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Plant Extracellular Vesicles Contain Diverse Small RNA Species and Are Enriched in 10 to 17 Nucleotide “Tiny” RNAs

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Short title: Plant EVs Contain Tiny RNAs

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22

23 **ABSTRACT**

24

25 Small RNAs (sRNAs) that are 21 to 24 nucleotides (nt) in length are found in most
26 eukaryotic organisms and regulate numerous biological functions, including transposon
27 silencing, development, reproduction, and stress responses, typically via control of the
28 stability and/or translation of target mRNAs. Major classes of sRNAs in plants include
29 microRNAs (miRNAs) and small interfering RNAs (siRNAs); sRNAs are known to travel
30 as a silencing signal from cell to cell, root to shoot, and even between host and pathogen.
31 In mammals, sRNAs are transported inside extracellular vesicles (EVs), which are mobile
32 lipid compartments that participate in intercellular communication. In addition to sRNAs,
33 EVs carry proteins, lipids, metabolites, and potentially other types of nucleic acids. Here
34 we report that plant EVs also contain diverse species of sRNA. We found that specific
35 miRNAs and siRNAs are preferentially loaded into plant EVs. We also report a previously
36 overlooked class of “tiny RNAs” (10 to 17 nt) that are highly enriched in EVs. This new
37 RNA category of unknown function has a broad and very diverse genome origin and might
38 correspond to degradation products.

39 INTRODUCTION

40 Small RNAs (sRNAs) are important 21 to 24 nucleotide (nt) non-coding signaling
41 molecules involved in a wide variety of processes, including plant development,
42 reproduction and defense (Samad et al., 2017). sRNAs can be divided into two
43 categories, microRNAs (miRNAs) and small interfering RNAs (siRNAs), based on the
44 differences in their biogenesis and mode of action. miRNAs originate from a single-
45 stranded, self-complementary, non-coding RNA that forms a hairpin structure. In contrast,
46 siRNAs originate from a double-stranded RNA molecule synthesized by RNA-
47 DEPENDENT RNA POLYMERASES (RDRs). siRNAs can further be divided into two
48 main categories, heterochromatic siRNAs (hc-siRNAs) and phased siRNAs, including
49 *trans*-acting siRNAs (tasi-RNAs). Both of these double-stranded RNA structures are
50 recognized by Dicer-like proteins (DCL), which cleave these RNAs into defined length
51 products. One strand of these products is then selectively loaded onto an ARGONAUTE
52 (AGO) protein and incorporated into the RNA-induced Silencing Complex (RISC). The
53 RISC uses the sRNA in a sequence-homology-dependent manner to negatively regulate
54 targets, typically mRNAs (Borges and Martienssen, 2015).

55 sRNAs are often mobile and function in non-cell autonomous silencing, which can
56 be either local or systemic. Local RNA silencing occurs among groups of adjacent cells
57 and can gradually spread from cell to cell (Marín-González and Suárez-López, 2012;
58 Dunoyer et al., 2013). Systemic silencing occurs over long distances and can spread
59 throughout an entire plant. While there are several documented cases of mobile small
60 RNAs in plants, the mechanisms by which these RNAs move has yet to be clearly
61 established. Local RNA silencing is thought to involve the transport of RNAs through
62 plasmodesmata (PD) with the aid of PD-interacting proteins. The same is thought to be
63 true for systemic silencing, although this process also likely requires transport of RNA into
64 and through the phloem. Systemic RNA silencing progresses in a vascular-like pattern
65 reminiscent of the phloem and is graft transmissible, moving bidirectionally up and down
66 the plant in a manner that is heavily dependent on sink and source relationships (Lee and
67 Frank, 2018).

68 Plant sRNAs are also known to move from plant cells into pathogens. Through a
69 phenomenon known as Host-Induced Gene Silencing (HIGS), small RNAs produced in a

70 plant cell can regulate the expression of genes in an invading pathogen or parasite. In
71 recent years, HIGS has become a powerful tool for engineering resistance in crop plants
72 to nematodes, insects, fungi, oomycetes and parasitic weeds (Cai et al., 2018). Although
73 multiple studies have developed artificial HIGS systems, the phenomenon can occur
74 naturally as well, such as in the transfer of microRNAs miR166 and miR159 from cotton
75 into the hemibiotrophic fungus, *Verticillium dahliae* (Zhang et al., 2016), and the transfer
76 of siRNAs from *Arabidopsis* into *Botrytis cinerea* (Cai et al., 2018). How this translocation
77 is mediated is not well understood.

78 In mammals, extracellular vesicles (EVs) are known to mediate long distance
79 transport of both coding and non-coding RNAs. RNAs protected within the lumen of an
80 EV are transported to distant cells, where they retain their function after delivery
81 (Ratajczak et al., 2006; Valadi et al., 2007; Mittelbrunn et al., 2011). The RNA contents
82 of mammalian EVs largely reflects that of their cell type of origin, but can show enrichment
83 for certain mRNAs and miRNAs. The overrepresentation of specific sequences inside
84 EVs suggests an active mechanism for loading RNAs into these compartments. Usually,
85 miRNAs make up a sizable percentage of the RNAs within EVs. However, other classes
86 of small non-coding RNAs are also present, including tRNAs, rRNAs and fragments
87 originating from both coding and non-coding RNAs. These other sRNAs are often highly
88 enriched in EVs compared to their cell of origin. The exact role of other non-coding RNAs
89 and RNA fragments in EVs has yet to be determined. They may function in signaling or
90 they may represent waste products that EVs remove from the cell (Bellingham et al.,
91 2012; Nolte'T Hoen et al., 2012; Huang et al., 2013; Chevillet et al., 2014; van Balkom et
92 al., 2015).

