformation. In
641 addition, we reported *MIR159* and ATP-binding cassette (ABC) transporters (AT3G13090,
642 AT3G13100 and AT4G39850) regulatory modules involved in the transport of xenobiotic

643 conjugates to the vacuole for storage, as well as *MIR159* and copper/zinc superoxide
644 dismutase (*CSD3*: AT5G18100) regulatory module, expected to reduce the extent of oxidative
645 stress and related damages on plant physiology.

646 In contrast, sensitive phenotypes were identified in FLAG_090F02 and FLAG_104E09 in
647 response to phe, with insertions in *Ath-MIR156e* (*Spa-MIR156e* and *Spa-MIR156j* homolog).
648 We then confirmed that the down-regulation of *Ath-MIR156e* in *Arabidopsis*, probably in
649 association with the up-regulation of negative stress tolerance regulators, is related with phe
650 sensitivity. In *Arabidopsis*, negative stress tolerance regulators targeted by *MIR156* include
651 SPLs or NAC transcription factors (Supplemental Table S8), which represent key genes involved
652 in plant development (Hyun et al., 2017; Li et al., 2017; Xu et al., 2016). Among *Ath-MIR156e*
653 target genes, interactions with *SPL2* and *SPL4* were experimentally validated by dual LUC
654 assays, and similar mechanisms for *MIR156*-NACs regulatory modules are expected according
655 to *in silico* target gene prediction. We also highlight the *MIR159*-MYB regulatory module
656 (validated on *Ath-MIR159b-MYB33* by dual LUC assays; Supplemental Fig. S5), because of their
657 impact reported in many stress responses (Roy 2016; Li et al. 2016; Chen et al. 2018).
658 Evidences that *MIR159* and *MIR156* interact with each other were reported by Guo et al.
659 (2017) who described that *MIR159-MYB33* regulatory modules impact the transcription of
660 *MIR156* and the expression patterns of target genes downstream.

661

662 Given the highly pleiotropic effects of *MIR156* and *MIR159* families in plants, we confirmed
663 based on primary root growth the impact of phe in *Arabidopsis* T-DNA mutants compared to
664 their control grown counterparts (tolerant and sensitive phenotypes for T-DNA lines with
665 insertion in *Ath-MIR159b* and *Ath-MIR156e* *MIR* genes respectively). Such results support that
666 *MIR156* and *MIR159* regulatory modules are targeted to adapt the xenome expression and

667 developmental plasticity in response to phe-induced stress in *Arabidopsis*. Although the
668 molecular responses to xenobiotics may differ between *Arabidopsis* and *Spartina* (Alvarez et
669 al., 2018; Cavé-Radet et al., 2019), we focused here on evolutionary conserved gene families
670 and miRNA regulatory networks within embryophytes (Taylor et al. 2014). Then, it is
671 reasonable to speculate similar mechanisms for *Spartina* phe-responsive miRNAs from
672 *MIR156* and *MIR159*, as we reported up-regulated *MIR156* and down regulated *MIR159* in
673 *Spartina* in response to phe, predicted to target SPLs and xenome genes. In contrast, potential
674 differences are observed as no target gene annotated among peroxidases, ROS scavenging
675 enzymes, or NAC transcription factors were associated with phe-responsive miRNAs identified
676 in *Spartina*.

677 While further work is needed to precisely confirm the molecular pathways of phe-responsive
678 miRNAs in *Spartina* (e.g. transcriptomic analysis of phe-responsive miRNA target genes and
679 TEs), we emphasize that most of phe-responsive miRNAs identified are novel miRNAs. Such
680 novel miRNAs specifically identified in *Spartina* may be effective in response to xenobiotics,
681 and could explain the divergent tolerance abilities between *Spartina* and *Arabidopsis* (*S.*
682 *anglica* tolerates up to 800 μ M phe, whereas the sub-lethal dose is 200 μ M phe in *Arabidopsis*;
683 see Dumas et al., 2016). Hence, further analyses focused on novel phe-responsive miRNAs
684 functional validation in *Spartina* are promising, and target mimicry (Franco-Zorrilla et al. 2007)
685 or artificial miRNAs (Eamens et al. 2011) represent useful tools for such purpose. For example,
686 phe-responsive novel miRNAs Spa-new_*MIR_207* and Spa-new_*MIR_458* (respectively
687 targeting *Spartina* contigs annotated as GTs and CYPs and specifically identified by comparing
688 *S. x townsendii* against the MPV) are candidates of interest in this perspective.

689

690 **5. Conclusion**

691 In this study, we explored the impact of phe in reprogramming the expression of *Spartina*
692 miRNAs in the context of recent allopolyploid speciation. The results shed light on the
693 regulatory mechanisms involved in the response to phe-induced stress, and highlight the role
694 of allopolyploidy in reshaping gene expression regulation and impacting the evolution of
695 xenobiotic tolerance. We show how divergent responses of miRNA regulatory modules are
696 involved in molecular responses to phe between *Spartina* species, and in this regard, the genus
697 *Spartina* provides a unique model system of recent natural speciation (besides resynthesized
698 allopolyploids) to decipher the early evolutionary changes associated to allopolyploidy. Such
699 changes are most likely impacting the plasticity and adaptive potential of species, and are
700 consistent with the enhanced stress tolerance reported following allopolyploidy (Cavé-Radet
701 et al. 2019).

702 In total, we identified 17 phe-responsive miRNAs from both conserved and novel miRNAs
703 annotated in *Spartina*. The results bring new insights to understand miRNA-mediated
704 responses involved in PAHs detoxification and tolerance, and provide useful data for
705 biotechnological perspectives in agronomy or phytoremediation. Using *Arabidopsis* as a
706 heterologous system, functional validation of *MIR156* and *MIR159* regulatory modules was
707 performed. Similar mechanisms were speculated in *Spartina* in response to genomic 'shock'
708 from allopolyploidy and phe, but further work is needed to precisely confirm the impact of
709 phe-responsive miRNA regulatory networks in such non-model plants. More particularly, we
710 emphasize on DE miRNAs in response to allopolyploidy under phe, undetected within phe-
711 responsive *Spartina* miRNAs but potentially involved in other detoxification metabolic
712 pathways as those we considered from the *Arabidopsis* xenome.

