Mobile Small RNAs Are Predominately Accumulated via Long-Distance Movement Rather than Local Biogenesis

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Research Article:

Mobile Small RNAs Are Predominately Accumulated via Long-Distance Movement Rather than Local Biogenesis

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Abstract

Long-distance RNA movement is important for plant growth and environmental responses; however, the extent to which RNAs move between distant tissues, their relative magnitude and functional significance remain to be elucidated on a genomic scale. Using a soybean (*Glycine max*)/common bean (*Phaseolus vulgaris*) grafting system, we identified 100 shoot-root mobile miRNAs and 32 shoot-root mobile phasiRNAs, which were predominantly produced in shoots but transported to roots, some of which enabled cleavage of their mRNA targets or their precursors. In contrast, most of the mobile mRNAs were transcribed in both shoots and roots and were truncated fragments, with the transported copies accounting for only a tiny portion of all copies accumulated in the recipient (shoots or roots) tissues. These findings suggest that the regulatory mechanisms for sRNAs movement are different from those for mRNA movement, and that the earlier is more strictly regulated, and likely, more functionally significant than the latter.

Introduction

Higher plants possess two specialized vascular tissues – the xylem and phloem, which conduct water and nutrients from the roots to the shoots and organic components produced by photosynthesis in the leaves throughout the plants as their respective basic functions. Unlike the

xylem, which is composed of primarily dead cells, the phloem consists of columns of living cells, including sieve elements and companion cells connected by plasmodesmata, allowing molecules such as carbohydrates, proteins/amino acids, and hormones to travel locally or over long distances[1,2,3,4,5]. The phloem also harbors a variety of RNAs such as messenger RNAs (mRNAs), miRNAs, and small interfering RNAs (siRNAs)[1,2,3,6,7]. Early studies demonstrated that several mRNAs produced in leaves were transported to distal tissues to exert physiological functions underlying specific traits[8,9,10,11,12]. Recent genomic analyses of transcripts in heterografted plants identified hundreds to thousands of mRNAs involved in shoot-to-root or *vice versa* trafficking[1,2,3,13]. In the past decade, several miRNAs have been identified as systemic signals mediating long-distance communications[14,15,16,17]. For example, miR399 in *Arabidopsis thaliana* and miR395 in *Brassica rapa* move from shoots to roots in response to nutrient deficiencies, while miR2111 in *Lotus japonicus* travels from shoots to roots to regulate the susceptibility of uninfected roots to the soil bacteria rhizobia as a mechanism to balance rhizobial infection and nodulation events[18,19,20,21]. More recently, a genomic analysis of grafted tissues identified numerous transposon-derived or heterochromatic siRNAs (hcsiRNAs) that are capable of trafficking from shoots to roots to modulate genome-wide DNA methylation in the recipient root cells[22,23].

Despite such a progress, surprisingly, genome-scale identification and characterization of mobile miRNAs – important regulators of gene expression – has not been conducted in any

organisms. Phased secondary siRNAs (phasiRNAs), a subclass of siRNAs that require a trigger miRNA for their biogenesis, were known to be capabale of cell-to-cell trafficking[24,25,26], but whether they move over long distance is yet to be determined. Shoots-to-roots mobile hcsiRNAs have been identified at the whole genome level, but whether hcsiRNAs also move from roots to shoots to regulate DNA methylation has not been investigated. Long-distance mobile mRNAs have been identified on a genome scale in several plants, but the integrity and abundance of mobile mRNAs in recipient tissues relative to those produced locally are unclear. Here, we integrate sRNA-seq, Illumina short-read RNA-seq, Nanopore long-read RNA-seq, and degradome-seq data from heterografted and autografted soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) – two economically important leguminous crops diverged from a common ancestor ~ 17 million years ago – to address these outstanding questions.

Results

Identification of mobile sRNAs using heterografted and autografted plants. To identify mobile RNAs and reveal the extent to which they move over long distance, we conducted grafting experiments that involved four distinct grafted scion/rootstock combinations – soybean/common bean and common bean/soybean heterografted plants, and soybean/soybean and common bean/common bean homografted plants. Grafting was conducted at the V1 developmental stage of

71 soybean plants when their first set of unfolded trifoliate leaves emerged, then four shoot samples
72 and four root samples were collected 10 days after grafting for RNA isolation and sequencing (Fig.
73 1a; see details in Methods). A total of 75,512 unique sRNAs ranging from 16 to 26-nt in size were
74 obtained from the eight samples. Of these, 21,141 (28.0%) were from soybean (Gm-sRNAs),
75 whereas 19,885 (26.3%) were from common bean (Pv-sRNAs). The origins of the remaining
76 sRNAs could not be determined as they were perfectly mapped to both the soybean and common
77 bean reference genomes[27,28] (Fig. 1b, Supplementary Dataset1). Comparison of the shoot and root
78 samples from the heterografted plants (see details in Methods) revealed 4,223 Gm-sRNAs and
79 7,063 Pv-sRNAs moving from shoots to roots, four Pv-sRNAs moving from roots to shoots, and
80 one Gm-sRNA moving bidirectionally (Fig. 1c, Supplementary Table 1). These mobile sRNAs
81 together, account for 27.5% of all the sRNAs distinguishable between the two crops.
82
83 **Long-distance movement and relative abundance of hcsiRNAs.** In either soybean or common
84 bean, the 24-nt sRNAs are most abundant and match primarily to transposable elements[27,28], and
85 were considered as hcsiRNAs (Fig. 1d). Of the 4,224 mobile Gm-sRNAs, 1,214 (28.7%) are Gm-
86 hcsiRNAs, which showed mobility from shoots to roots. Of the 7,067 mobile Pv-sRNAs, 4,018
87 (56.9%) are Pv-hcsiRNAs, which were capable of moving from shoots to roots. None of the Gm-
88 hcsiRNAs or Pv-hcsiRNAs was detected to have moved from roots to shoots (Supplementary

89 Table 1). Of the 1,214 shoot-to-root mobile Gm-hcsiRNAs and 4,018 shoot-to-root mobile Pv-
90 hcsiRNAs, 1,146 (94.4%) and 3,883 (96.6%) were not detected in the heterografted soybean roots
91 and heterografted common bean roots, respectively (Fig. 1e,f and Supplementary Table 2). Even
92 though the remaining 68 Gm-hcsiRNAs and 135 Pv-hcsiRNAs were detected in the heterografted
93 soybean roots and heterografted common bean roots, their relative abundances were substantially
94 lower than detected in the homografted soybean roots and homografted common bean roots,
95 respectively, indicating that mobile hcsiRNAs are predominantly produced in shoots instead of
96 roots (Fig. 1e,f and Supplementary Table 2). Overall, the relative abundance of the mobile Gm-
97 hcsiRNAs in heterografted common bean roots was similar to that observed in homografted
98 soybean roots and *vice versa* (Fig. 1g,h), suggesting that the biogenesis and mobility of the
99 hcsiRNAs were likely not affected by the recipient tissues.
100
101 **Long-distance movement, relative abundance, and miRNA-mediated systemic regulation.** A
102 total of 622 miRNAs were identified in the eight samples, including 161 Gm-miRNAs, 72 Pv-
103 miRNAs, and 389 miRNAs sharing identical sequences between soybean and common bean. Of
104 the 161 Gm-miRNAs, 67 were detected to be mobile from shoots to roots, one was detected to be
105 mobile bidirectionally, and the remaining 93 were immobile between shoots and roots. Of the 72
106 Pv-miRNAs, 33 were detected to be mobile from shoots to roots and 39 were immobile between

shoots and roots (Supplementary Table 1). Of the 67 shoot-to-root mobile Gm-miRNAs and 33 shoot-to-root mobile Pv-miRNAs, 63 (94.0%) and 25 (75.8%) were not detected in the heterografted soybean roots and heterografted common bean roots, respectively (Fig. 2a,b and Supplementary Table 3). Of these mobile miRNAs, six (miR166i, miR1509a, miR1510a, miR1510b, miR5770a, and miR5770b) were detected to have moved from shoots to roots in both soybean and common bean (Supplementary Table 4). Overall, the relative abundance of the mobile Gm-miRNAs in heterografted common bean roots was similar to that observed in homograft soybean roots and *vice versa*, but exceptions were also observed (Fig. 2c,d and Supplementary Table 3). The mobility of several miRNAs was validated by stem-loop PCR (Supplementary Fig. 1). In contrast to the mobile miRNAs, their precursors were expressed at similar levels between the homografted roots and heterografted roots of a same species (Fig. 2e,f). These observations indicate that the mobile Gm-miRNAs were predominantly produced in shoots, and accumulated in heterografted roots to a level that is similar to what was detected in the homografted roots of the same species, although there are exceptions, particularly, for some of the Pv-miRNAs (Fig. 2g).