93 Although the RNA content of mammalian EVs has been extensively characterized,
94 the RNA content of plant EVs has not been carefully assessed. To address this
95 knowledge gap, we optimized methods for purifying EVs from plant apoplastic wash fluid
96 (Rutter and Innes, 2017), performed sRNA-seq analysis on EV RNA, and compared the
97 resulting data to sRNAs in the source leaf tissue and EV-depleted apoplastic wash fluid.
98 These analyses revealed that plant EVs are enriched in single-stranded small RNAs,
99 especially a class of RNAs ranging in size from 10 to 17 nts. These RNAs seem to
100 represent degradation products of small RNA production. Their abundance in plant EVs

101 raises the question of whether they serve a function in either intercellular and/or inter
102 kingdom communication.

103

104

105 **RESULTS AND DISCUSSION**

106

107 To analyze the sRNA content of EVs, we constructed sRNA libraries from three biological
108 replicates of *Arabidopsis thaliana* rosette leaves (“total RNA”, henceforth), EVs, and
109 apoplastic wash fluids depleted of EVs (“apoplast” henceforth), as previously described
110 ([Rutter and Innes 2017](#)). After an initial step of standard read trimming (adaptor removal
111 and size selection from 18 to 34 nt), we observed that only 20% of the reads from EV
112 samples were retained. This very low rate of retention was due to a large proportion of
113 very short reads, from 10 to 17 nt long, present in the EV samples. We named these
114 unusually short reads “tiny RNAs” or tyRNAs. Changing the trimming criteria to retain any
115 read from 10 to 34 nt allowed us to achieve an average of ~60% read retention
116 ([Supplemental Table 1](#)). The size distribution of the EV and apoplast samples was
117 unexpected ([Figure 1A](#)). Total RNA samples had the typical size distribution of
118 *Arabidopsis* small RNAs, a peak at 24 nt followed by secondary peaks at 21 and 22 nt.
119 However, EV samples had a high proportion of tyRNA reads between 10 and 15 nt and
120 no peak in the 20 nt range, while apoplast samples had a high proportion of longer reads,
121 between 30 and 32 nt ([Figure 1A, left panel](#)). When considering only distinct reads, where
122 tags with the exact same sequence were counted only once ([Figure 1A, right panel](#)), EV
123 samples continued to show an enrichment for tyRNAs. This suggests that the tyRNAs
124 present in EVs were not only abundant, but also highly diverse in their sequence
125 composition. Comparatively, apoplastic sRNAs were not enriched for tyRNA sequences.
126 This strongly argues that tyRNA reads were not a result of degradation occurring during
127 RNA isolation or library preparation, as these steps were performed in parallel for all RNA
128 samples.

129

130 **Tiny RNAs mostly originate from CDSs, TEs and intergenic regions**

131 To understand the genomic origin of these tyRNAs, we mapped these reads to different
132 features of the *Arabidopsis* genome, including MIRNAs (genes encoding miRNA
133 precursors), TAS genes (genes giving rise to tasiRNAs), transposable elements (“TEs”),
134 rRNAs, tRNAs, small nuclear and small nucleolar RNAs (snRNAs and snoRNAs), coding
135 sequences (“CDSs”), potential RNA Polymerase IV products (“Pol4”) (as defined in
136 [Blevins et al., 2015](#)) and intergenic regions ([Figure 1B](#)). For reads that mapped to more
137 than one feature, we counted each feature once. We performed the same analysis with
138 the sRNA reads (18 to 34 nt) and observed that relative to sRNAs, tyRNAs were enriched
139 in sequences that map to MIRNAs and TEs ([Figure 1B](#)). While the EV samples contained
140 a higher abundance of tyRNA reads, tyRNAs from both total RNA and EV samples
141 mapped to a similar distribution of features. In both samples, the majority of tyRNAs
142 mapped back to TEs, CDSs and intergenic regions, which combined, comprise the largest
143 proportion of the *Arabidopsis* genome ([Figure 1B](#)). We also detected high levels of
144 tyRNAs derived from Pol4 precursors and rRNA regions, which represent 14% and 0.12%
145 of the genome, respectively. Typically, rRNA represents around 80% of total leaf RNA,
146 so the relatively low frequency of rRNA reads in each library indicates that they were not
147 significantly contaminated with degraded cellular RNA. Together, these observations
148 strongly suggest that EVs are highly enriched for tyRNAs and that this new RNA category
149 might correspond to specific degradation products of sRNA precursors.