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1030 **7. Statements & Declarations**

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1042 (INRA Versailles) for providing *Arabidopsis* T-DNA mutant lines.

1043

1044 **Data accessibility**

1045 Raw sequencing files of sRNAs reads are publicly available and deposited on NCBI under
1046 accession number PRJNA552944.

1047

1048 **Author contributions**

1049 A.C.-R., A.S, M.A., and A.E. designed the experiments. A.E., O.L., R.M., L.T., L.S.P. and A.C.-R.
1050 performed the experiments. A.S., M.A., A.E., L.T., and A.C-R. analysed data. A.C-R., M.A, A.S.,
1051 and A.E. wrote the article. Conflicts of interest: 'none'.

1052 **8. Figures**

1053

1054 **Figure 1. Venn Diagrams of conserved (a), and novel (b) miRNAs expressed under phe-**
1055 **induced stress across *Spartina* species.** The reticulate origin of the F1 interspecific hybrid, *S.*
1056 *x townsendii* and of *S. anglica* resulting from genome doubling of the former is presented on
1057 the right.

1058

1059 **Figure 2. Heatmaps from miRNA differential expression analysis in *Spartina* under phe-**
1060 **induced stress.** MicroRNA differential expression analysis included (a) intraspecific
1061 comparisons in response to phe (alt: *S. alterniflora*; mar: *S. maritima*; town: *S. x townsendii*;
1062 ang: *S. anglica*) and (b) pairwise interspecific comparisons under phe. Here, DE miRNAs under
1063 phe-induced stress in response to hybridization (*S. x townsendii* compared to the MPV),
1064 genome doubling *per se* (*S. anglica* compared to *S. x townsendii*) and allopolyploidy (*S. anglica*
1065 compared to the MPV) are presented. DE miRNAs were clustered according to their expression
1066 patterns between comparisons (*: p.value < 0.05; **: p.value < 0.01; -: miRNA undetected).

1067

1068 **Figure 3. Impact of allopolyploidy on *Spartina* miRNA expression patterns under phe-**
1069 **induced stress.** Transcriptional changes of miRNAs are presented as percentages (proportions
1070 of miRNA expression changes) between parental species (*S. alterniflora* compared to *S.*
1071 *maritima*), in response to hybridization (*S. x townsendii* compared to the MPV), genome
1072 doubling *per se* (*S. anglica* compared to *S. x townsendii*), and allopolyploidy (*S. anglica*
1073 compared to the MPV) are provided and compared to results previously obtained under
1074 control condition (Cavé-Radet et al. 2020). Venn diagrams represent the number of DE
1075 miRNAs (up-regulated in red and down-regulated in blue) between control (green circles) and
1076 phe-induced stress conditions (yellow circles) among comparisons.

1077

1078 **Figure 4. Functional validation of phe-responsive miRNAs *Spa-MIR159d* (*Ath-MIR159b***
1079 **homolog) and *Spa-MIR156e* (*Ath-MIR156e* homolog) in *Arabidopsis*.** On the left, insertions
1080 of T-DNA lines SAIL_770_G05 and SALKseq_030399.2 (in *MIR* gene AT1G18075) are presented
1081 (a). After 14 (b) and 20 days (c) cultivated under 0 and 200 μ M phe respectively, primary root
1082 lengths (\pm SE) of mutants were plotted and compared to compared to their control grown
1083 counterparts (Col-0). On the right, insertions of T-DNA lines FLAG_090F02 and FLAG_104E09
1084 (in *MIR* gene AT5G11977) are presented (d). After 14 (e) and 20 days (f) cultivated under 0
1085 and 200 μ M phe respectively, primary root lengths (\pm SE) of mutants were plotted and
1086 compared to their control grown counterparts (Ws). The rosette leaves of FLAG_090F02
1087 cultivated under moderate xenobiotic concentration (50 μ M phe) for 14 days were
1088 photographed. Bars annotated with different letters are significantly different according to
1089 pairwise Kruskal-Wallis tests (Bonferroni adjusted p.value < 0.05).

1090

1091 **Figure 5. Experimental validation by dual LUC assay for computational interactions of**
1092 ***Arabidopsis* Ath-MIR156e (homologous to phe-responsive Spa-MIR156e) and its predicted**
1093 **targets.** The significant repression when Ath-MIR529b was co-infiltrated with the control
1094 perfect match (PM) target construct was used as a positive control (a). The controls consist of
1095 assays where the miRNA precursor culture were replaced with a precursor construct culture
1096 that does not match with the target sequence of interest, and significant differences in dual
1097 LUC assays are used to validate miRNAs and target genes interactions. Interactions tested are
1098 Ath-MIR156e with target genes *SPL2*: (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 2;
1099 AT5G43270), *SPL4* (AT1G53160), and *SPL13B* (AT5G50670) (b). Data were depicted as box and
1100 whisker plots with interquartile range boxes and min/max whiskers (n=6). Asterisks represent
1101 statistically significant differences using a simple two-tailed unpaired *t*-test with ****p.value
1102 < 0.0001.

1103

9. Tables

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Table 1. Phe-responsive miRNAs retained in *Spartina*. Mature miRNA sequences (6 conserved and 11 novel miRNAs), target gene annotations from the *Arabidopsis* xenome, and expression patterns of miRNAs in response to phe are provided (the corresponding pairwise comparisons are included).