Of the 67 mobile Gm-miRNAs and 33 mobile Pv-miRNAs, 32 and 10 were detected to enable the cleavage of their putative mRNAs in either soybean, or common bean, or both by degradome-seq (Supplementary Table 5). When RNA-seq data from the eight tissues were integrated, five soybean and four common bean genes, whose mRNAs were cleaved by corresponding common

bean and soybean mobile miRNAs, showed a reduced level of expression in heterografted soybean and common bean roots, respectively. By contrast, such cleavages and reduction in mRNA abundance were not detected in homografted soybean and common bean roots. In addition, 12 soybean genes were detected to be targeted by soybean miRNAs and meanwhile exhibit reduced expression in homografted soybean roots compared with heterografted soybean roots, in which no cleavages of those mRNAs were detected (Fig. 2h,i,j). One of the mobile miRNAs, gma-miR4415a/b-3p, which was produced in soybean shoots and moved to the homografted soybean roots and heterografted common bean roots was able to target soybean gene *Glyma.20G051900* and common bean gene *Phvul.006g011700*, respectively, resulting in reduced expression of respective targets in the two roots.

Long-distance movement, relative abundance, and phasiRNA-mediated systemic regulation. Based on the degradome-seq and sRNA-seq data, a total of 29 soybean *PHAS* loci and 13 common bean *PHAS* loci were detected to have generated phasiRNAs. Of all the phasiRNAs generated from the 42 loci, 61 were Gm-phasiRNAs, 18 were Pv-phasiRNAs, and 25 were not distinguishable between soybean and common bean. Of the 61 Gm-phasiRNAs, 23 were detected to move from shoots to roots, and 38 were immobile. Of the 18 Pv-phasiRNAs, 14 were detected to move from shoots to roots and 4 were immobile (Fig. 3a,b and Supplementary Table 6). The relative

abundance of the mobile Gm-phasiRNAs in heterografted common bean roots was similar to that observed in homografted soybean roots and *vice versa* (Fig. 3c,d and Supplementary Table 6). Few of these mobile Gm-phasiRNAs and Pv-phasiRNAs were detected in heterografted soybean and common bean roots, although their phasiRNA precursor transcripts showed similar levels of abundance in heterograft and homograft roots of either soybean or common bean (Fig. 3a,b,e,f and Supplementary Table 6). These observations suggest that the phasiRNAs were nearly exclusively produced in the shoots instead of in the roots (Fig. 3h). Biogenesis of phasiRNAs from their precursor transcripts ("*PHAS*" loci) requires trigger miRNAs. As exemplified in Figure 3g, gma-miR1510b-3p was detected to have triggered the production of phasiRNAs from the transcripts of *Glyms.04G219600* and *Glyma.15G232600* in soybean shoots but no such phasiRNAs were detected in heterografted soybean roots, in which both gma-miR1510b-3p and the transcripts of two soybean genes were present. Degradome-seq revealed that three of the mobile phasiRNAs produced from their precursor – the transcripts of *Glyms.04G219600*, enabled *cis*-directed cleavage of the precursor in the homografted soybean roots, and one of the three sites were also further confirmed by 5' Rapid Amplification of cDNA Ends (RACE)-PCR. By contrast, such cleavage sites were not detected in the heterografted soybean roots (Fig. 3g,h), exemplifying phasiRNA-mediated systemic regulation of their own precursors.

According to the degradome-seq data, mRNAs from 19 genes were detected to be the targets

of 17 phasiRNAs including four mobile Gm-phasiRNAs, one mobile Pv-phasiRNA, seven immobile Gm-phasiRNAs, and five phasiRNAs that were not distinguishable between the two plants (Supplementary Table 7). Of the four mobile Gm-phasiRNAs, one was able to target two soybean genes (*Glyma.16G050500*, *Glyma.19G100200*) in homografted roots and a common bean gene (*Phvul.001G087000*) in heterografted roots, and three were detected to target four soybean genes in homografted roots. The mobile Pv-phasi-RNA was detected to target a common bean gene (*Phvul.001G087000*) in homografted roots and a soybean gene (*Glyma.19G100200*) in heterografted roots. As exemplified in Figure 3h, some phasiRNAs were not only able to target their precursor *PHAS* loci, but also non-phasiRNA-producing genes.

Long-distance movement, relative abundance, and integrity of mobile mRNAs. Thousands of plant mRNAs capable of moving between shoots and roots have been previously identified through RNA-sequencing of grafted tissues in multiple heterograft systems; however, large proportions of short RNA-seq reads from heterografted species were unassignable to either species, thus, the integrity of the mobile mRNAs remains largely unknown. In an attempt to assess the relative completeness of mobile mRNAs, we decoded the transcriptomes of the eight grafted samples by Illumina short-read RNA-seq and Oxford Nanopore long-read RNA-seq, which together detected expression of 38,241 soybean genes and 24,062 common bean genes. The short-read RNA-seq

179 data revealed the mobility of 1,322 mobile soybean mRNAs and 874 common bean mRNAs. Of
180 the 1,322 soybean mRNAs, 1,167 moved from shoots to roots, 130 moved from roots to shoots,
181 and 25 moved bidirectionally. Of the 874 common bean mRNAs, 684 moved from shoots to roots,
182 153 moved from roots to shoots, and 37 moved bidirectionally (Fig. 4a and Supplementary Table
183 1 and 8). The long-read RNA-seq data revealed the mobility of 163 soybean mRNAs and 129
184 common bean mRNAs, of which, ~56% were detected to cover the "full-length" of respective
185 coding sequences) (Supplementary Table 8). Of the 163 soybean mRNAs, 117 moved from shoots
186 to roots, 35 moved from roots to shoots, and 11 moved bidirectionally. Of the 129 common bean
187 mRNAs, 90 moved from shoots to roots, 29 moved from roots to shoots, and 10 moved
188 bidirectionally. Totaling five pairs of orthologous genes in soybean and common bean were
189 detected to have produced mobile mRNAs in both species. The trafficking of mRNAs from a
190 number of genes including *Glyma.11G114000*, *Glyma.11G228000*, *Phvul.001G259000*,
191 *Phvul.001G113800*, *Phvul.001G229500*, *Phvul.010G023000* and *Phvul.008G285100*, which are
192 predicted to be involved in various signaling pathways underlying plant growth, photosynthesis,
193 and/or vesicle-mediated transport, was further confirmed by qRT-PCR (Supplementary Fig. 2).
194 Remarkably, only eight mobile soybean mRNAs and 14 mobile common bean mRNAs were
195 detected by both the short-read RNA-seq and long-read RNA-seq.