150

151 **MIRNA-derived tyRNAs are not randomly distributed**

152 To determine whether tyRNAs are products of sRNA processing, we next analyzed their
153 relative position in non-coding RNAs. The abundance of tyRNAs derived from miRNA
154 precursors was almost twice that of snRNAs, snoRNAs, and tRNAs. Since this posits a
155 clear preferential accumulation of MIRNA-derived tyRNAs in EVs, we analyzed the
156 location of tyRNA sequences within miRNA precursors. In addition to a 5' cap and a 3'
157 poly-A tail, miRNA precursors can be subdivided into the following segments: the 5'
158 region, miR-5p, the loop region, miR-3p and the 3' region ([Figure 2A](#)). sRNAs (18 to 34
159 nt) and tyRNAs (10 to 17 nt) mapped to these regions with different patterns of abundance
160 ([Figure 2B](#)). In all of our samples, sRNAs originated almost exclusively from the miR-3p
161 and miR-5p regions, which correspond to the mature miRNA/miRNA* duplex. tyRNAs,

162 however, originated mostly from the loop region of the precursor, followed in abundance
163 by the 5' and 3' regions (Figure 2B). These results suggest that MIRNA-derived tyRNAs
164 are largely products of the remnant pieces following cleavage by DICER-LIKE 1 (DCL1).
165 The terminal regions of miRNA precursors are known to undergo exonucleolytic decay
166 via a process that includes RISC-INTERACTING CLEARING 3'- 5'
167 EXORIBONUCLEASES (RICE1) to clear 5' fragments (Zhang et al., 2017; Yu et al.,
168 2017b) and EXORIBONUCLEASE 4 (XRN4) to clear 3' fragments (Souret et al., 2004).
169 However, the process by which the leftover loop region is degraded is less well examined,
170 but apparently results in the production of abundant tyRNAs.

171 We also analyzed tyRNA abundances in a *dcl234* triple mutant, which is deficient
172 for the production of 22-nt and 24nt siRNAs, including transacting siRNAs (tasiRNAs;
173 Henderson et al., 2006), but is unaffected in miRNA biogenesis. Consistent with
174 expectations, MIRNA-derived tyRNA accumulation did not differ between the *dcl234* triple
175 mutant and wild-type Col-0. However, we observed a clear reduction in TAS-derived
176 tyRNAs (Figure 2C), indicating that these tyRNAs are likely derived from tasiRNA
177 precursors.

178 Next, we examined data previously generated from *hen1*, *sdn1/sdn2* and
179 *hen1/sdn1/sdn2* mutants (Yu et al., 2017a; samples included in the PRJNA251351 GEO
180 dataset). HEN1 (HUA ENHANCER 1) is a methyl transferase that stabilizes specific
181 sRNAs by adding a methyl group at the 3' end, which protects them from degradation (Li
182 et al., 2005). When HEN1 is absent, sRNAs undergo an uridylation process that allows
183 exonucleases encoded by *SDN1* (SMALL RNA DEGRADING NUCLEASE) and *SDN2* to
184 degrade them. We observed that MIRNA-derived tyRNAs increased in *hen1* mutants
185 relative to wild-type Col-0, when miRNAs are destabilized, but remained at wild-type
186 levels in *sdn1/sdn2* and *hen1/sdn1/sdn2* mutants, which lack the exonucleases
187 responsible for miRNA degradation (Figure 2C). These results support our hypothesis
188 that MIRNA-derived tyRNAs are degradation products.

189 tyRNAs also mapped to the miRNA/miRNA* duplex, and we next checked the
190 position of the tyRNAs within these ~21 or 22 nt molecules (Figure 2D). Since the miRNA
191 and miRNA* designations were removed from miRBase, we treated both sides of the
192 duplex equally. The majority of EV tyRNAs that mapped to miRNAs originated from the

193 center of the miRNA sequence (i.e. nucleotides 3 to 12) ([Figure 2D](#)). In contrast, tyRNAs
194 from total leaf libraries and apoplast mostly started at position 1 of the miRNA, followed
195 by position 11, and they have an end position more widely distributed, from positions 13
196 to 21. These observations suggest that EV tyRNAs are not a random sampling of total
197 cell tyRNAs, but are instead derived from a specific packaging process.

198

199 **1-hit tyRNAs map to coding CDSs and intergenic regions**

200 Due to the short length of tyRNAs, most tyRNAs map to multiple locations in the genome.
201 To better determine their origin, we repeated the previously described analyses using
202 only reads that map once to the genome (hereafter “1-hit” reads). These 1-hit tyRNAs
203 have a different size distribution, ranging from 13 to 17 nt long, with the majority being 16
204 to 17 nt ([Supplemental Figure 1A](#)).

205 The majority of the 1-hit tyRNAs mapped to coding (CDS) and intergenic regions
206 ([Figure 3A](#)). We examined in more detail the exact origin of the CDS-derived tyRNAs by
207 dividing each gene into 5’UTR, exons, introns and 3’UTR. The 1-hit tyRNAs originated
208 mostly from the sense strand of exons ([Figure 3B](#)), with no difference regarding the size
209 of the tyRNAs ([Supplemental Figure 1B](#)). These results were independent of the sample
210 type, suggesting that most of the CDS-derived tyRNAs originate from mature mRNAs.