miRNA name	miRNA sequence	Arabidopsis annotation of target genes	Target genes description	DE miRNA expression patterns in response to phe
Spa-MIR156c	UUGACAGAAGAUAGAGAGCAC	AT5G43270	SPL	Up-regulated: → <i>S. x townsendii</i> (phe vs. control) → <i>S. anglica</i> (phe vs. control) → <i>S. x townsendii</i> compared to <i>S. maritima</i> (phe) → <i>S. anglica</i> compared to <i>S. maritima</i> (phe)
Spa-MIR156d	UUGACAGAAGAGAGUGAGCAC	AT5G43270; AT5G50670; AT1G53160	SPL	Up-regulated: → <i>S. alterniflora</i> compared to <i>S. maritima</i> (phe) → <i>S. x townsendii</i> compared to <i>S. maritima</i> (phe) → <i>S. anglica</i> compared to <i>S. maritima</i> (phe)
Spa-MIR156e	UGACAGAAGAGAGUGAGCAC	AT5G43270; AT5G50670; AT1G53160	SPL	Up-regulated: → <i>S. alterniflora</i> (phe vs. control) → <i>S. alterniflora</i> compared to <i>S. maritima</i> (phe)
Spa-MIR156h	UGACAGAAGAGAGCGAGCAC	AT5G43270; AT5G50670; AT1G53160	SPL	Up-regulated: → <i>S. alterniflora</i> compared to <i>S. maritima</i> (phe) → <i>S. x townsendii</i> compared to <i>S. maritima</i> (phe) → <i>S. anglica</i> compared to <i>S. maritima</i> (phe)
Spa-new_MIR_3	CGGCGGCAGAGCUCGAGGCUC	AT1G53160	SPL	Up-regulated: → <i>S. alterniflora</i> compared to <i>S. maritima</i> (phe)
Spa-MIR159d	UUUGGAUUGAAGGGAGCUCUU	AT1G13080	cytochrome P450 family	Down-regulated: → <i>S. alterniflora</i> compared to <i>S. maritima</i> (phe) → <i>S. x townsendii</i> compared to <i>S. maritima</i> (phe) → <i>S. anglica</i> compared to <i>S. maritima</i> (phe)

Spa-MIR159j	UUUGGAUUGAAGGGAGCUCU	AT1G13080	cytochrome P450 family	Down-regulated: → <i>S. alterniflora</i> compared to <i>S. maritima</i> (phe)
Spa-new_MIR_151	AAAAUGUUGUCAUUAGAACAA	AT3G26300	cytochrome P450 family	Down-regulated: → <i>S. anglica</i> (phe vs. control)
Spa-new_MIR_181	UAACGACAACAAUUUCAGAAC	AT4G34310; AT3G26300	alpha/beta-Hydrolases superfamily protein and cytochrome P450 family	Down-regulated: → <i>S. anglica</i> (phe vs. control)
Spa-new_MIR_1	AUCUCUGAGAAGCUCUUUAUAGGA	AT1G53290	glycosyltransferase gene family	Down-regulated: → <i>S. alterniflora</i> compared to <i>S. maritima</i> (phe)
Spa-new_MIR_207	AUGAGAUUGGAACAUUGCCA	AT4G31780	glycosyltransferase gene family	Down-regulated: → <i>S. x townsendii</i> compared to the MPV (phe)
Spa-new_MIR_316	GAAAUUGUUGUCGCUAGGACA	AT4G34310	alpha/beta-Hydrolases superfamily protein	Down-regulated: → <i>S. anglica</i> compared to <i>S. maritima</i> (phe)
Spa-new_MIR_426	UUGGAUGAGAGAUGAAAGACC	AT3G24040; AT3G15350; AT3G13730	glycosyltransferase gene family and cytochrome P450 family	Down-regulated: → <i>S. x townsendii</i> compared to <i>S. maritima</i> (phe) → <i>S. anglica</i> compared to <i>S. maritima</i> (phe)
Spa-new_MIR_447	UAAAUUGUUGUCAUUAGGACA	AT4G34310; AT3G26300	alpha/beta-Hydrolases superfamily protein and cytochrome P450 family	Down-regulated: → <i>S. alterniflora</i> compared to <i>S. maritima</i> (phe) → <i>S. anglica</i> compared to <i>S. maritima</i> (phe)
Spa-new_MIR_458	UAAAUUGUUGUUGUAGGACA	AT3G26300	cytochrome P450 family	Down-regulated: → <i>S. x townsendii</i> compared to the MPV (phe)
Spa-new_MIR_56	UCUGAAAUUGUUGUCAUUAGG	AT4G34310; AT3G26300	alpha/beta-Hydrolases superfamily protein and cytochrome P450 family	Down-regulated: → <i>S. anglica</i> (phe vs. control)
Spa-new_MIR_72	UAAAUUGUUGUCGGUAGGACA	AT4G34310; AT3G26300	alpha/beta-Hydrolases superfamily protein and cytochrome P450 family	Down-regulated: → <i>S. x townsendii</i> compared to <i>S. maritima</i> (phe) → <i>S. anglica</i> compared to <i>S. maritima</i> (phe)

1109 **Supplementary Information:**

1110 **Figure S1.** Plasmids used for transient assay systems to validate computationally predicted targets of
1111 miRNAs.

1112 **Figure S2.** Expression patterns obtained for 11 miRNAs in response to phe within *Spartina* species.

1113 **Figure S3.** Identification process of phe-responsive miRNAs in *Spartina*.

1114 **Figure S4.** *De novo* prediction of a putative primary *MIR156* transcript in *Arabidopsis* (*MIR-ncRNA*),
1115 homologous to Spa-*MIR156e* and Spa-*MIR156j*.

1116 **Figure S5.** Additional experimental validation by dual LUC assay of computational interactions of
1117 *Arabidopsis* miRNAs (homologous to phe-responsive miRNAs in *Spartina*) and their predicted targets.

1118

1119 **Table S1.** Pairwise comparisons between treatments and species performed for miRNA differential
1120 expression analysis.

1121 **Table S2.** TAIR10 gene families potentially involved in xenobiotic detoxification.

1122 **Table S3.** Primers used for RT-qPCR of Spa-miRNAs.

1123 **Table S4.** Target site adaptors used to amplify the target sequences used for the cloning in the plasmid
1124 pGrDL_SPb and in dual LUC assays.

1125 **Table S5.** Summary of small RNA-seq data processing in *Spartina*.

1126 **Table S6.** *Spartina* annotated target genes of DE miRNAs in response to hybridization and
1127 allopolyploidy.

1128 **Table S7.** *Spartina* miRNAs and homologous *MIR* genes identified in *Arabidopsis*.