196 It is proposed that soybean underwent a whole genome duplication event ~13 million years
197 ago, leaving ~16,500 duplicated gene pairs retained in the current genome. We found that, of the
198 1,477 mobile mRNAs in soybean, 300 were from singletons, 1,075 from one copy of duplicated
199 gene pairs, and 51 from both copies of duplicated gene pairs (Supplementary Table 9). Thus, no
200 apparent biases of the mobility against either duplicates or singletons were observed (3.6% in
201 duplication vs 2.0% in singleton). Among all mobile mRNAs detected in soybean and common
202 bean, only 72 were from orthologous genes in the two species, although enriched biological
203 pathways of the mobile mRNAs in the two species are similar (Supplementary Fig. 3 and
204 Supplementary Table 10).
205 In contrast to the predominant accumulation of sRNAs in the recipient tissues (mainly roots),
206 the relative abundance of mobile mRNAs in the recipient tissues (both shoots and roots), as
207 detected by comparison between the heterografted and homografted tissues, was extremely low
208 (Fig. 4b and 4c). For example, most of the soybean mRNAs detected in heterografted common
209 bean roots showed high levels of abundance in both homografted soybean roots and heterografted
210 soybean roots (Supplementary Table 8), whereas most of the soybean sRNAs detected in
211 heterografted common bean roots were not detected in the heterografted soybean roots
212 (Supplementary Table 3). These observations indicate that most mobile mRNAs detected in the
213 recipient tissues (either shoots or roots) were actually produced locally, but most mobile sRNAs

214 accumulated in the roots were transported from shoots.

215

216 **Discussion**

217 Accumulating evidence has demonstrated the importance of plant mobile RNAs as signal
218 molecules in shoot-root communications, but no previous studies have investigated the mobility
219 of multiple types of RNAs simultaneously at the whole genome level, perhaps, partly due to the
220 limitations of the grafting systems used in those studies. For example, grafting of the wild-type
221 Arabidopsis shoots with the roots of Arabidopsis mutants lacking the functional RNA polymerase
222 IV required for 24-nt sRNA biogenesis identified mobile 24-nt hcsiRNAs[29,30], but it would be
223 unable to detect other types of mobile RNAs. Similarly, grafting of different Arabidopsis ecotypes
224 was able to identify a large number of mobile mRNAs[7], but it would be ineffective in detecting
225 mobile sRNAs. Although a number of heterografting systems with highly diverged plant species
226 were used to identify genome-wide mobile mRNAs, the effectiveness of those systems in
227 identifying mobile sRNAs was not explored. In our study, the high degree of sequence divergence
228 between the two leguminous crops enabled identification of 5,232 mobile hcsiRNAs, 100 mobile
229 miRNAs, and 41 mobile phasiRNAs, as well as 2,466 mobile mRNAs throughout the two genomes.
230 Given that less than a dozen mobile miRNAs and phasiRNAs have been previously reported[3], the
231 effectiveness of our experiments in identifying mobile sRNAs, particularly miRNAs and

phasiRNAs, is laudable. We would like to point out that our approach also has its own limitations. For example, it was only able to determine the mobility of 233 miRNAs that are unambitiously assigned to the soybean or common bean genomes, which account for ~37% of all the miRNAs detected in the investigated shoot and root tissues. Whether this subset of miRNAs is representative of all the miRNAs expressed in the two crops remains unclear. However, several individual miRNAs showing sequence polymorphisms between soybean and common bean and shoot-to-root trafficking are also shared by other legumes, in which they have been demonstrated to play important roles in root and nodule development. For example, in *Medicago truncatula*, miR166 and miR1509 were found to regulate the root and nodule development by targeting mRNAs encoding HD-ZIP transcription factors and by triggering the production of phasiRNAs from transcripts of an *APETALA2* homolog, respectively,[31,32]. In addition, miR1510 was found to be a young miRNA specific to the Phaseoleae tribe of legumes and a predominant trigger for the production of phasiRNA from transcripts of *NB-LRR* genes that may underlie plant's responses to biotic stresses[31,33,34]. Thus, although these miRNA sequences are diverged among species, they appear to be functionally conserved among these legumes and execute their functional roles systemically through long-distance trafficking.

It is noteworthy that our study revealed several contrasting features between mobile sRNAs and mobile mRNAs. One of such features is the directionality of RNA movement. The mobile

sRNAs, including hcsiRNAs; miRNAs; and phasiRNAs, were detected to move from shoots to roots only, with few exceptions, whereas ~13% of the mobile mRNAs were detected to move from roots to shoots. Movement of mRNAs from shoots and roots and *vice versa* was commonly observed in other grafting experiments, but the numbers of mobile mRNAs as well as the proportions of mobile mRNAs trafficking in the two directions vary greatly among different experiments[35]. Such variation may be associated with growth conditions and developmental stages of the grafted plants, the time points after grafting for tissue collection, and sequencing coverages[3]. Another feature is the relative abundance of transported RNA copies versus all copies accumulated in the recipient tissues. In general, the mobile sRNAs transported to the recipient tissues (i.e., roots) were highly abundant, and not produced locally, whereas the mRNAs transported to the recipient tissues (either shoots or roots) were detected to be minimal and account for small proportions of all the mRNAs from the same set of genes detected in the same tissues. This may explain why only a small portion of mobile mRNAs were detected by both the short-read sequencing and long-read sequencing of mRNAs. Lastly, a subset of the mobile sRNAs such as miRNAs and phasiRNAs have been validated to play regulatory roles in the recipient tissues, whereas most of the mobile mRNAs were truncated fragments, which apparently cannot be translated into functional proteins. Together, these features suggest that the long-distance trafficking of sRNAs is more strictly regulated than that of mRNAs. Given such a high level of species-specificity and low level of

abundance, most of the mobile mRNAs detected in this and other studies[3,35] are likely non-functional in their recipient tissues.

Methods

Plant materials and growth conditions. Seeds of soybean variety Williams 82 and common bean variety Jiulibai were soaked in water for one day and then sowed in the soil to grow in the growth chamber under the cycles of 12 h light at 28 °C / 12 h darkness at 24 °C, with humidity at 30%. The stems of the seedlings were cut seven days (V0 stage) after sowing for the grafting experiments following a protocol described previously[36]. The grafting experiments include four distinct scion/rootstock combinations – soybean/common bean and common bean/soybean heterografted plants, and soybean/soybean and common bean/common bean homografted plants, from which eight tissues, homografted *G. max* (soybean) shoots (*hoGmSt*), homografted *G. max* roots (*hoGmRt*), heterografted *G. max* shoots (*heGmSt*), heterografted *G. max* roots (*heGmRt*), homografted *P. vulgaris* (common bean) shoots (*hoPvSt*), homografted *P. vulgaris* roots (*hoPvRt*), heterografted *P. vulgaris* shoots (*hePvSt*), and heterografted *P. vulgaris* roots (*hePvRt*) were sampled for sequencing and PCR analyses.

Sequencing of sRNA, mRNA and degradomes and processing of raw data. Total RNAs were isolated from grafted shoots and roots samples using TRIzol Reagent (Invitrogen/Life Technologies, CA). The sRNA-seq libraries were constructed using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA.) following the manufacturer's manual, and then sequenced using the Illumina Hiseq 2500 platform to generate the 50-bp single-end reads. The raw reads were processed with the fastx-toolkit (version 0.0.14, http://hannonlab.cshl.edu/fastx_toolkit) for removal of low-quality reads and adaptor sequences, and the processed reads were mapped to the soybean and common bean reference genomes (version 12.1, phytozome) using the Bowtie program[37] (version 1.1.1) with 0 mismatches (-v 0). The abundance matrix of sRNAs was normalized to CPM (counts per million reads) based on the mapping results using in-house Perl scripts.

