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Identification of mRNAs that Move Over Long Distances Using an RNA-Seq Analysis of Arabidopsis/*Nicotiana benthamiana* Heterografts

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Phloem is a conductive tissue that allocates nutrients from mature source leaves to sinks such as young developing tissues. Phloem also delivers proteins and RNA species, such as small RNAs and mRNAs. Intensive studies on plant systemic signaling revealed the essential roles of proteins and RNA species. However, many of their functions are still largely unknown, with the roles of transported mRNAs being particularly poorly understood. A major difficulty is the absence of an accurate and comprehensive list of mobile transcripts. In this study, we used a hetero-graft system with *Nicotiana benthamiana* as the recipient scion and Arabidopsis as the donor stock, to identify transcripts that moved long distances across the graft union. We identified 138 Arabidopsis transcripts as mobile mRNAs, which we collectively termed the mRNA mobilome. Reverse transcription–PCR, quantitative real-time PCR and droplet digital PCR analyses confirmed the mobility. The transcripts included potential signaling factors and, unexpectedly, more general factors. In our investigations, we found no preferred transcript length, no previously known sequence motifs in promoter or transcript sequences and no similarities between the level of the transcripts and that in the source leaves. Grafting experiments regarding the function of *ERECTA*, an identified transcript, showed that no function of the transcript mobilized. To our knowledge, this is the first report identifying transcripts that move over long distances using a hetero-graft system between different plant taxa.

Keywords: Graft • Long-distance transport • Mobile mRNA • Phloem • RNA-Seq.

Abbreviations: ddPCR, droplet digital PCR; qPCR, quantitative real-time PCR; RNA-Seq, RNA sequencing; RT–PCR, reverse transcription–PCR.

Introduction

Systemic signaling is an essential mechanism for multicellular organisms to co-ordinate bodily functions and act as a single living unit. In contrast to the animal nervous systems, plants lack a similar electrical network of tissues; besides, plants evolved vascular tissues that run throughout the plant body connecting different organs. Plant vascular tissues, like those of animals, serve as conduits for water and nutrients. However, recent studies have discovered that they also act as long-distance communication pathways. Xylem tissues form a tract for water and micronutrient transport, carry secreted peptides from roots to shoots and transmit information on soil conditions to shoots (Okamoto et al. 2013, Tabata et al. 2014). Phloem tissues form a tract to transport saccharide nutrients generated by photosynthesis in source leaves, as well as phytohormones, RNA species and proteins (Lough and Lucas 2006). Thus, plant vascular systems serve not only to conduct water and nutrients, but also as a signaling superhighway connecting the entire body.

Phloem tubes consist of living cells, called sieve elements, and their function is maintained by the surrounding companion cells and parenchyma. To understand systemic signaling in plants further, phloem exudates have been analyzed, with a special interest in macromolecules. These studies have suggested that phloem tubes contain hundreds of proteins and hundreds of RNA species, including small RNA, non-coding RNA and mRNA (Lough and Lucas 2006, Spiegelman et al. 2013). Furthermore, some of these proteins and RNA species have roles as systemic signals, such as FT proteins, encoded by the *FLOWERING LOCUS T* (*FT*) gene, which serves as florigen signals (Corbesier et al. 2007, Jaeger and Wigge 2007, Lin et al. 2007, Mathieu et al. 2007, Tamaki et al. 2007, Notaguchi et al. 2008) and small RNAs that trigger systemic silencing (Brosnan and Voinnet 2011).

For >15 years, research has focused on the biological functions of phloem-mobile mRNAs. By using gain-of-function transcripts, including *KNOTTED1-like homeobox* (*KNOX*), *BEL1-like* (*BELL*), *GIBBERELLIC ACID INSENSITIVE* (*GAI*) and *Auxin/Indole-3-Acetic Acid* (*Aux/IAA*) transcription factor transcripts, it was demonstrated that mobile transcripts can impact development in their destination tissues, such as leaf shape, fruit size, tuberization and root architectures (Kim et al. 2001, Haywood et al. 2005, Banerjee et al. 2006, Mahajan et al. 2012, Notaguchi et al. 2012). Because the phenotypes can be explained by their translated proteins, the phloem-mobile transcripts may exert their

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functions through their translated proteins. Grafting experiments have provided strong evidence for their mobility over long distances. Thus, it has been shown that plant phloem tube tissues translocate mRNA species. However, the lack of an available knock-out mutant phenotype for genes of interest did not allow the conclusive investigation of their biological functions. Therefore, the experiments did not clarify their biological necessity for mRNA movement (Notaguchi 2015). The current information on phloem-associated mRNAs was constructed by phloem exudate analyses that do not exclude the possibility of contaminants from other tissues. Hence, information that concentrated on mobile mRNAs is required.

Evidence suggests that mobile transcripts have some unexplained roles in plants, with the same transcripts being transported in a broad range of plants. The transport of *GAI* mRNA was identified in pumpkin originally (Haywood et al. 2005), and subsequently the transport of homologous transcripts was reported in Arabidopsis, tomato and apple (Haywood et al. 2005, Roney et al. 2007, Xu et al. 2010). This is also true for *Aux/IAA* mRNAs, with the transport of two members reported in melon, one in tomato, one in apple and two in Arabidopsis (Omid et al. 2007, Roney et al. 2007, Kanehira et al. 2010, Notaguchi et al. 2012). In addition, phloem sap contains no detectable RNase activity (Sasaki et al. 1998, Doering-Saad et al. 2002), suggesting that RNA molecules are intact inside the phloem translocation stream. These facts imply that the plants have an evolutionarily conserved, mRNA transport mechanism. Although the mRNA transport phenomenon appears important, neither the biological roles of the mobile transcripts nor their influence on their targeted tissues have been revealed. In addition, we are not absolutely sure whether they function in their RNA or protein form.