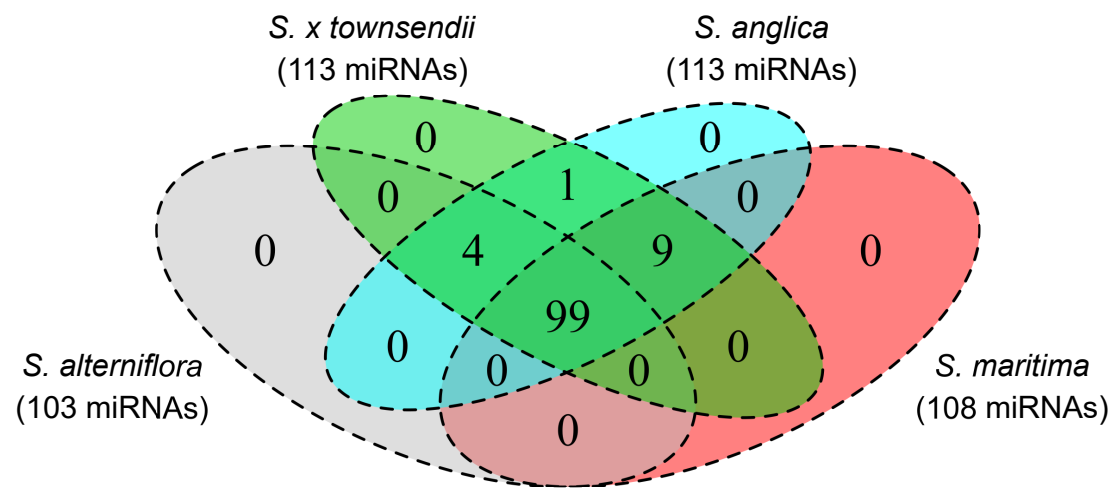
1129 **Table S8.** Target prediction results of phe-responsive homologous miRNAs in *Arabidopsis*.

1130 **Table S9.** *Spartina* homologous *MIR* genes in *Arabidopsis* and their corresponding T-DNA mutant lines.

1131

1132 **Note S1.** Detailed method followed for the experimental validation of miRNAs and target genes
1133 interactions from *A. thaliana* by dual LUC assays.

a



b

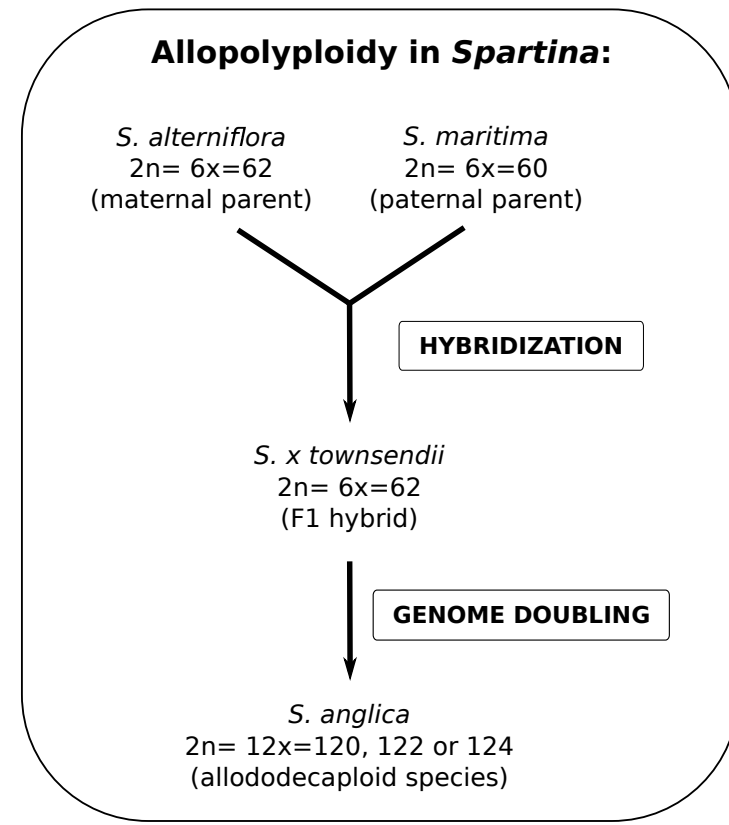
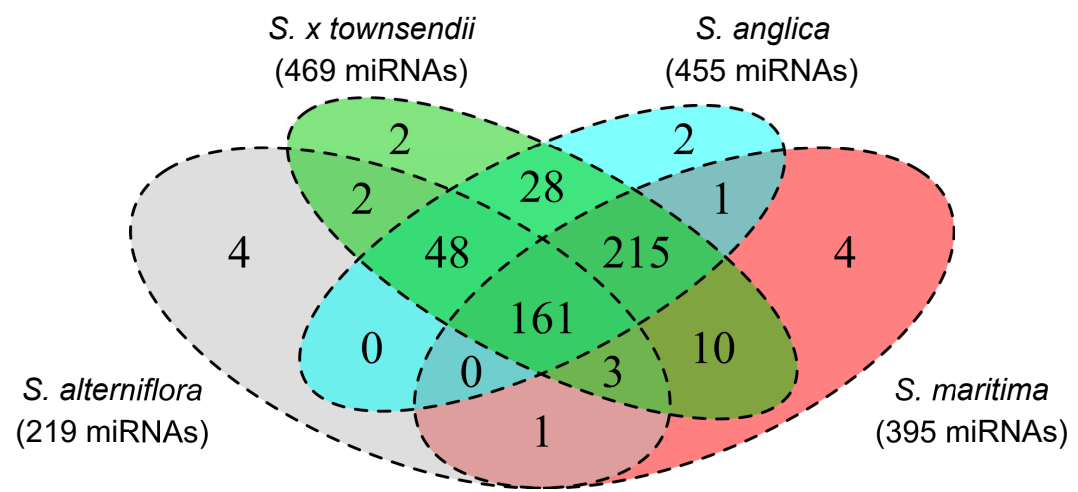