Total RNAs were processed using the Epicentre Ribo-zero™ rRNA Removal Kit (Epicentre, USA) to deplete ribosomal RNAs, and the processed RNA samples were used to construct RNA-seq libraries using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, USA). Then the RNA libraries were sequenced using the Illumina Hiseq 4000 platform to generate 150bp paired-end reads. The raw reads were processed also with the fastx-toolkit program for removal of low quality reads and adaptor sequences, and the processed reads from each libraries were subsequently mapped to the soybean or common bean reference genomes[27, 28] using

303 STAR[38](version 2.5.4b) with the default parameters. The reads uniquely mapped to individual
304 genes were extracted using Samtools[39] (version 1.8), and then the abundance of mRNAs from each
305 gene was counted and normalized to CPM using the Bedtools program[40] (version 2.29.0) and the
306 "EdgeR" packages in R[41] (version 3.28), based on the recommendation by its User's Guide for
307 experiments without biological replicates.

308 To detect full-length mobile RNAs, the Oxford Nanopore Sequencing of cDNAs with a strand-
309 switching method was employed using the cDNA-PCR Sequencing Kit (SQK-PCS 109) and PCR
310 Barcoding Kit (SQK-PBK004), following the manufacturer's manuals. The base-called reads
311 were collected and converted to FASTA format using the MinKNOW software
312 (https://nanoporetech.com). The Nanopore reads were mapped to the soybean and common bean
313 genomes using the minimap2 program[42] (version 2.11) with the parameters (-ax splice -uf -k14 --
314 secondary=no --splice-flank=no). The gene expression level was quantified using Stringtie and
315 Cuffmerge (https://www.biostars.org).

316 Degradome-seq, also referred as to parallel analysis of RNA ends (PARE), is a modified
317 5'RACE with high-throughput short-read sequencing method for target mRNA confirmation and
318 cleavage site detection, and was conducted following the protocol described previously[43] with
319 modifications made by LC-BIO (Hangzhou, China). The 47-bp single-end PARE sequences were
320 sequenced using the Illumina Hiseq 2500 platform. A combination of the degradome-seq data, with

321 the transcriptome data and the sRNA reads from soybean and common bean were used to detect
322 cleavage sites within mRNAs using the Paresnip2 program[44] (version 4.5).
323
324 **Sequence analyses for detection of mobile RNAs.** sRNAs with sizes ranging from 16 nt to 26 nt
325 and copy numbers ≥1 CPM in at least one of the eight samples were kept for detection of mobile
326 sRNAs. The 24-nt sRNAs were defined as hcsiRNAs, the miRNAs were identified by searching
327 against miRbase – the microRNA database[45], with a focus on previously identified soybean and
328 common bean miRNAs[33]. The phasiRNAs precursor genes and phasiRNAs were identified using
329 the PhaseTank program[46] (version 1.0) with two reference genomes (soybean and common bean)
330 and other default parameters. Putative miRNA triggers for production of phasiRNAs were
331 predicted using degradome data and psRNAtarget – a plant small RNA target analysis server[47].
332 mRNAs were identified by comparison with annotated genes in the soybean and common bean
333 genomes[27,28]. The variety of mobile RNAs showing distinguishable sequences between the two
334 crops were identified following the criteria described below: i) Soybean RNAs (i.e., Gm-RNAs
335 including Gm-hcsiRNAs, Gm-miRNAs, Gm-phasiRNAs, and Gm-mRNAs) were defined to be
336 mobile from shoots to roots when they were detected in hoGmRt, hoGmSt, heGmSt and hePvRt,
337 but not detected in hoPvSt and hoPvRt; ii) Gm-RNAs were defined to be mobile from roots to
338 shoots when they were detected in hoGmRt, hoGmSt, heGmRt and hePvSt, but absent in hoPvSt

and HoPvRt; iii) common bean RNAs (i.e., Pv-sRNAs including Pv-hcsiRNAs, Pv-miRNAs, Pv-phasiRNAs, and Pv-mRNAs) were defined to be mobile from shoots to roots when they were detected in hoPvSt, hoPvRt, hePvSt and heGmRt, but not detected in hoGmSt and hoGmRt; iv) Pv-RNAs were defined to be mobile from roots to shoots when they were detected in hoPvSt, hoPvRt, hePvRt and heGmSt, but not detected in hoGmSt and hoGmRt; v) mobile RNAs were defined as bi-directional when they were detected to be mobile both from shoots to roots and from roots to shoots. Relative abundance of transported versus all RNAs accumulated in the recipient tissues was roughly estimated based on the CPM of soybean/common bean RNAs in heterografted common bean/soybean tissues versus CPM of soybean/common bean RNAs in homografted soybean/common bean tissues.

qRT-PCR, stem-loop qRT-PCR, and 5' RACE-PCR and sequencing and analysis of PCR fragments. Reverse transcription PCR (RT-PCR), quantitative RT-PCR (qRT-PCR), stem-loop RT-PCR, stem-loop qRT-PCR, and 5'RACE-PCR were performed as previously described[48,49]. In addition to RNA-seq, The relative abundance of mRNA from chosen genes was evaluated by qRT-PCR, in which the soybean gene Gm*Cons4* (GenBank ID BU578186)[50] and common bean gene *PvActin11* (*Phvul.008G011000*)[51] was used as an respective internal reference to quantify the relative expression levels of the soybean and common bean genes from three biological replicates.

All the primers used in this study are listed in Supplementary Table 11.

Gene set enrichment and pathway analysis. Gene ontology enrichment analysis of a given gene group, such as, differentially expressed genes and mobile mRNAs was performed and visualized with the clusterProfiler R package[52] (version 3.10). The clusterProfiler's input files were formatted using in-house Perl scripts. GO term information of soybean and common bean was extracted from the gene annotation files in the Phytozome database. The GO terms, with FDR-adjusted p value < 0.05, were considered as significant enrichment.

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Author contributions

JM, SL and XW designed the research; XW analyzed the data; SL, XW, WX, TL, CC, LC, and CC performed the research; JM wrote the manuscript with input from SL and XW.

Conflicting interest

The authors declare no conflict of interest.

Data availability

The raw read sequences are deposited in the National Center for Biotechnology Information Sequence Read Achieve (http://www.ncbi.nlm.nih.gov/sra/) under the accession number PRJNA648759.