211 To further characterize the origin of the exon-derived tyRNAs, we analyzed their 5’
212 positions relative to the full-length mRNA, expressed as a percentage of the total length.
213 In all samples, we observed a consistent peak originating at the center of the mRNA
214 ([Figure 3C](#)). This persistent peak was more prominent in the EV samples than in whole
215 leaf and apoplast samples. This pattern suggests that mRNAs may be first degraded by
216 5’ and 3’ exonucleases, with tyRNAs being derived from what is left over.

217

218 **EVs preferentially load specific miRNAs**

219 We next investigated whether sRNAs accumulate differentially in EVs relative to the
220 apoplast and total leaf samples, focusing on three major classes of sRNAs
221 (heterochromatic siRNAs or hc-siRNAs, phasiRNAs, and miRNAs) ([Figure 4](#)). The most
222 differentially represented sRNAs were miRNAs ([Figure 4A](#)). Group I consisted of miRNAs
223 that were more abundant in EVs and apoplast samples compared to total RNA. While the

224 miRNAs within this group seem to be secreted from the cell, there is no specific
225 accumulation in EVs. Group II miRNAs accumulated to high levels in EVs and were
226 present at slightly lower levels in the apoplast samples, suggesting some accumulation
227 in EVs. The miRNAs in group III were depleted in the apoplast samples relative to total
228 RNA and EVs, suggesting that group III RNAs are preferentially secreted via EVs. The
229 miRNAs in group IV had an opposite accumulation pattern, low in EVs compared to
230 apoplast and high in apoplast compared to total RNA samples. These results indicate that
231 a distinct subset of miRNAs are preferentially loaded into the EVs.

232 The four patterns of preferential miRNA secretion described above did not
233 correlate with total miRNA abundance in leaves. miRNAs with similar abundances in the
234 total leaf sample were represented in both groups III and IV (i.e. those that accumulated
235 to both low and high levels in the EV samples; Figure 4C). For example, miRNAs
236 miR390b-3p, miR167c-3p, miR8166 and miR8180 had a similar abundance in total RNA
237 samples, but the first two were abundant in EV samples while the latter two displayed a
238 low abundance in EVs.

239 Combined, our findings hint at two separate pathways for the secretion of miRNAs.
240 While miRNAs present in groups I and II may be secreted through either pathway, with a
241 slight preference for EV-dependent secretion in group II, miRNAs in group III are strongly
242 dependent on EVs for secretion. In contrast, miRNAs in group IV are secreted primarily
243 through an EV-independent pathway. There may be specific sorting mechanisms for
244 loading miRNAs into these separate pathways. Whether such specificity might be directed
245 by the miRNA sequence or duplex structure, or something about its target or function
246 remains unknown.

247

248 **siRNAs are poorly represented in EVs**

249 We next assessed the abundance of full-length siRNAs in EVs, including phased siRNAs
250 (phasiRNAs), tasiRNAs and heterochromatic siRNAs (hc-siRNAs). For phasiRNAs, we
251 analyzed all members of families of genes encoding nucleotide-binding site leucine-rich
252 repeat proteins (NLRs), pentatricopeptide repeat proteins (PPR) and SUPPRESSOR OF
253 AUXIN RESISTANCE proteins (SAR), which are the primary sources of phasiRNAs in
254 *Arabidopsis* ([Supplemental Table 2](#)). We were able to identify 21 phasiRNA producing

255 loci that were differentially accumulated in one of our three comparisons (Fig. 4B). These
256 loci can be divided into two groups; group one had high accumulation in EV and apoplast
257 samples, compared to total RNA, and group II had a low accumulation in EV samples
258 compared to apoplast and total RNA samples, and a high accumulation in apoplast
259 compared to total RNAs. Similarly, we analyzed the accumulation of the 24 different
260 *Arabidopsis* tasiRNAs that have been identified (Zhang et al., 2014) (Supplemental Table
261 2), and found only two that were differentially accumulated (Figure 4C – upper panel),
262 with low accumulation levels in EVs. We also analyzed 21- and 22-nt RNAs derived from
263 the eight *TAS* genes identified in the *Arabidopsis* genome for their specific differential
264 accumulation in our samples (Figure 4C – lower panel). We observed that none of these
265 *TAS*-derived sRNAs accumulated in EVs. Instead, all of the differentially accumulated
266 sRNAs preferentially accumulated in the apoplast. This may indicate that phasiRNAs and
267 tasiRNAs are preferentially directed to an apoplastic secretion pathway that is
268 independent of EVs.

269 hc-siRNAs largely originate from transposable elements (TEs) and are thus
270 repetitive in their nature, confounding analyses that require knowing their exact origin.
271 Some hc-siRNAs may map to more than one TE with all the mapping locations
272 corresponding to a single TE superfamily. Using hc-siRNAs that mapped within single
273 superfamilies (Supplemental Table 2 - Raw reads all sRNAs), we measured hc-siRNA
274 levels and observed no clear patterns of accumulation in EV samples. Therefore, unlike
275 miRNAs that demonstrated specificity in their EV accumulation, there was no evidence of
276 specificity in EV-localized sRNAs from loci generating either hc-siRNA or any other siRNA
277 analyzed here.