Fig. 2 a \log_2 fold change: -10 -5 0 5 10

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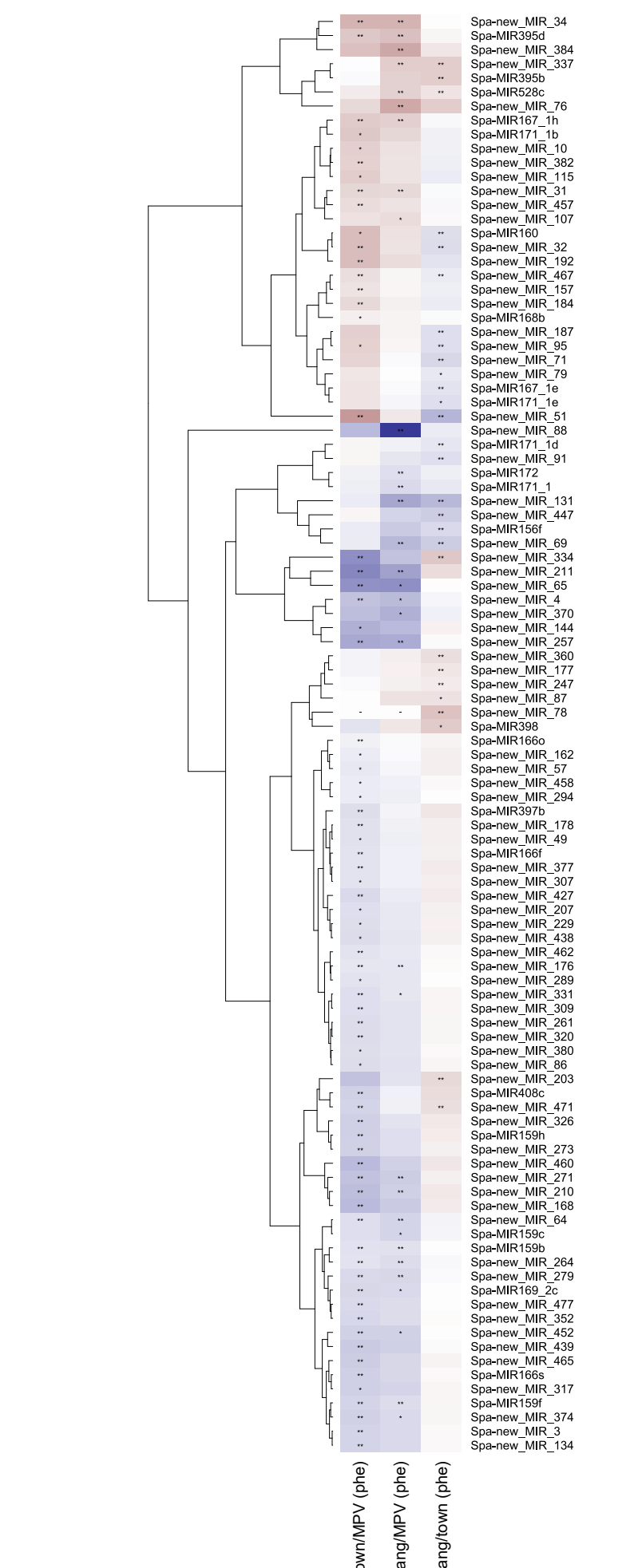