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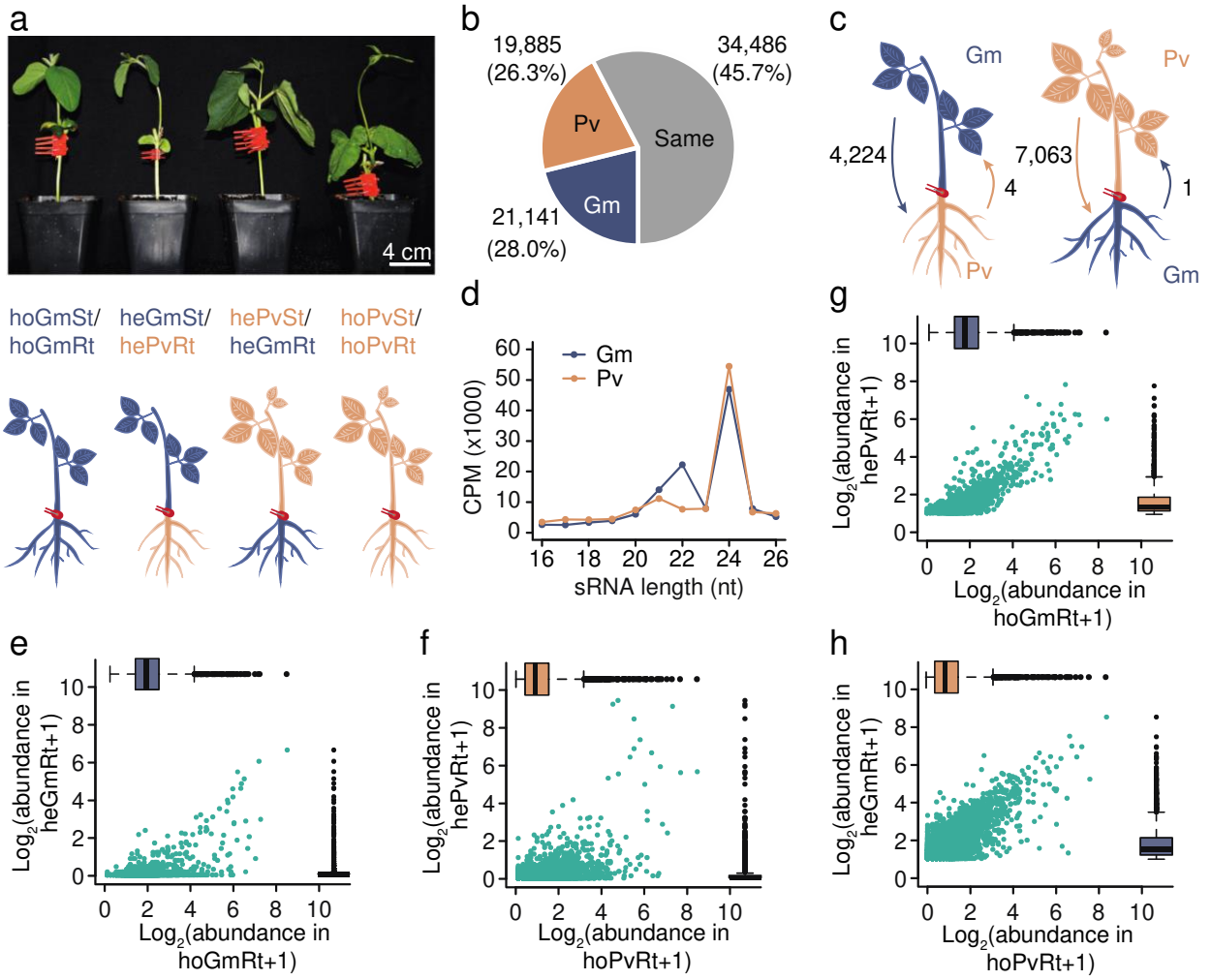

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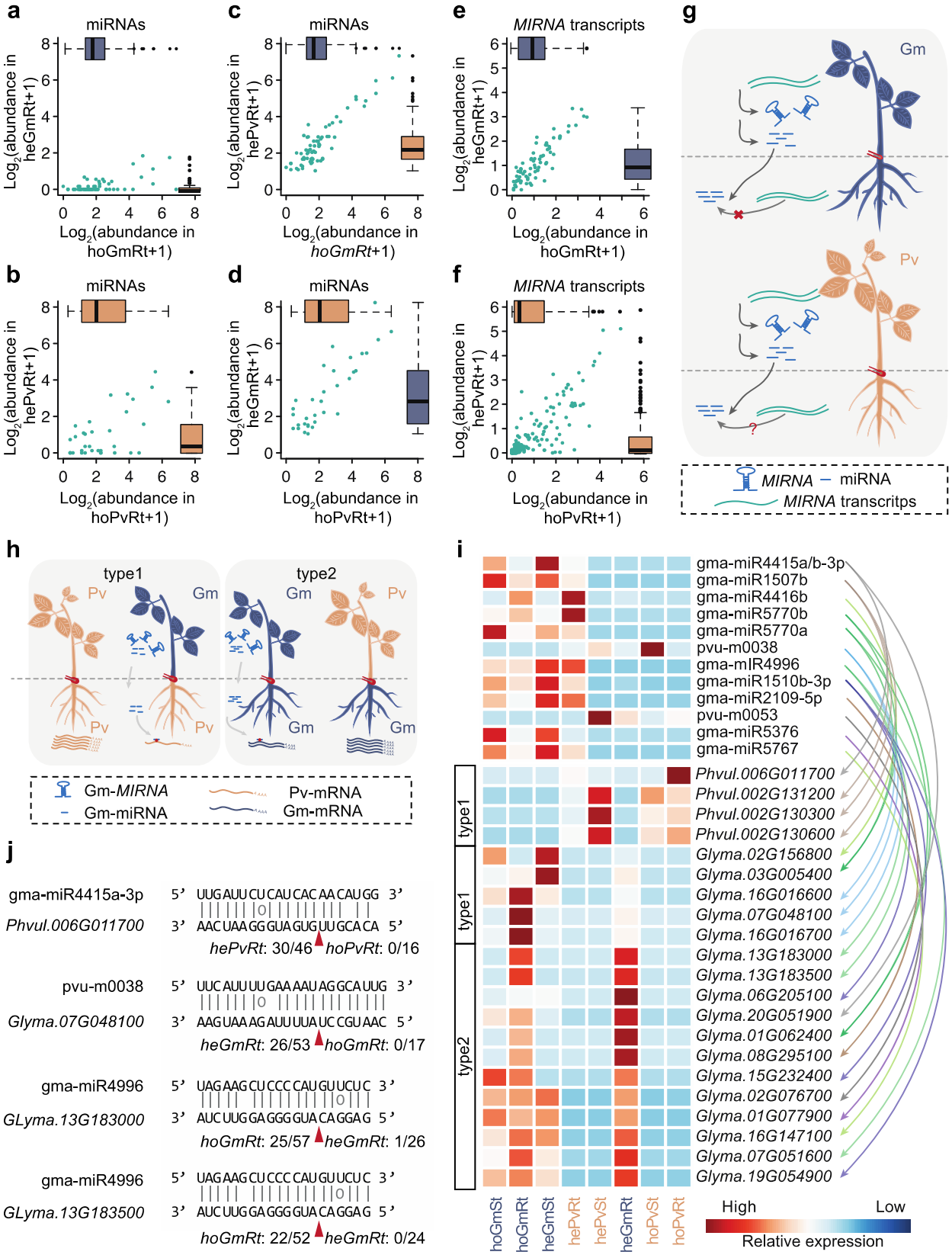
a
miRNAs
Log2(abundance in heGmRt+1)
Log2(abundance in hoGmRt+1)
b
miRNAs
Log2(abundance in hePvRt+1)
Log2(abundance in hoPvRt+1)
c
miRNAs
Log2(abundance in hePvRt+1)
Log2(abundance in hoGmRt+1)
d
miRNAs
Log2(abundance in heGmRt+1)
Log2(abundance in hoPvRt+1)
e
MIRNA transcripts
Log2(abundance in heGmRt+1)
Log2(abundance in hoGmRt+1)
f
MIRNA transcripts
Log2(abundance in hePvRt+1)
Log2(abundance in hoPvRt+1)
g
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MIRNA – miRNA
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Gm
Gm-MIRNA
Gm-miRNA
Pv-mRNA
Gm-mRNA
i
gma-miR4415a/b-3p
gma-miR1507b
gma-miR4416b
gma-miR5770b
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Glyma.15G232400
Glyma.02G076700
Glyma.01G077900
Glyma.16G147100
Glyma.07G051600
Glyma.19G054900
type1
type1
type2
hoGmSt
hoGmRt
heGmSt
hePvRt
hePvSt
heGmRt
hoPvSt
hoPvRt
High
Low
Relative expression
j
gma-miR4415a-3p 5' UUGAUUCUCAUCACAACAUGG 3'
Phvul.006G011700 3' AACUAAGGGUAGUGUUGCACA 5'
hePvRt: 30/46 hoPvRt: 0/16
pvu-m0038 5' UUCAUUUUGAAAAUAGGCAUUG 3'
Glyma.07G048100 3' AAGUAAAGAUUUAUCCGUAAC 5'
heGmRt: 26/53 hoGmRt: 0/17
gma-miR4996 5' UAGAAGCUCCCCAUGUUCUC 3'
GLyma.13G183000 3' AUCUUGGAGGGGUACAGGAG 5'
hoGmRt: 25/57 heGmRt: 1/26
gma-miR4996 5' UAGAAGCUCCCCAUGUUCUC 3'
GLyma.13G183500 3' AUCUUGGAGGGGUACAGGAG 5'
hoGmRt: 22/52 heGmRt: 0/24