278

279 CONCLUSIONS

280

281 Here we have shown that EVs are highly enriched in small RNAs 10 to 17 nucleotides in
282 length, which we have named tiny RNAs. tyRNAs appear to be degradation products
283 derived from multiple sources, including mRNAs, primary miRNAs, siRNAs, tasiRNAs and
284 hcRNAs. Whether this new class of RNAs have a cellular function, or represent a waste-
285 or by-product of cellular metabolism, remains unknown.

286 One potential function of tyRNAs could be as small activating RNAs (saRNAs).
287 RNA activation is a mechanism by which small RNAs, usually 18 to 21 nt long, can
288 activate gene transcription, and was first identified in human cells more than a decade
289 ago (Li et al., 2006). More recently, several studies have identified animal saRNAs that
290 are able to positively regulate gene expression by targeting UTRs (Vasudevan and Steitz,
291 2007; Rocha et al., 2007; Ørom et al., 2008). Since then, more than 15 examples of
292 saRNAs have been described in animal model systems, such as rats and primates, *in*
293 *vivo* and *in vitro* (Huang et al., 2010). saRNAs bind to promoter regions in a wide range
294 of positions relative to their transcription starting site (TSS) (Li et al., 2006; Janowski et
295 al., 2007), as well as the 3' UTR (Yue et al., 2010).

296 Here we also described that some miRNAs are specifically loaded into EVs, which
297 reinforces the theory that sRNAs use EVs for long distance movement through the plant,
298 and possibly as a cross-kingdom delivery system. Recently it has been described that
299 plants secrete EVs to transfer sRNAs into fungal pathogens (Cai et al., 2018). Specifically,
300 it has been shown that two plant-derived tasiRNAs from *TAS1c* and *TAS2* genes are
301 transferred to *Botrytis cinerea* via detergent-sensitive particles to target genes involved in
302 vesicle trafficking. We also found that some TAS1- and TAS2-derived siRNAs are
303 secreted out of the cell, but mainly through an apoplastic secretion mechanism. The
304 discrepancies between our findings and those of Cai et al. (2018b) may be due to
305 differences in isolation methods, as Cai et al. used 100,000 x g as the relative centrifugal
306 force rather than 40,000 x g to isolate EVs and they did not purify the vesicles away from
307 any contaminating protein complexes. It may be that the tasiRNAs detected in Cai et al.
308 were associated with other extracellular particles separate and distinct from EVs. This
309 makes sense if, as our data suggests, plants employ multiple mechanisms for the
310 secretion and long-distance transport of sRNAs.

311

312 **METHODS**

313 **Plant materials and growth conditions**

314 *Arabidopsis* seeds were germinated on 0.5X Murashige and Skoog (MS) medium
315 containing 0.8% agar as previously described (Rutter and Innes 2017). The seeds were
316 stored at 4°C for two days and then moved to short day conditions (9 hour days, 22°C,

317 150 $\mu\text{Em}^{-2}\text{s}^{-1}$). The seedlings were transferred after one week to Pro-Mix B Biofungicide
318 potting mix supplemented with Osmocote slow-release fertilizer (14-14-14).

319

320 **EV isolation and purification**

321 *Arabidopsis* EVs were isolated from apoplastic wash and purified on an iodixanol density
322 gradient, as previously described (Rutter and Innes 2017). Briefly, 6 week-old *Arabidopsis*
323 rosettes were vacuum infiltrated with Vesicle Isolation Buffer (VIB; 20 mM MES, 2 mM
324 CaCl_2 , 0.01 M NaCl, pH 6.0). The rosettes were gently blotted to remove excess buffer
325 and packaged inside needless, 30 ml syringes. The syringes were centrifuged for 20 min
326 at 700 x g (2°C , JA-14 rotor, Avanti™ J-20 XP Centrifuge, Beckman Coulter, Indianapolis
327 IN). The apoplastic wash collected from the rosettes was then filtered through a 0.22 μm
328 membrane and centrifuged successively at 10,000 x g for 30 min to remove any large
329 particles, 40,000 x g for 60 min to pellet the EVs and again at 40,000 x g to wash and re-
330 pellet the EVs (2°C , SW41, Optima XPN-100 Ultracentrifuge, Indianapolis IN). After the
331 first 40,000 x g step, 5 mLs of the supernatant were retained on ice for RNA isolation. The
332 washed EV pellet was resuspended in 1 ml of cold, sterile VIB and loaded on top of a
333 discontinuous iodixanol gradient (5%, 10%, 20% and 40%) (Optiprep™; Sigma-Aldrich,
334 St. Louis MO). The gradient underwent centrifugation at 100,000 x g for 17 hours (2°C ,
335 SW41, Optima XPN-100 Ultracentrifuge, Indianapolis IN). After centrifugation, the first 5
336 ml of the gradient were discarded. The next three fractions of 0.8 ml were collected. The
337 fractions were diluted in cold, sterile VIB and re-pelleted at 100,000 x g for 60 min (2°C ,
338 TLA100.3, Optima™ TLX Ultracentrifuge, Beckman Coulter, Indianapolis IN). The pellets
339 were resuspended in cold, sterile VIB, combined and brought up to a total volume of 100
340 μl using VIB.