To gain knowledge of phloem-mobile transcripts, we decided to collect an accurate and comprehensive profile of transcripts that move over long distances by analyzing hetero-grafted plant species, Arabidopsis and *Nicotiana benthamiana*, which belong to different taxa, rosids and asterids, respectively. The large difference in genomic information allows for a clear discrimination when classifying the transcript derivatives. The usefulness of this hetero-graft system was demonstrated by identifying several phloem-mobile mRNAs in a previous study (Notaguchi et al. 2012). In this report, we first ensured the suitability by testing the mobility of two other kinds of phloem-mobile transcripts. Then, by analyzing these hetero-graft samples using RNA sequencing (RNA-Seq), we established mobile mRNA-omics data.

Results

Identification of Arabidopsis mobile transcripts through the RNA-Seq analysis of hetero-grafts

A hetero-graft system, in which Arabidopsis was the donor stock and *N. benthamiana* was the recipient scion, was used to identify transcripts that moved across the graft union (**Fig. 1A**). In our preliminary test, symplasmic transport was established between the stock and the scion plants, and *N. benthamiana* scions did not show obvious phenotypic changes except for their growth delay (data not shown). In this system, if some Arabidopsis transcripts move to the *N. benthamiana* scion across the graft union, they can be identified as distinct transcripts from those of *N. benthamiana* based on their sequence differences. In fact, two groups of transcription factor transcripts, *GAI* and *Aux/IAA*, were identified as phloem-mobile transcripts in a previous study (Notaguchi et al. 2012). First, we confirmed the usefulness of this hetero-graft system by testing the mobility of two transcripts, *eukaryotic initiation factor 5A* (*eIF5A*) and *rubisco small subunit* (*RBCS*), which was previously suggested in several plant species (David-Schwartz et al. 2008, Deeken et al. 2008, Westwood et al. 2009, Ma et al. 2010). Reverse transcription–PCR (RT–PCR) analysis was conducted on the three *eIF5A* and four *RBCS* homologs in Arabidopsis using gene-specific primers. The prepared primers specifically amplified the targets from Arabidopsis, but not from intact *N. benthamiana* stem samples. In our test, one out of three *eIF5A* homologs, *eIF5A2*, and one of four *RBCS* homologs, *RBCS1A*, were detected from the *N. benthamiana* scion grafted onto Arabidopsis. We also performed RT–PCR using a second set of primers designed to detect those not previously amplified, except *eIF5A3*, which has a high homology that made the designing of a specific primer difficult; but no additional homologs were amplified. Thus, this hetero-graft system can be used to identify Arabidopsis mobile transcripts (**Fig. 1B**).

To obtain an exhaustive profile of mobile transcripts, we conducted transcriptomics using an RNA-Seq analysis on the same hetero-grafted samples. We sequenced cDNA libraries derived from the scions of hetero-grafted samples, as well as Arabidopsis rosette leaves as the positive control and intact *N. benthamiana* stems as the negative control, using an Illumina Genome Analyzer IIx (GAIIx) sequencer (Illumina, Inc.). As we had experienced tissue contamination, consisting mostly of pollen grains from offshoots, in our preliminary experiments, we took great care in the preparation of the samples. To avoid tissue contamination, the entire *N. benthamiana* scion was covered with a plastic bag until harvested (**Supplementary Fig. S1**). Sampling was done by two persons; one manipulated/touched the outside of the plastic bag and the other one manipulated/sampled the scion inside the plastic bag. Thus, we were able to avoid pollen contamination as confirmed by the pollen transcriptome data in our laboratory.

Sequencing using the Illumina GAIIx allowed us to extend the read lengths with high accuracy to approximately 120 nucleotides (nt) using three reagent kits, while the use of only one kit provided a maximum length of approximately 45 nt. As longer reads gave us more significant results, we used three reagent kits for sequencing and obtained 119 nt reads for the *N. benthamiana* scion samples. The total number of reads was 327 million, which is 10 times more than a normal transcriptomic analysis.

The reads were stringently mapped to Arabidopsis transcript references, and the reads having <3 nt mismatches were collected. All collected reads were manually checked for quality, and, if the reads were mapped to repeat sequences or detected single nucleotide polymorphisms (SNPs) usually