Figure 2. Relative abundance and systemic gene regulation of mobile miRNAs. a. Comparison of soybean miRNA abundances between heGmRt and hoGmRt. **b,** Comparison of common bean miRNA abundances between hePvRt and hoPvRt. **c**, Comparison of soybean miRNA abundances between hePvRt and hoGmRt. **h**, Comparison of common bean miRNA abundances between between heGmRt and hoPvRt. **e**, Comparison of soybean *MiRNA* abundances between heGmRt and hoGmRt. **f,** Comparison of common bean *MiRNA* abundances between hePvRt and hoPvRt. Boxplots displayed the distribution of the log2 transformed abundances of miRNAs or *MiRNA*s in individual samples. **g**, Modes of mobile miRNA biogenesis and systemic regulation of target genes. **h** and **i**, Exemplification of mobile miRNA-mediated down-regulation of target genes in recipient tissues revealed by comparison of mRNA abundances between homografted and hetergrafted roots of a same crop, e.g., down-regulation of Pv-mRNAs (or Gm-mRNAs) by mobile Gm-miRNAs (or Pv-miRNAs) in hePvRt (or heGmRt) (type 1), and down-regulation of Gm-mRNAs by mobile Gm-*miRNAs in hoGmRt (type* 2). The curved lines with arrowheads connect miRNAs and respective target genes. **j**, Exemplification of confirmed cleavages (sites and frequencies indicated by arrows and ratios, respectively) of mRNAs by mobile miRNAs in recipient tissues of hetergrafted and homografted plants.

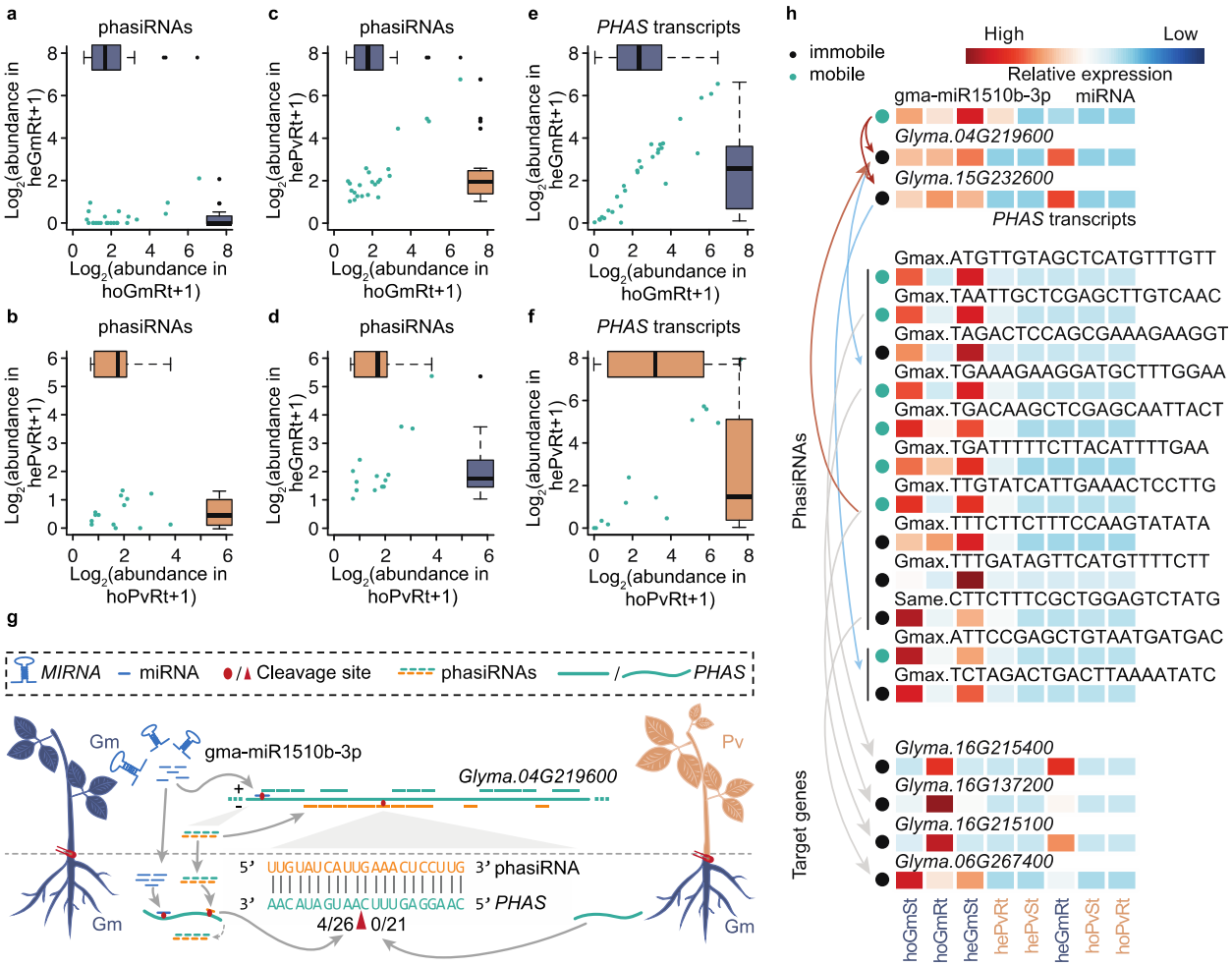


Figure 3. Relative abundance and systemic gene regulation of mobile phasiRNAs. a. Comparison of soybean phasiRNA abundances between heGmRt and hoGmRt. **b,** Comparison of common bean phasiRNA abundances between hePvRt and hoPvRt. **c**, Comparison of soybean phasiRNA abundances between hePvRt and hoGmRt. **d**, Comparison of common bean phasiRNA abundances between heGmRt and hoPvRt. **e**, Comparison of soybean *PHAS* transcript abundances between heGmRt and hoGmRt. **f,** Comparison of common bean *PHAS* transcript abundances between hePvRt and hoPvRt. Boxplots displayed the distribution of the log2 transformed abundances of phasiRNAs or *PHAS* transcripts in individual samples. **g.** Exemplification of regulatory cascades involving mobile phasiRNAs. gma-miR1510b-3p triggered production of a cluster of phasiRNAs from *Glyma.04G219600* in soybean shoots, three of which enabled cleavage of their own precursor, with one validated to have implemented the cleavage in hoGmRt but not in heGmRt. Cleavage site and frequency are indicated by arrows and ratios, respectively. **h**, Relative abundance of gma-miR1510b-3p, all phasiRNAs produced by the *PHAS* loci, and the *PHAS* loci in the eight tissues. The curved lines with arrowheads connect the miRNA and its *PHAS* targets, *PHAS* loci and phasiRNAs from the loci, or phasiRNAs and their respective target genes.