341

342 **RNA Sample Preparation**

343 RNA was isolated from three different sources: leaf tissue, EV-depleted supernatant and
344 purified EVs. Leaf RNA was isolated from 100 mg of leaf tissue harvested after collecting
345 apoplastic wash, frozen and ground to a powder using liquid nitrogen and suspended in
346 1 ml of TRIzol™ Reagent (Thermo Fischer Scientific, Waltham, MA). Supernatant was
347 collected after the first 40,000 x g step, as described above, and combined with 0.1

348 volumes of 3 M sodium acetate and one volume of cold isopropanol. The supernatant
349 sample was mixed briefly by vortexing and kept at -20°C for one hour. After one hour,
350 the sample was centrifuged for 30 min at 12,000 x g and 4°C. The resulting pellet of RNA
351 was resuspended in 1 ml of TRIzol™ Reagent. EV RNA was isolated from EVs purified
352 on a discontinuous iodixanol gradient as described above. 100 µl of EVs was added to 1
353 ml of TRIzol™ Reagent. Once all three samples were in TRIzol™ Reagent, RNA was
354 extracted following the manufacturer's instructions.

355

356 **Small RNA libraries**

357 Small RNA libraries were constructed as described ([Mathioni et al., 2017](#)) using the
358 NEBNext® Small RNA Library Prep Set (NEB #E7330S). Due to the low abundance of
359 RNA present in extracellular vesicles and apoplast samples, 500 ng of total RNA was
360 used as the starting material. Libraries were sequenced on a NextSeq instrument with
361 single-end 75-bp reads at the Indiana University Center for Genomics and Bioinformatics
362 (Bloomington, IN).

363

364 **Data analysis**

365 Small RNA sequencing libraries were trimmed for adaptors using cutadapt v1.16 ([Martin,](#)
366 [2011](#)) with a minimum insert size of 10 nt and a maximum of 34 nt. Sequence quality was
367 assessed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).
368 Clean reads were aligned to the *Arabidopsis* genome (TAIR version 10), and all
369 subsequent analysis were done using Bowtie2 ([Langmead and Salzberg, 2012](#)). For
370 miRNA and tasiRNA analyses, the latest versions of miRBase (v22 - ([Kozomara and](#)
371 [Griffiths-Jones, 2014](#)) and tasiRNA database ([Zhang et al., 2014](#)) were used,
372 respectively. Mapping positions for miRNAs were assessed using the Bowtie2 output file.
373 Differential accumulation analyses were performed using DEseq2 with default
374 parameters, using not normalized reads as input ([Love et al., 2014](#)) and graphical
375 representations using ggplot2 ([Wickham, 2009](#)) in the R statistical environment. For box
376 and whisker plots produced by ggplot2, the upper whisker represents the maximum value,
377 or the top of the box plus 1.5 IQR, whichever is less (IQR = the length of the box), while

378 the lower whisker represents the minimum value, or the bottom of the box minus 1.5 IQR,
379 whichever is greater.

380

381 **Micrococcal nuclease protection assays**

382 EVs were isolated from 10 mL of apoplastic wash fluid collected from 36 *Arabidopsis*
383 rosettes, as described above. The EV pellet obtained after the second centrifugation step
384 at 40,000 x g was re-suspended in 250 μ L of sterile VIB (pH 7.5) supplemented with 1X
385 micrococcal nuclease reaction buffer (New England BioLabs; 50 mM Tris-HCl, 5 mM
386 CaCl_2 , pH 7.9). An equal amount of re-suspended EVs (50 μ L) was used for each
387 treatment with or without membrane permeabilization prior to micrococcal nuclease
388 addition. To permeabilize EVs, 0.075% (v/v) Triton X-100 was added to EVs and vortexed
389 for 1 minute. For nuclease treatments, 0.5 μ L (1000 units) of micrococcal nuclease (New
390 England BioLabs catalog number M0247S) was added and incubated at 37°C for 5
391 minutes. Digestion was terminated by addition of EGTA (final concentration: 20 mM) and
392 RNA was immediately isolated using TRIzol reagent (Invitrogen) as described above.
393 Both microRNAs and tyRNAs levels were quantified using stem-loop qRT-PCR as
394 described by [Varkonyi-Gasic et al. \(2007\)](#), starting with same volume of sample from each
395 treatment. Primers used for first strand cDNA synthesis and qRT-PCR are provided in
396 [Supplemental Table 3](#).

397

398 **Accession Numbers**

399 All RNA sequencing data has been deposited into the Gene Expression Omnibus (GEO)
400 under the accession code GSE114696.

401

402 **Supplemental Data**

403 **Supplemental Figure 1:** Characterization of 1-hit tyRNAs.

404 **Supplemental Table 1:** small RNA sequencing summary statistics.

405 **Supplemental Table 2:** Raw read count by sRNA class.

406 **Supplemental Table 3:** Primers used for qRT-PCR analyses.