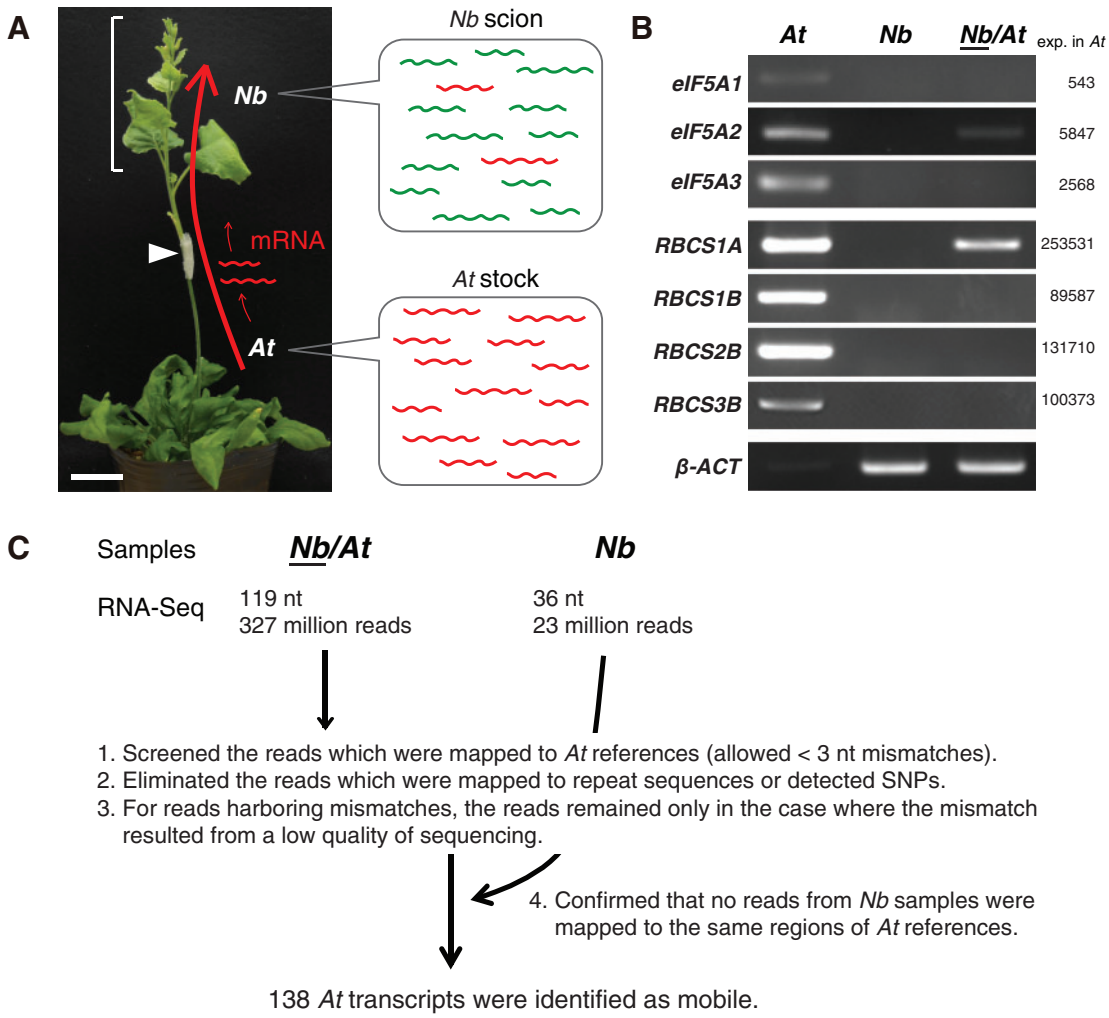


Fig. 1 *Nicotiana benthamiana*/Arabidopsis hetero-graft system for identification of transcripts that move over long distances. (A) A hetero-grafted plant of *N. benthamiana* (*Nb*)/Arabidopsis (*At*) at 3 weeks after grafting. If *At* transcripts (red) moved to the *Nb* scion across the graft union (arrowhead), the transcripts can be distinguished from the *Nb* transcripts (green) based on the differences in genomic information between these two plants. The scion stems were sampled by cutting at 3 cm above the graft union (parenthesis) for further analyses. The scale bar indicates 3 cm. (B) Identification of *At* mobile transcripts using RT–PCR analysis of the hetero-graft system. RNA was extracted from *At* rosette leaves, intact *Nb* stems and the *Nb* scion stems grafted onto *At* bolting stems (*Nb*/*At*, the underlined *Nb* shows the sample for RNA extraction). The accumulation of gene transcripts from two families, *eukaryotic initiation factor 5A* (*eIF5A*) and *rubisco small subunit* (*RBCS*), was analyzed. The target specificity of designed primers was confirmed by the presence of amplified bands in the *At* samples and the absence of corresponding bands in the *Nb* samples. Note that *eIF5A2* and *RBCS1A* transcripts were detected from the *Nb*/*At* sample. *β-ACT* was amplified as a reference. The expression in the *At* rosette leaves (exp. in *At*) represented by a number of reads in RNA-Seq analysis is indicated on the right. (C) RNA-Seq analysis workflow to identify *At* mobile transcripts. cDNA libraries were constructed from RNAs extracted from *Nb*/*At* and *At*. As a result, 138 *At* transcripts were identified as mobile.

found in conserved regions between species, then the reads were eliminated as candidates. For reads harboring mismatches, the reads remained only in the case where mismatches resulted from a low quality of sequencing. We also obtained 23 million reads of 36 nt length from intact *N. benthamiana* stem samples and mapped them to Arabidopsis transcript references. Although the shorter reads have a greater chance of being cross-mapped to the Arabidopsis references, no reads from intact *N. benthamiana* stems were mapped to the same regions as those detected from the *N. benthamiana* scion samples. This confirms the significance of our data. As a result, 138 Arabidopsis transcripts were identified from the *N. benthamiana* scions grafted onto Arabidopsis stocks as candidates for mobile transcripts (**Fig. 1C**).

Independent confirmation of mobility by RT–PCR, qPCR and ddPCR

To investigate further the mobility of the identified transcripts, RT–PCR analysis was conducted on 70 transcripts, around half of those identified. Designed primer sets for 63 out of 70 transcripts specifically amplified Arabidopsis targets from Arabidopsis rosette leaf samples but not from intact *N. benthamiana* stems (**Supplementary Fig. S2**). As a result,

15 transcripts were detected from the grafted *N. benthamiana* scions in the first trial. Because primers are an important factor for efficient PCRs, we designed additional primer sets for the two transcripts that had not been detected. Then, these two transcripts were successfully detected (**Supplementary Fig. S2**). Thus, the chance of detection increases under the appropriate PCR conditions. The amplification of the proper Arabidopsis targets was confirmed by subsequent cloning of PCR products into a plasmid vector and sequencing. The mobility of 17 transcripts was indicated by RT–PCR analysis (**Fig. 2**).

We next applied two other methods, quantitative real-time PCR (qPCR) and droplet digital PCR (ddPCR), to confirm the mobility of the transcripts. ddPCR offers highly precise absolute quantification without requiring a calibration curve and is used for gene expression analysis for rare transcripts, rare sequence detection, etc. We tested for three genes as representatives, *eIF5A2*, *RBCS1A* and a leucine-rich repeat (LRR) receptor-like serine/threonine kinase gene, *ERECTA*. *eIF5A2* and *RBCS1A* were successfully detected by RT–PCR analyses, but *ERECTA* was not. For the qPCR analysis, because the amount of mobile transcripts is expected to be low, standard curves were drawn using a dilution series of DNA fragments to a calculated limit of one DNA molecule (0.1 attogram). cDNA derived from the *N. benthamiana* scions, as well as Arabidopsis rosette leaves and intact *N. benthamiana* stems, were used for the qPCR analyses. A significant signal was detected from the *N. benthamiana* scion samples for *RBCS1A* but not for *eIF5A2* or *ERECTA* (**Fig. 3A**). For the ddPCR analysis, using the QX100 system (Bio-Rad Laboratories), we performed experiments with maximum *N. benthamiana* scion sample inputs, as well as Arabidopsis rosette leaf and intact *N. benthamiana* stem samples as the positive and negative controls, respectively. Each sample of cDNA was partitioned into multiples of 20,000 droplets, with target and background DNA randomly distributed among the droplets. PCRs were executed in each droplet until saturation level was reached, followed by detection of an independent signal from each. For *eIF5A2* detection, the number of droplets providing positive signals was 23,008, four and 0 for samples of Arabidopsis rosette leaves, the *N. benthamiana* scion stems and intact *N. benthamiana* stems, respectively (see the Materials and Methods for the starting amounts of each cDNA sample and the technical replicates). Similarly, positive counts for *RBCS1A* and *ERECTA* were also detected from the samples of Arabidopsis rosette leaves and the *N. benthamiana* scions, but never from intact *N. benthamiana* stem samples. Thus, gene-specific signals were successfully detected for all three tested genes (**Fig. 3B, C**). These experiments confirm the significance of our experimental workflow in its ability to identify mobile transcripts.