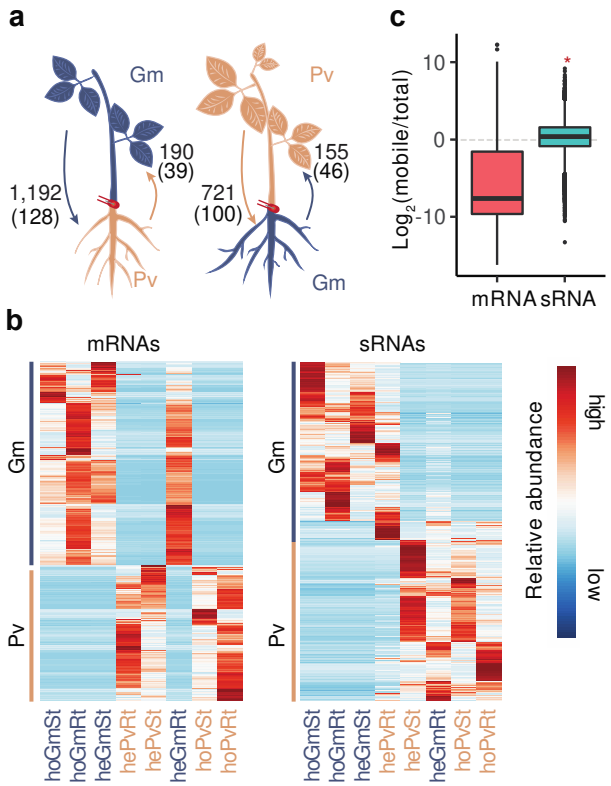
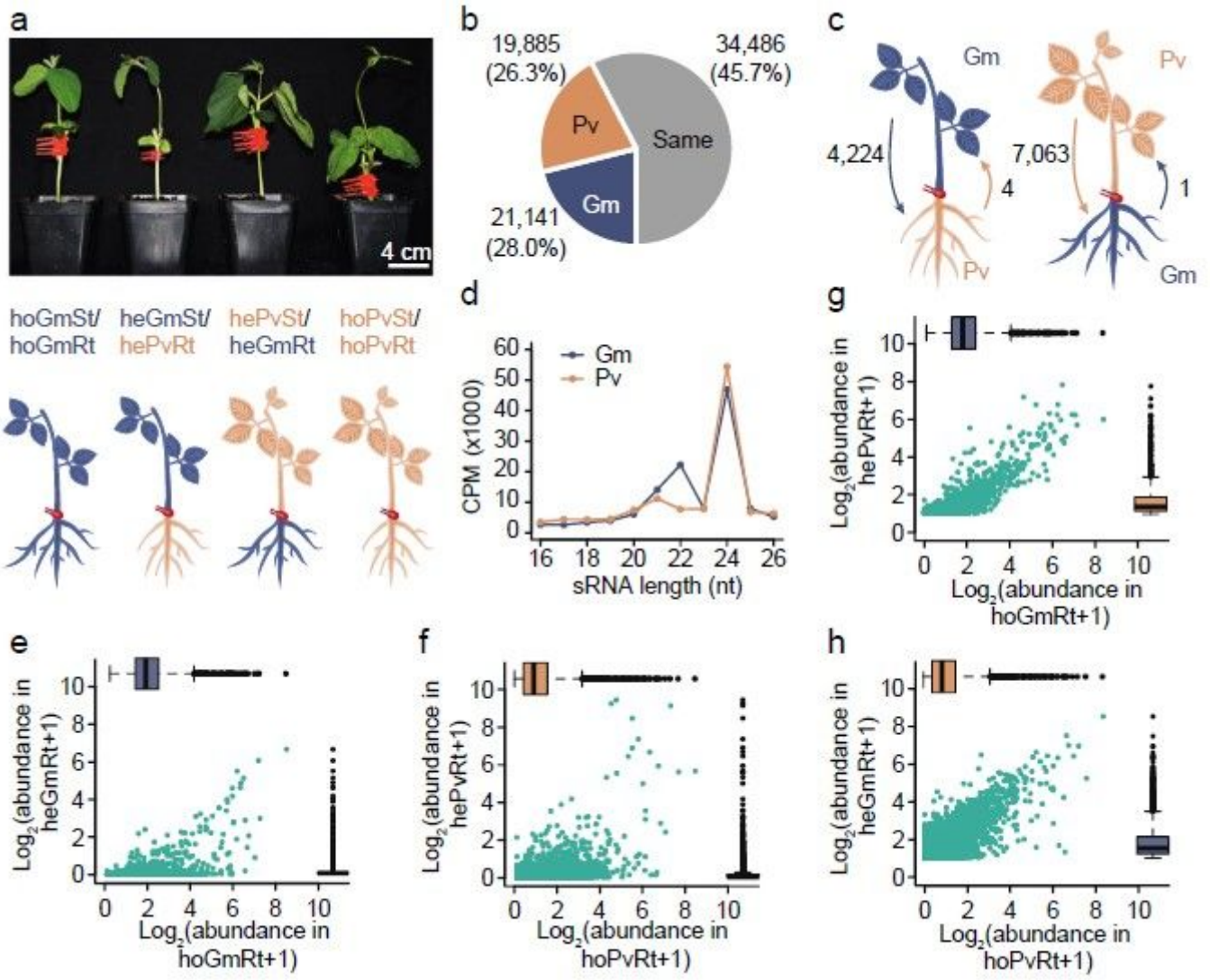


Figure 4. Differences in directionality and relative abundance between mobile sRNAs and mRNAs. a, Numbers of mobile soybean and common bean mRNAs detected by short-read Illumina RNA-seq (out of the brackets) and long-read Nanopore RNA-seq (within the brackets) and the directionality of mRNA movement. **b**, Relative abundances of mobile mRNAs and sRNAs shown by heatmaps. The of blue and orange bars beside the heatmaps represent the RNAs from soybean (Gm) and common bean (Pv), respectively. **c**, Abundances of transported RNAs relative to abundances of respective RNAs accumulated (transported and locally produced) in recipient tissues shown by the distribution of the log2 transformed values. The grey dotted line indicates similar abundance between the transported RNAs in recipient tissues and locally produced RNAs. The red asterisk indicates significant difference (p value <0.001, *K-S* test).