407

408

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416

417

418 AUTHOR CONTRIBUTIONS

419 P.B., B.D.R., R.W.I. and B.C.M. designed the research. P.B., B.D.R. and H.Z. performed
420 the research. P.B. and R.P. analyzed the data. P.B., B.D.R. and R.W.I. wrote the article.
421 All authors read and commented on the manuscript.

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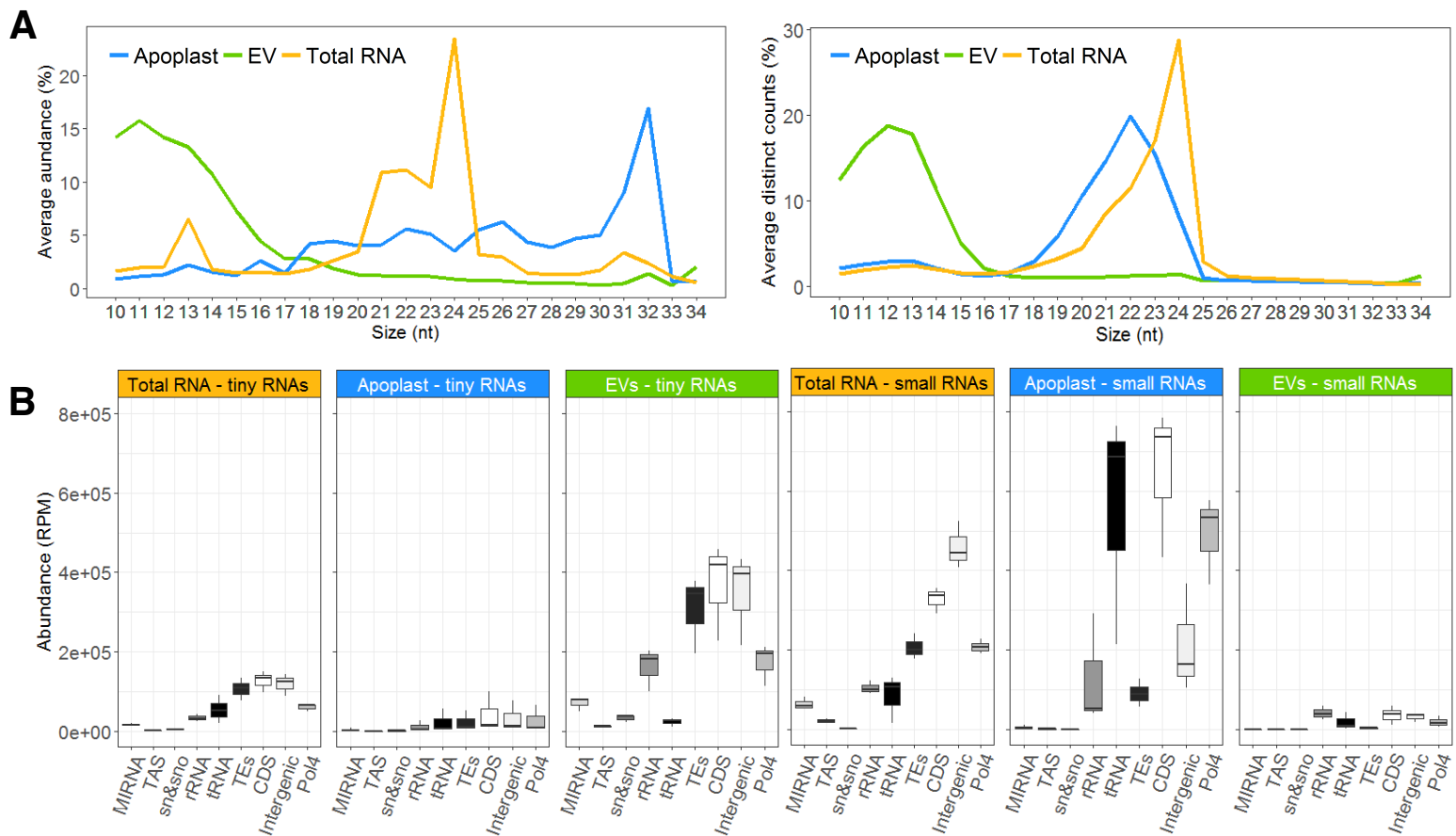


Figure 1. EVs are Enriched in Tiny RNA Sequences Derived from Diverse Sources.

(A) Size distribution of sRNAs mapping to the genome; the percentage of each size class was calculated for each source of data, represented by the average of three replicate libraries. The x axis indicates the sRNA size and the y axis indicates its proportion. The panel at the left indicates the sRNA sizes and proportions, as measured by total abundance of the reads, and at right, as calculated by distinct reads (unique sequences).

(B) The abundance of reads mapping to nine different features of the *Arabidopsis* genome. These include the following from left to right: *miRNA* precursors, transacting siRNAs (TAS) precursors, small nuclear RNAs (snRNA and snoRNAs); ribosomal RNAs; tRNAs; transposable elements (TEs); coding sequences (including introns and UTRs; CDS); intergenic sequences; and siRNA precursors dependent on RNA Polymerase IV (Pol4).