Quantitative information can be obtained by qPCR and ddPCR. In the qPCR analysis of *RBCS1A*, a quantitative estimation was achieved to a minimum of 0.0001 pg of DNA template (the correlation coefficient, $R^2 > 0.99$, of the standard curve occurs for standards higher than the 0.00001 pg DNA template). Because the target amount in the grafted scions was below this level, we could only estimate that the transcript levels were $<10^{-6}$ compared with those in the source leaves (**Fig. 3A**). The difficulty of estimating the amount of transcripts also occurred in the ddPCR analysis. More than 10 positive counts per 1 µl reaction mixture were required for an accurate quantification, but tested transcripts in the grafted scions occurred at a lower concentration. Based on tentative calculations of the data presented, the transcript levels in the grafted scions were approximately 10^{-5}–10^{-6} compared with those in source leaves (**Fig. 3D**). Thus, an accurate quantification of the mobile transcripts was not possible, but we estimated the level

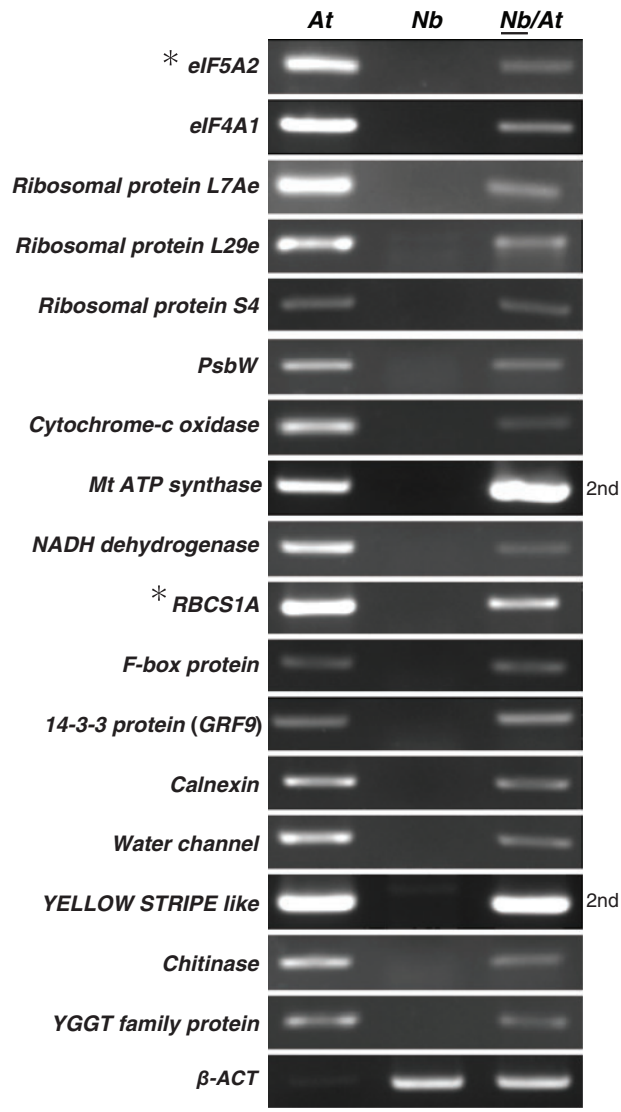
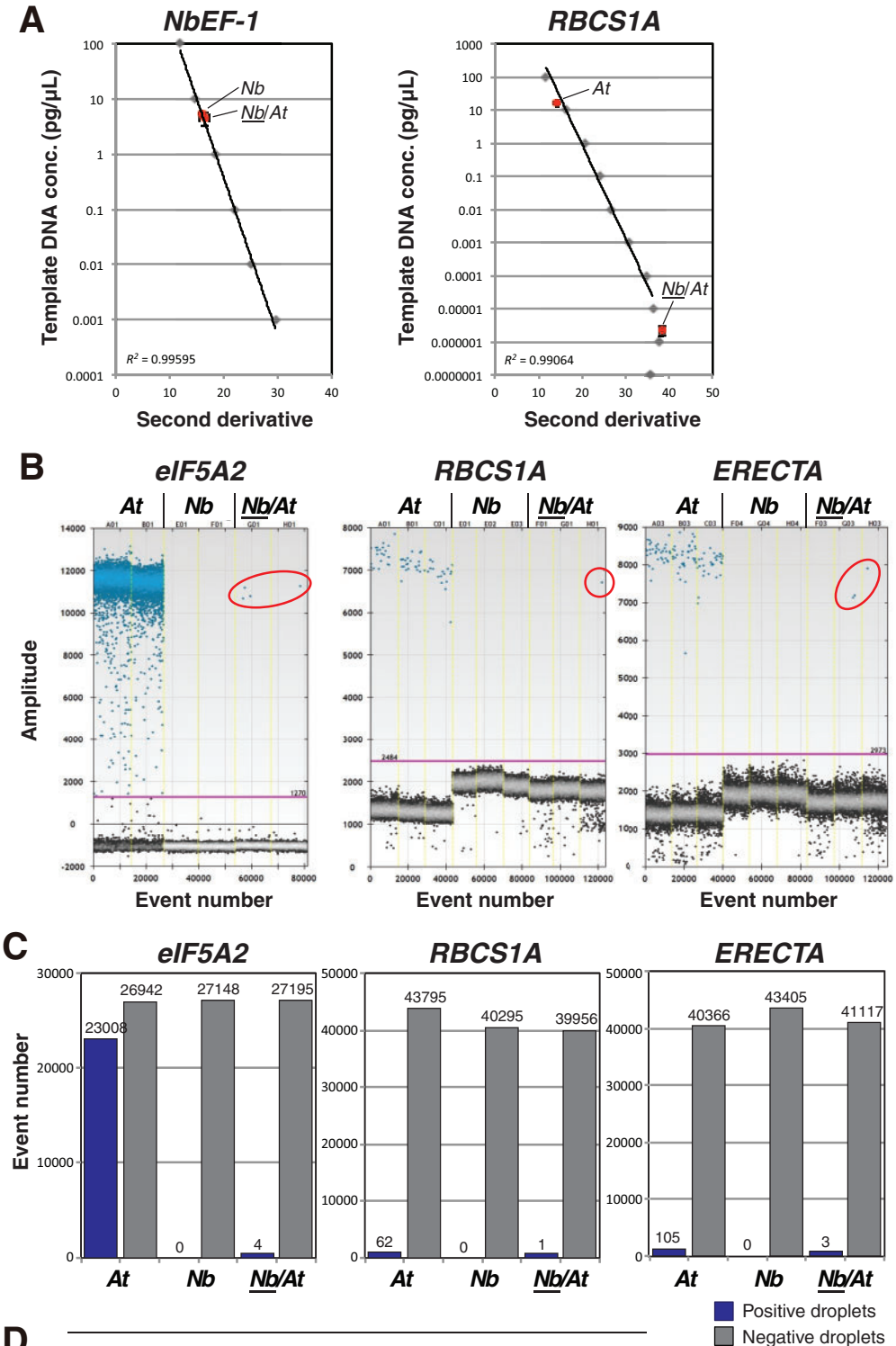