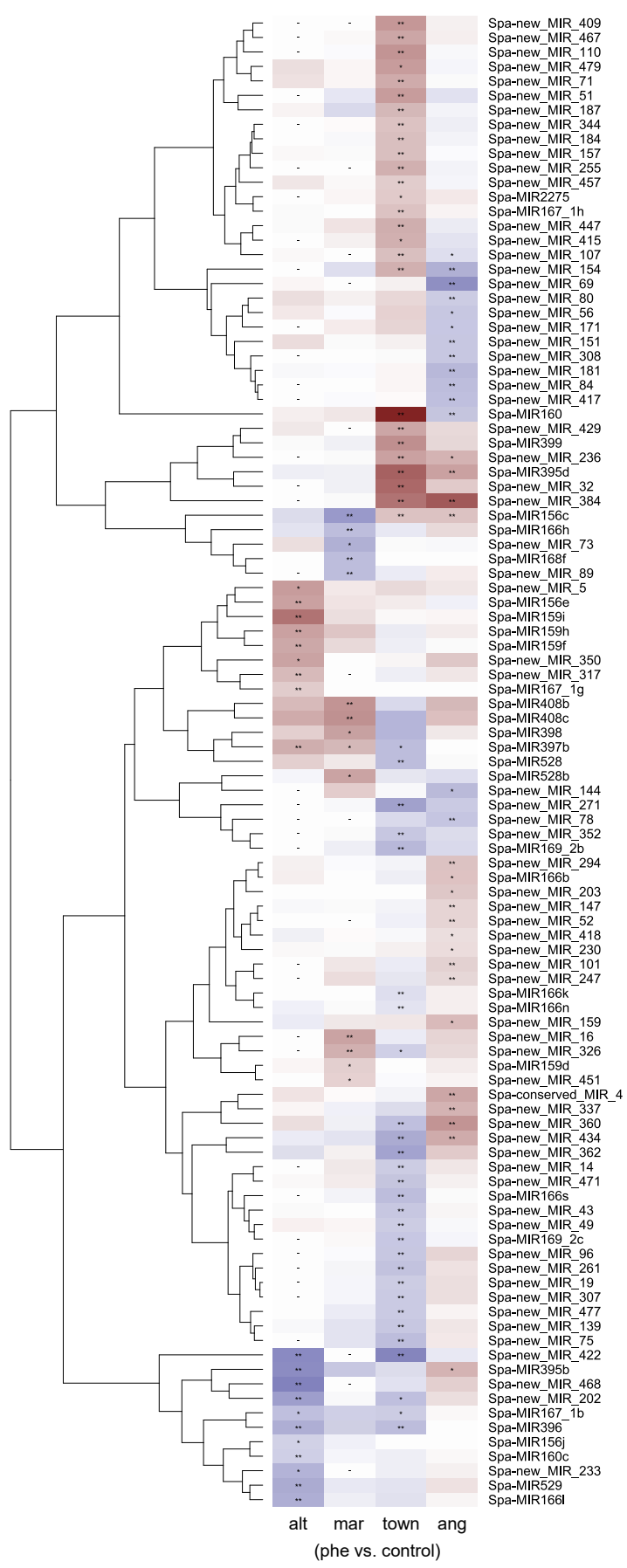
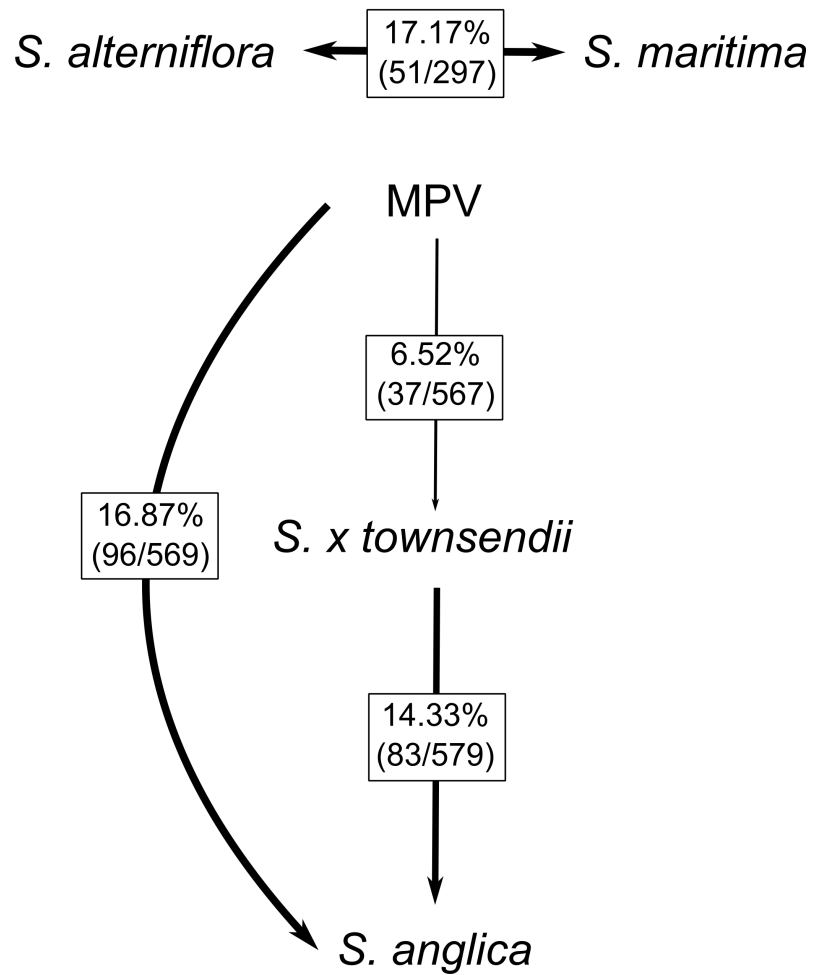
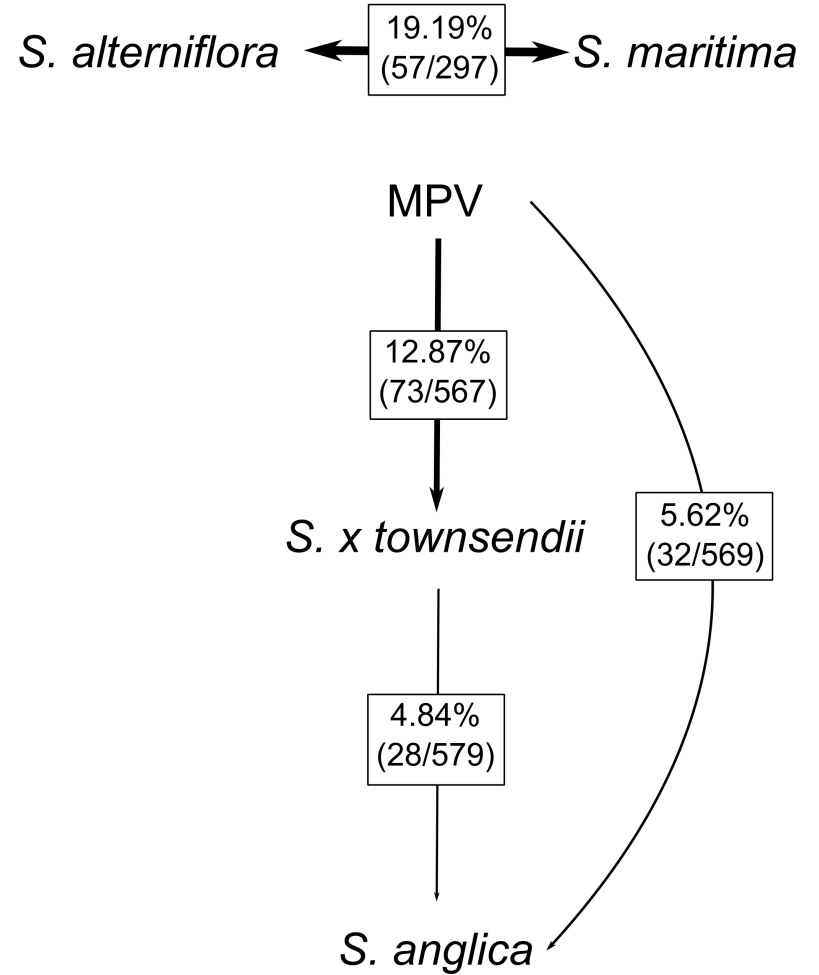