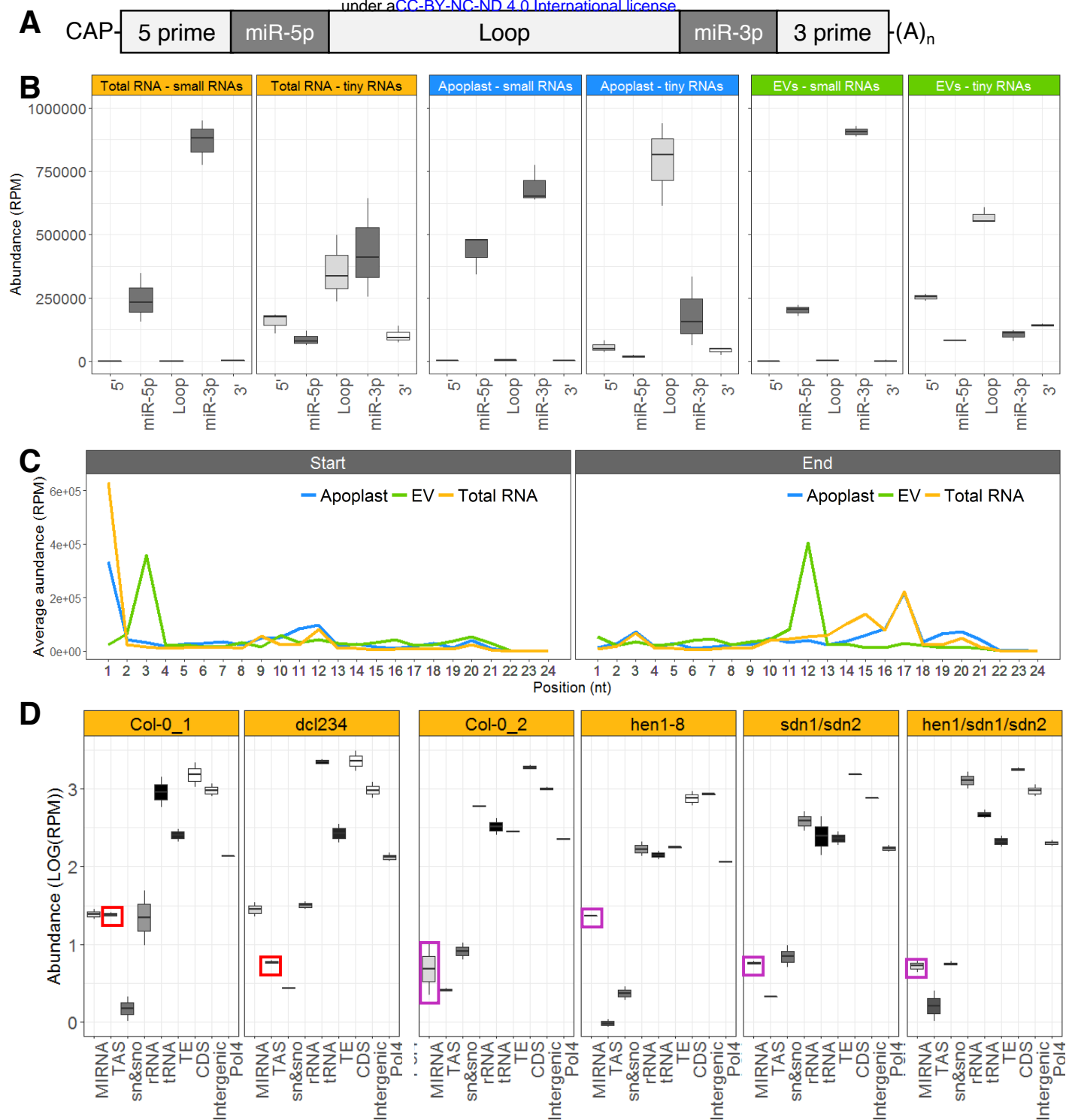


Figure 2. EV tyRNAs are Derived from Specific Regions of Precursor RNAs.

(A) microRNA precursor structure, including from left to right, 5' region, mature miRNA miR-5p, loop region, mature miRNA miR-3p, and 3' region.

(B) EV tyRNAs are enriched in sequences that map to the loop region of miRNA precursors. The abundance of small RNAs and tiny RNAs mapping to different regions of a miRNA precursor, as described in panel A. The y axis represents abundance, in reads per million (RPM).

(C) EV tyRNAs mapping to mature miRNAs are cut out of the central region. Plots show the relative position of 5' ends (left plot) or 3' ends (right plot) of tyRNAs relative to nucleotide position within the parent miRNA.

(D) Mutations that affect abundance of specific subclasses of sRNAs have a corresponding influence on tyRNA abundance. The abundance of total leaf tyRNAs mapping to different features of the *Arabidopsis* genome. These include the following from left to right: *miRNA* precursors, *TAS* precursors, snRNA and snoRNAs; ribosomal RNAs; tRNAs; transposable elements; coding sequences (including introns and UTRs); intergenic sequences; and known Pol IV precursors. The y axis represents abundance, in reads per million (RPM), in a logarithmic (LOG₁₀) scale. Colored boxes highlight tvRNAs affected by mutations.