Fig. 2 Confirmation of the identification of mobile Arabidopsis transcripts from a *Nicotiana benthamiana*/Arabidopsis hetero-graft system using RT–PCR. RT–PCR analyses of represented transcripts were conducted on the Arabidopsis rosette leaf (*At*), intact *N. benthamiana* stem (*Nb*) and the grafted *N. benthamiana* scion stem (*Nb*/*At*, the underlined *Nb* shows the sample for RNA extraction) samples. Note that *eIF5A2* and *RBCS1A* transcripts were reproducibly identified as mobile transcripts (asterisks). All transcripts were detected from the grafted scion sample after the first round of PCR. The second round of PCR was performed for some bands to be clearly shown (marked on the right). *β-ACT* was amplified as a reference.



D

Copies / cDNA derived from 10 ng total RNA			
	At	*Nb/At*	Ratio of *Nb/At* to *At*
eIF5A2	0.130	21100	6.14×10^{-6}
RBCS1A	0.010	312	3.13×10^{-5}
ERECTA	0.029	572	4.98×10^{-5}

Fig. 3 Confirmation of the identification of mobile Arabidopsis transcripts from a *Nicotiana benthamiana*/Arabidopsis hetero-graft system using qPCR and ddPCR. (A) qPCR analysis of *Nicotiana benthamiana elongation factor-1α* (*NbEF-1α*) as an internal reference was conducted on
(continued)

of the transcripts in the grafted *N. benthamiana* scion samples as $\leq 10^{-6}$ compared with the Arabidopsis source leaf samples.

Profiles of the 138 identified transcripts

All identified transcripts are listed in **Supplementary Table S1**, which includes potential factors related to signaling. Our list includes several transcription factor transcripts, including a BEL1-like homeodomain (BEL10), which has a potato homolog, *StBEL5*, that is mobile in its mRNA form (Banerjee et al. 2006), and Aux/IAA (IAA9) transcripts, which have homologs that were transported across graft unions in melon and Arabidopsis (Omid et al. 2007, Notaguchi et al. 2012). In addition, the mobility of a homeobox protein, ATHB34, an ABA-responsive element (ABRE) binding factor, ABRE4, and an AUXIN RESPONSE FACTOR, ARF7, was revealed. Other proteins related to cellular processes and regulation were also discovered to be mobile. For examples, two 14-3-3 protein, five receptor kinase, four G-protein and four WD-repeat protein transcripts, as well as cell modification enzyme, intrinsic protein, calcium and stress/defense-related transcripts were identified in the present experiments (**Table 1**). It is noteworthy that there still remains controversy over involvement of *FT* mRNA as a mobile signal (e.g. McGarry and Kragler 2013), although Mathieu et al. (2007) and Notaguchi et al. (2008) have disproved the mRNA hypothesis by using artificial microRNA against *FT* mRNA and a synthetic *FT* gene with extensive nucleotide substitutions throughout the open reading frame (ORF) and foreign untranslated regions (UTRs), respectively. In our test, the mobility of *FT* mRNA was not found.

In addition, there were transcripts identified that were not related to signaling. For example, ribosomal protein transcripts are general factors. Our list also includes fundamental transcripts for cell homeostasis, such as the synthesis and metabolism of proteins and peptides. More strikingly, several organelle-localized protein transcripts were identified. As almost all cells have mitochondria and plastids; these components must be ubiquitously expressed. Thus, our list shows that the pool of mobile transcripts includes those for many general factors (**Table 2**).

To investigate whether identified transcripts share some characteristics in their mRNA length, primary sequence motif(s) and expression levels in source leaves, we analyzed each characteristic by comparing entire gene sets from the Arabidopsis genome. In our test, the distribution of mRNA lengths was similar (**Supplementary Fig S3**). We tested for enriched sequences in the transcripts using the MEME Suite analysis tools (http://meme.nbcr.net/meme/), but no obvious structural motifs were found. The expression levels in source leaves were variable (**Supplementary Table S2**). For the transport of transcripts via phloem, expression in companion cells is a possible requirement. However, in our motif search of the promoter region (−2,000 to −1 upstream region from the ATG of each gene) using the MEME tools, we did not find previously identified motifs, including a reported phloem *cis*-element (CT–GA-rich repeats; Ruiz-Medrano et al. 2011). To date, no obvious characteristic has been found to identify mobile transcripts.