Fig. 3

Control:

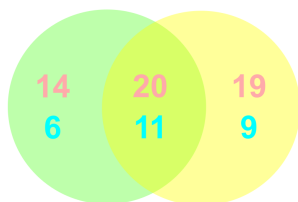


Phe-induced stress:

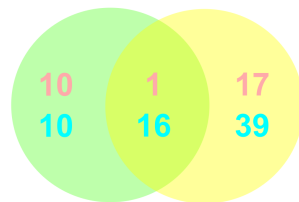


DE miRNAs between control (green) and phe-induced stress (yellow):

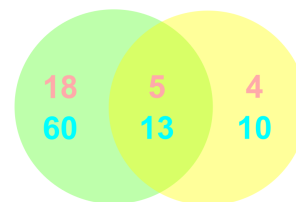
→ up-regulated
→ down-regulated



S. alterniflora vs. *S. maritima*



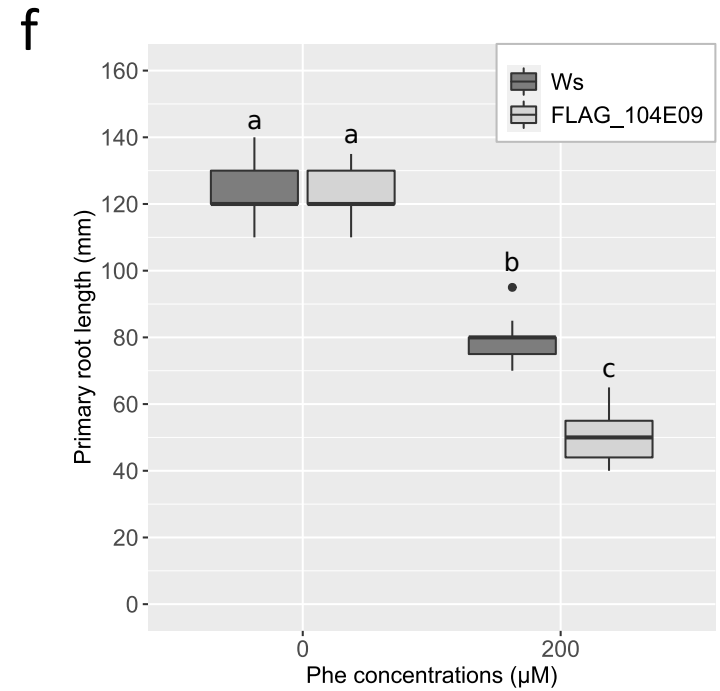
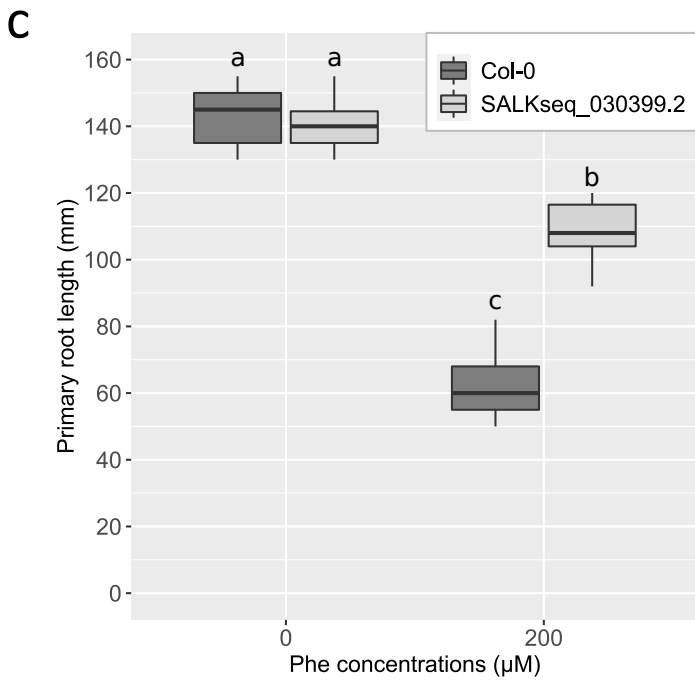
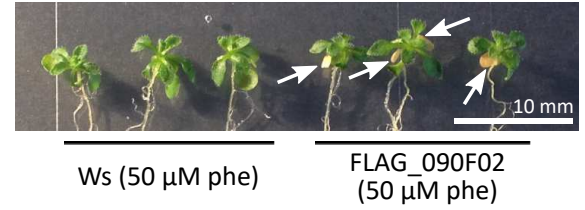
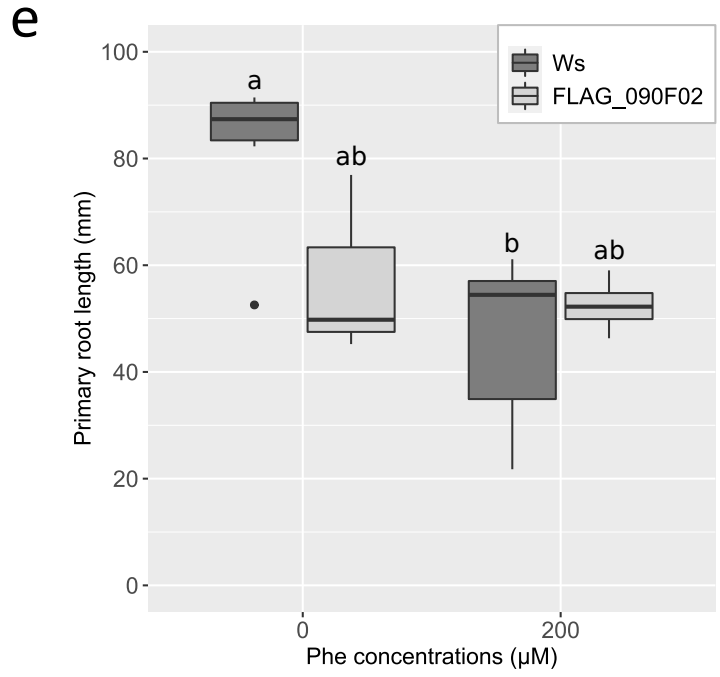
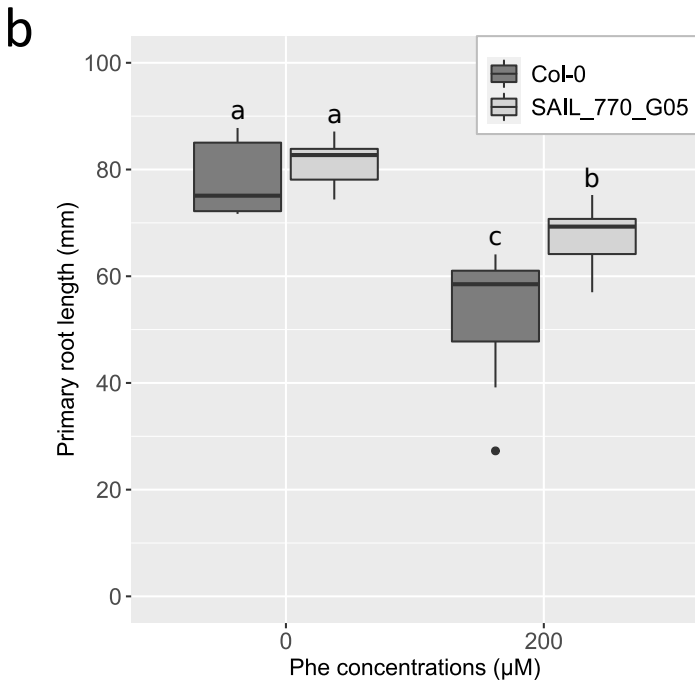
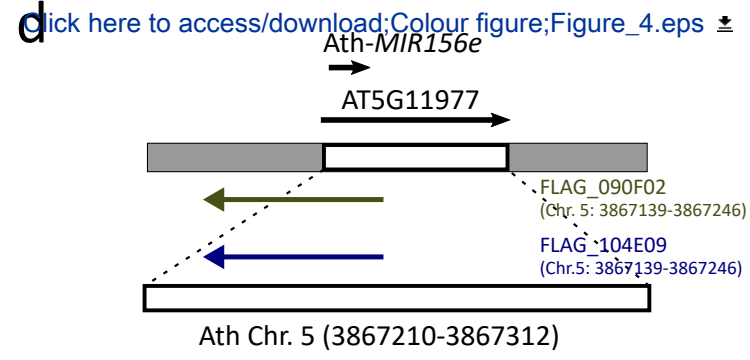
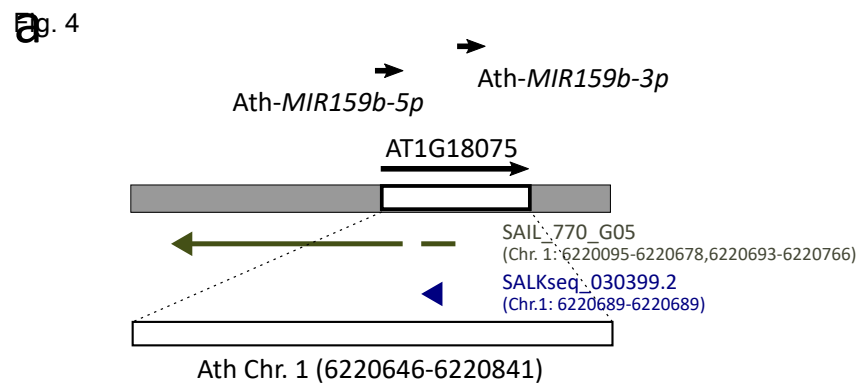
S. x townsendii vs. MPV