Figures



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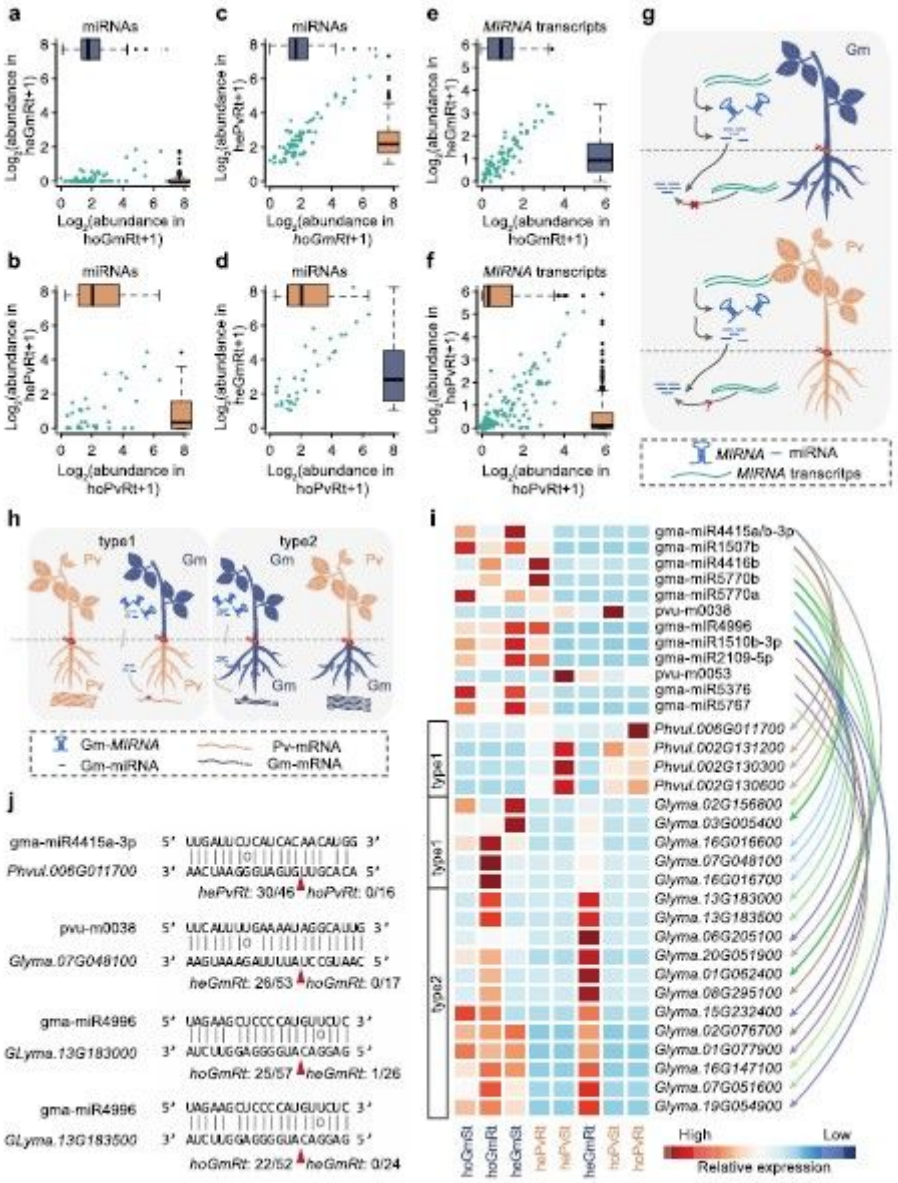

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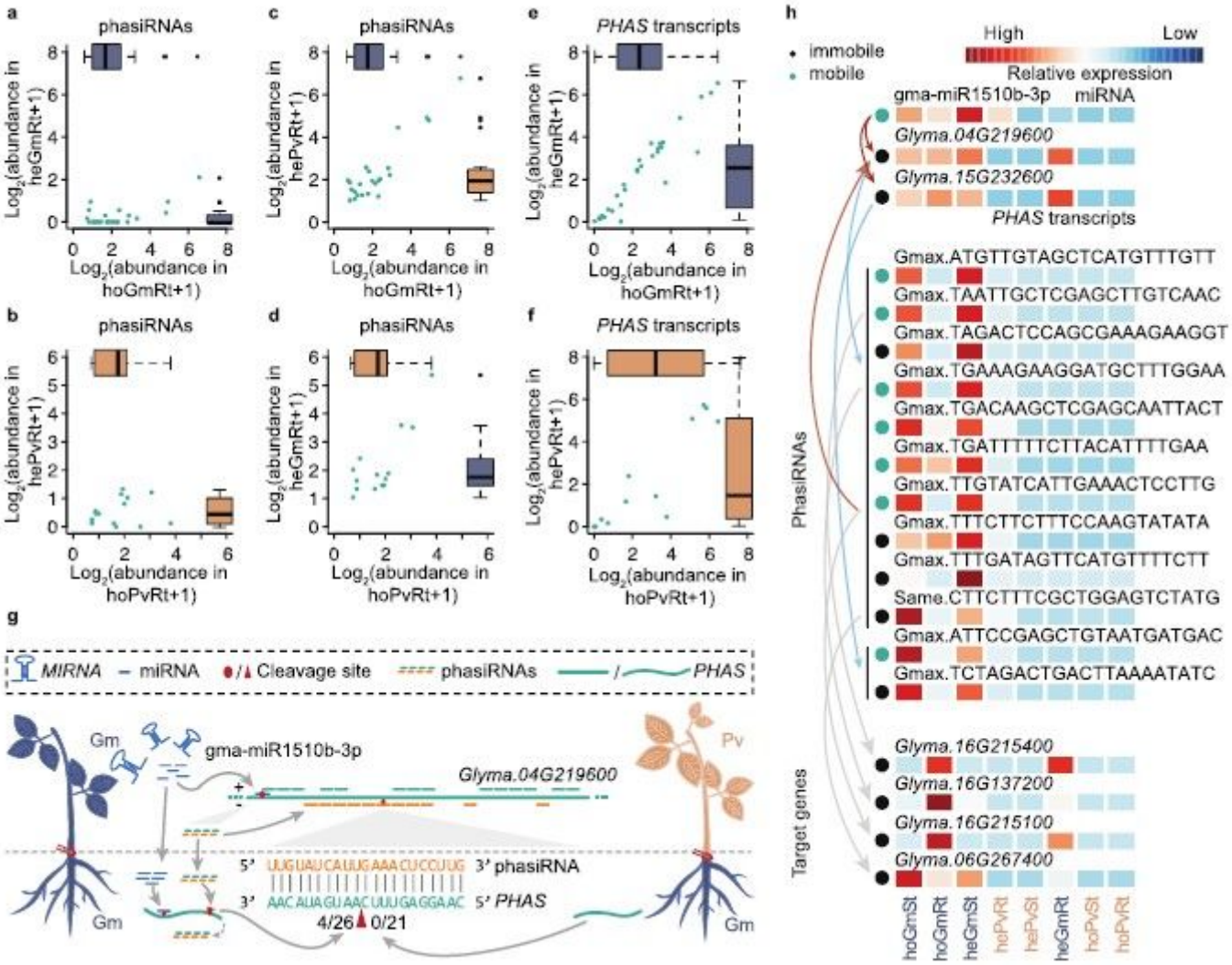

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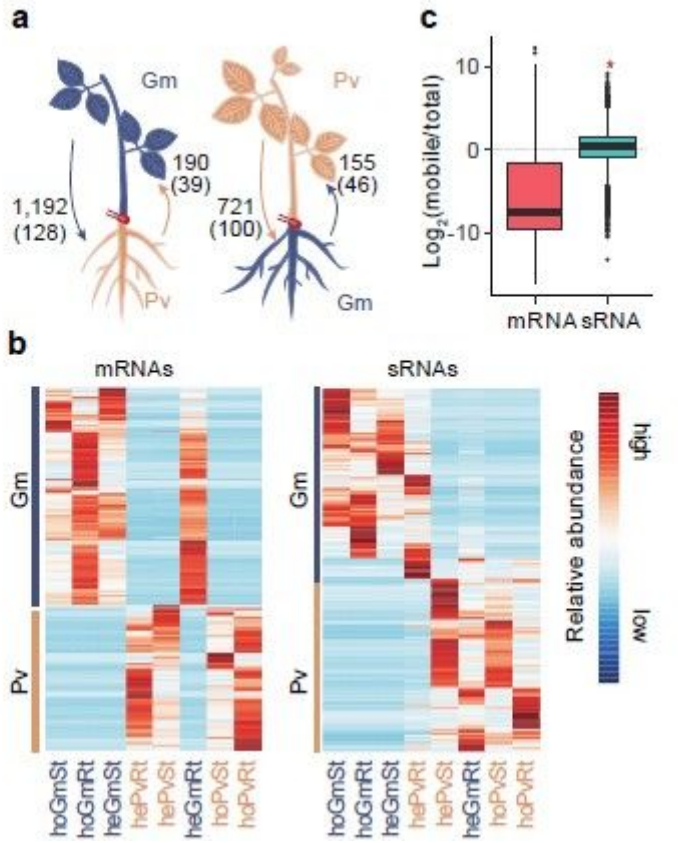

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- SupplementaryTable10.xlsx
- SupplementaryTable11.xlsx
- SupplementaryDataset1.xlsx
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