We performed a data comparison between our data and the transcriptome data of Arabidopsis phloem exudates presented by Deeken et al. (2008), where 2,417 phloem exudate transcripts were identified. As expected, the data overlapped, with 56 out of 138 transcripts being previously identified (**Supplementary Table S3**). Thus, we successfully showed the mobility of transcripts detected from phloem exudates over long distances.

Distinction of local gene actions from transcript transport

Because many transcripts we identified are highly expressed in a broad range of tissues, including young sink tissues, we hypothesized that the low levels of transported transcripts may not explain all of the gene functions. To test this, we focused on the *ERECTA* gene, which is expressed in many tissues and has multiple biological roles, such as inflorescence structure, shoot length and shoot apical meristem maintenance (Torii et al. 1996, Uchida et al. 2013). We performed stem-grafting experiments using a knockout mutant of *ERECTA*, *erecta-105* (Torii et al. 1996), which has a shortened inflorescence phenotype. The mutant inflorescence stems were grafted onto the wild-type stocks, and, as controls, the mutant and the wild-type self-grafts were also performed. As a result, the *erecta* mutant phenotypes in lengths of pedicels, fruits and internodes between fruits were not rescued by grafting onto the wild-type stock (**Supplementary Fig. S4**), suggesting that these *ERECTA* gene actions are dependent on its onsite gene expression rather than its transcript mobility.

Discussion

We presented a profile of Arabidopsis mobile transcripts, an mRNA mobilome, by applying an RNA-Seq analysis to a

Fig. 3 Continued

intact *N. benthamiana* stem and the grafted *N. benthamiana* scion stem samples. Note that the expression levels of *NbEF-1α* in these two samples were comparable. qPCR analysis for Arabidopsis *RBCS1A* transcripts was conducted on Arabidopsis rosette leaf (*At*), intact *N. benthamiana* stem (*Nb*) and the grafted *N. benthamiana* scion stem (*Nb*/*At*, the underlined *Nb* shows the sample for RNA extraction) samples. The target specificity was confirmed by the absence of signal in intact *N. benthamiana* stem samples. The *RBCS1A* transcripts were detected in the grafted *N. benthamiana* scions, but outside of the range for an accurate standard curve ($R^2 > 0.99$). Bars represent the standard deviations of three biological replicates. (B–D) ddPCR analyses of three Arabidopsis transcripts, *eIF5A2*, *RBCS1A* and *ERECTA*, were conducted on the same sample set as above. (B) The signal amplitude for each drop was measured and drops over the threshold (pink lines) were positive (blue). A few positive drops were detected from the grafted scion samples (encircled in red). (C) Summary of the number of positive and negative drops. (D) Summary of the data quantification.

Table 1 Potential regulatory factors identified as mobile Arabidopsis transcripts from a *Nicotiana benthamiana*/Arabidopsis hetero-graft system

	Gene	Annotation
Transcription	AT1G12860	bHLH DNA-binding superfamily protein (SCRM2)
	AT1G19700	BEL1-like homeodomain 10 (BEL10)
	AT3G17590	Transcription regulatory protein SNF5, putative (BSH)
	AT3G19290	ABRE-binding factor 4 (ABRE4)
	AT3G28920	Homeobox protein 34 (ATHB34)
	AT5G20730	Transcriptional factor B3 family protein (ARF7)
	AT5G65670	Indole-3-acetic acid-inducible 9 (IAA9)
	AT5G48385	FRIGIDA-like protein
	AT5G12400	DNA binding; zinc ion binding
	AT1G18080	Transducin/WD40 repeat-like superfamily protein
	AT4G11270	Transducin/WD40 repeat-like superfamily protein
	AT5G54200	Transducin/WD40 repeat-like superfamily protein
	AT4G33260	Transducin family protein/WD-40 repeat family protein
	AT2G42590	14-3-3 protein, GF14 mu, GRF9
	AT1G35160	14-3-3 protein, GF14 phi, GRF4
Receptor kinase	AT2G26330	Leucine-rich receptor-like protein kinase family protein (ERECTA)
	AT5G01890	Leucine-rich receptor-like protein kinase family protein
	AT1G29720	Leucine-rich repeat transmembrane protein kinase
	AT1G31420	Leucine-rich repeat protein kinase family protein
	AT3G51550	Malectin/receptor-like protein kinase family protein (FERONIA)
G protein	AT5G57960	GTP-binding protein, HflX (ARFA1F)
	AT1G07940	GTP-binding elongation factor Tu family protein (ARFA1D)
	AT1G10630	ADP-ribosylation factor A1F
	AT1G70490	Ras-related small GTP-binding family protein
Cell modification	AT2G36850	Glucan synthase-like 8
	AT5G09870	Cellulose synthase 5
	AT5G59310	Lipid transfer protein 4
	AT5G11740	Arabinogalactan protein 15
	AT3G57690	Arabinogalactan protein 23
	AT4G12730	FASCICLIN-like arabinogalactan 2
	AT2G05920	Subtilase family protein
	AT4G30020	PA-domain-containing subtilase family protein
Intrinsic protein	AT4G00430	Plasma membrane intrinsic protein 1;4
	AT4G35100	Plasma membrane intrinsic protein 3
	AT2G36830	Gamma tonoplast intrinsic protein
Calcium	AT5G61790	Calnexin 1
	AT1G56340	Calreticulin 1a
	AT5G55530	Calcium-dependent lipid-binding family protein
Stress/defense	AT1G01800	NAD(P)-binding Rossmann-fold superfamily protein
	AT1G17890	NAD(P)-binding Rossmann-fold superfamily protein
	AT2G02050	NADH-ubiquinone oxidoreductase B18 subunit, putative
	AT1G15690	Inorganic H pyrophosphatase family protein
	AT1G66880	Protein kinase superfamily protein
	AT3G18490	Eukaryotic aspartyl protease family protein
	AT4G03960	Phosphotyrosine protein phosphatases superfamily protein
	AT4G13940	*S*-Adenosyl-L-homocysteine hydrolase (SAHH1)
	AT5G24780	Vegetative storage protein 1
	AT1G05850	Chitinase family protein

hetero-graft system where Arabidopsis was used as the donor stock plant. Our data include many types of protein transcripts, including transcription-related, stress-induced, enzymatic and metabolic, as well as organelle-associated molecules, and we expediently categorized potential regulatory and general factors. We did not identify any common features in the mobile transcripts, but we determined that not all of their gene actions rely on the transcript's mobility.