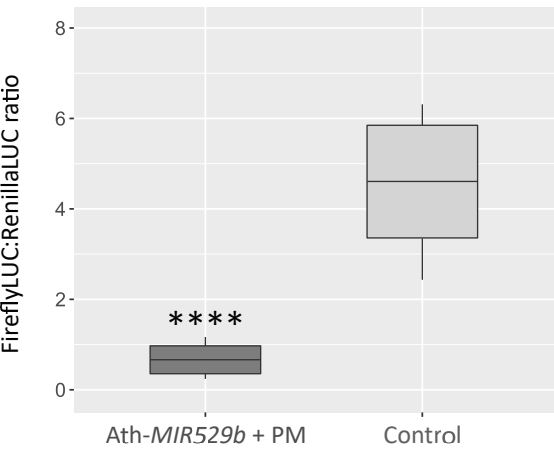
S. anglica vs. MPV



S. anglica vs. *S. x townsendii*



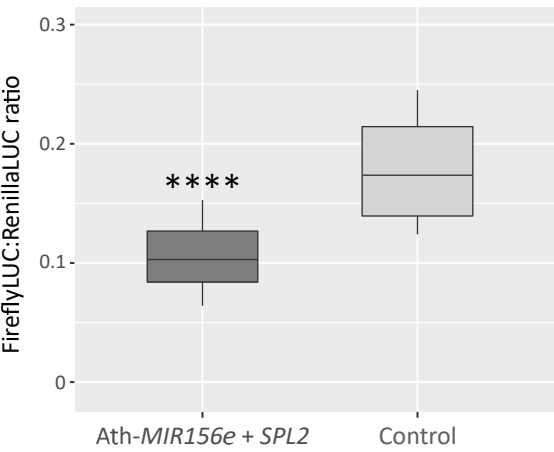
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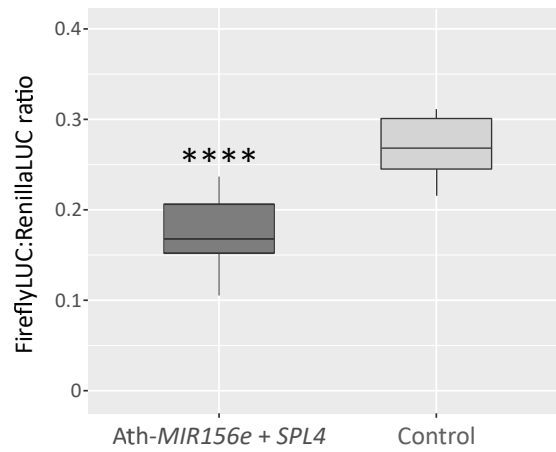
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b



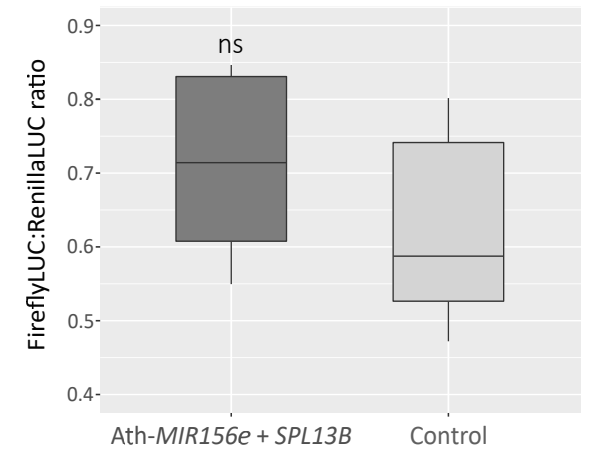
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SPL13B
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 Ath-MIR156e 20 CACGAGUGAGAGAAGACAGU 1