The significance of the RNA-Seq data analysis was confirmed by RT–PCR, qPCR and ddPCR analyses (**Figs. 2**, **3**). That we truly identified mRNA is supported by the following: (i) cDNA was synthesized using oligo(dT) primers in all experiments; (ii) some RNA-Seq reads were positioned over two exons without potential intron gaps; and (iii) many of the primer sets bridged intron(s) to distinguish genomic DNA and cDNA (**Supplementary Table S4**). The present RNA mobilome data set may not include all mobile transcripts. The data include

Table 2 General factors identified as mobile Arabidopsis transcripts from a *Nicotiana benthamiana*/Arabidopsis heterograft system

	Gene	Annotation
Ribosomal protein	AT3G04840	Ribosomal protein S3Ae
	AT5G15200	Ribosomal protein S4
	AT5G18380	Ribosomal protein S5 domain 2-like superfamily protein
	AT4G00100	Ribosomal protein S13A
	AT1G77940	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
	AT2G47610	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
	AT2G40510	Ribosomal protein S26e family protein
	AT2G39390	Ribosomal L29 family protein
	AT3G06700	Ribosomal L29e protein family
	AT5G02450	Ribosomal protein L36e family protein
Protein synthesis/metabolism	AT1G26630	Eukaryotic translation initiation factor 5A-1
	AT3G13920	Eukaryotic translation initiation factor 4A1
	AT1G48400	F-box/RNI-like/FBD-like domains-containing protein
	AT3G51530	F-box/RNI-like/FBD-like domains-containing protein
	AT3G08690	Ubiquitin-conjugating enzyme 11
	AT1G64230	Ubiquitin-conjugating enzyme 28
Peptidase	AT4G33410	SIGNAL PEPTIDE PEPTIDASE-LIKE 1
	AT3G10410	SERINE CARBOXYPEPTIDASE-LIKE 49
	AT1G44820	Peptidase M20/M25/M40 family protein
	AT3G05350	Metallopeptidase M24 family protein
Chloroplast	AT4G12800	Photosystem I subunit l
	ATCG00340	Photosystem I PsaA/PsaB protein
	ATCG00020	Photosystem II reaction center protein A
	AT2G30570	Photosystem II reaction center W
	AT1G67090	Ribulose bisphosphate carboxylase small chain 1A
	ATCG00490	Ribulose bisphosphate carboxylases
	ATCG00480	ATP synthase subunit beta
	AT1G16410	Cytochrome p450 79f1
	AT3G07430	YGGT family protein
	AT4G25080	Magnesium-protoporphyrin IX methyltransferase
Mitochondria	AT4G29480	Mitochondrial ATP synthase subunit G protein
	AT5G13450	Delta subunit of Mt ATP synthase
	AT4G21105	Cytochrome-*c* oxidases; electron carriers
	AT5G13430	Ubiquinol-cytochrome C reductase iron–sulfur subunit
	AT3G07480	2Fe–2S ferredoxin-like superfamily protein
	AT5G63510	Gamma carbonic anhydrase like 1
	AT3G29290	Pentatricopeptide repeat superfamily protein
	AT4G11010	Nucleoside diphosphate kinase 3

eIF5A2 and *RBCS1A*, which had been previously identified as mobile (**Fig. 1B**), and an *Aux/IAA*, which was different from those identified in a previous study (Notaguchi et al. 2012), but not *GAI* (Haywood et al. 2005, Notaguchi et al. 2012), which is known to be mobile. Unexpectedly, our RT–PCR analysis of the same samples again failed to detect *GAI* and the two *Aux/IAA* transcripts (data not shown) that had been routinely detected previously (Notaguchi et al. 2012). Thus, we assumed that the current analytical depth was not sufficient to identify the entire set of mobile transcripts. Additionally, the population of mobile transcripts may change depending on the growth conditions and experimental circumstances. The procedure using hetero-grafs between different plant taxa could reflect the result.

We identified the transcripts of shootward movement at the bolting stage. To gain insights on the rootward mobile transcripts at the seedling stage, which may be different from those identified this time, we also tested the micrografting between an Arabidopsis shoot scion and an *N. benthamiana* rootstock by following a procedure for Arabidopsis micrografting (Notaguchi et al. 2009). However, it has not been successful (Notaguchi et al. 2012). We instead performed RNA-Seq analysis on the micrografts between Arabidopsis and other *Brassicaceae* plant species, *Capsella rubella* or *Cardamine hirsuta*. However, when we discriminated the raw sequenced reads, we found false-positive results, possibly because of underdeveloped genome information (data not shown). Thus, we do not at present have accurate information for the transcripts of rootward movement. It has long been hypothesized that phloem transport proceeds by turgor-driven mass flow from the source mature leaves to the sink tissues, such as the shoot apical region and the root. In fact, both shootward and rootward transport of several phloem-mobile transcripts was observed (Notaguchi et al. 2012). Therefore, we assume that the cargos in the phloem translocation stream possibly move along with the phloem mass flow.

If transported transcripts exert their biological functions in their destination tissues, the amount targeted to these tissues could be a fundamental factor influencing accumulation. RNA-Seq, qPCR and ddPCR methods, which generally allow for the quantification of target transcript levels, did not provide sufficiently quantifiable results in the present study because of the small number of reads mapped to each reference, the results being outside the accurate standard curve range and the low numbers of positive droplets, respectively (**Supplementary Table S1**; **Fig. 3**). In our tentative estimations, based on the data of qPCR and ddPCR, the amounts of transcripts transported into the grafted scions was around $\leq 10^{-6}$ compared with the source leaves (**Fig. 3**). Because the transcripts of many genes that are expressed only in a small number of cells are detected in low quantities, the mobile transcripts could target only specific regions. Additionally, early turnover might occur at their destinations to maintain the responsiveness to dynamically changing cues in the sink tissues.

Thus, increasing the analytical depth is the next challenge. The RNA-Seq and ddPCR techniques performed better in detecting rare targets than the RT–PCR and qPCR methods, owing to the unlimited depth of sequencing and droplet PCRs, respectively. We sequenced several 100 million reads for the grafted scion samples, which reached a saturation point as indicated by the repeated detection of identical sequence reads for some transcripts (**Supplementary Table S1**), indicating that new library construction and further sequencing are required to gain information. Together with the expensive running costs for ddPCR, further analyses will be costly. If the mobile transcripts target only specific cells/tissues, using all of the tissues diluted the target, resulting in the low-level detection of mobile transcripts. Thus, improving sample preparation could be helpful in performing a detailed investigation of the mobile transcripts. The combination of fluorescence reporter marker lines for phloem tube cells (Thompson and Wolniak 2008, Ernst et al. 2012) and a cell-sorting technique could enrich the cells harboring targeted mobile transcripts.

Although we did not find common structural motifs in the primary sequence of the identified transcripts, they may share some feature, such as the animal mRNA guidance system, that is targeted by the transport machinery (Martin and Ephrussi 2009). If we enrich the mobile transcripts, Structural-Seq, a recently developed technique to investigate RNA secondary structure (Ding et al. 2014), may be worth applying. An interesting hypothesis is that only certain members of a gene family may have the ability to move in mRNA form even though they are expressed in similar quantities (**Fig. 1B**) (Huang et al. 2009, Notaguchi et al. 2012), implying that there is an underlying mechanism to transport typical populations of transcripts preferentially. However, our list includes ubiquitously expressed gene transcripts, such as a rubisco protein and eukaryotic transcription factor transcripts, which are expressed in almost all green tissues at high levels. Thus, we theorize that the selection may occur both on purpose and incidentally. Our stem grafting results on *ERECTA* (**Supplementary Fig. S4**) suggest that not all gene actions are explained by transcript transport. Additionally, the analysis on phloem exudates identified both target-specific (Ham et al. 2009) and non-target-specific RNA-binding proteins (Xoconostle-Cázares et al. 1999, Ma et al. 2010), leading us to hypothesize that mRNA long-distance transport could occur in both a selective and a non-selective manner (Notaguchi 2015).

We developed a procedure to collect mobile transcriptomics data, which could be applied to screen systemic signals generated under specific environmental or internal conditions by performing the same analysis on the grafted plants under particular environmental conditions or at different developmental stages. This would increase the understanding of the mechanisms behind long-distance signaling in plants and on how plants adapt their growth and development to the surrounding environment.

Materials and Methods

Grafting

Nicotiana benthamiana/Arabidopsis stem grafting procedures were described in a previous report (Notaguchi et al. 2012). In brief, wedge grafts were performed by inserting an *N. benthamiana* stem scion into a slit made into an Arabidopsis bolting stem. To avoid tissue contamination from the Arabidopsis stock to the *N. benthamiana* scions, entire scions were covered with plastic bags until the sampling day. Sampling was performed 3 weeks after grafting. To form a strong sink, *N. benthamiana* scions were defoliated 3 d before sampling. After defoliation, each scion was covered with a new plastic bag (**Supplementary Fig. S1**). To reduce humidity inside the plastic bags, the upper part of the bags was cut using scissors after the first week of grafting. This period allowed for tissue attachment between the scion and the stock plants. Sampling of the scions was performed by two persons, taking great care not to contaminate the samples. A stem section >3 cm above the graft union was harvested using clean forceps. Expanded flowers and maturated fruits were discarded, and stems harboring only small flower buds on the apical region were harvested.

Arabidopsis stem grafting (**Supplementary Fig. S4**) procedures were described in a previous report (Tsukaya et al. 1993). The Arabidopsis ecotype Columbia (Col) was used as the wild type. The *erecta-105* mutants in the Col background (Torii et al. 1996) were used in the experiments. The lengths of enlarged fruits, pedicels and internodes produced on newly grown sections of the scion stems were measured 3 weeks after grafting.

RNA-Seq analysis

Total RNA was extracted from the grafted *N. benthamiana* scion stems and similar aged intact *N. benthamiana* stems, using TRIzol reagent (Life Technologies), and then treated with RNase-free DNase I (Life Technologies), according to the manufacturer's instructions. A TruSeq RNA Sample Preparation kit (Illumina) was used to construct cDNA libraries according to the manufacturer's instructions. The single ends of cDNA libraries were sequenced for 119 and 36 nt from grafted *N. benthamiana* scion and intact *N. benthamiana* stem samples, respectively, using the Illumina Genome Analyzer IIx. The resulting sequence data were deposited in the DDBJ Sequence Read Archive (DRA) at the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) under the accession number DRA002825. The reads were mapped to the Arabidopsis reference (TAIR10) using Bowtie (Langmead et al. 2009) with the following options '-v 3 -m 1 -all -best -strata', and the number of reads mapped to each reference was counted. See text and **Fig. 1C** for further mobile transcript screening processes.

RT–PCR

Total RNA was extracted from Arabidopsis rosette leaves, intact *N. benthamiana* stems and grafted *N. benthamiana* scion stems, as described above. cDNA was synthesized in a 20 µl reverse transcription reaction mixture using Superscript III (Life Technologies). For intact *N. benthamiana* and grafted *N.*

benthamiana scions, 5 μg of total RNA was used as a template. For Arabidopsis rosette leaf samples, 0.5 μg of total RNA was used as a template. After reverse transcription, the mixture of Arabidopsis rosette leaf samples was diluted with 30 μl of distilled water, and 1 μl aliquots were used for 40 cycles of PCRs with an annealing temperature of 55–60°C. The mixture of intact *N. benthamiana* and grafted *N. benthamiana* scions was directly used for PCR assays without dilution. For the second PCRs, 1 μl aliquots of the first PCR products were used for another 30 PCR cycles. Primers for **Fig. 1B** are provided in **Supplementary Table S4**. Primers for **Fig 2** and **Supplementary Fig. S2** are provided in **Supplementary Table S5**. PCR products were resolved by electrophoresis on agarose gels and visualized by ethidium bromide staining. For the grafted samples tested in **Figs. 1B** and **2**, and **Supplementary Fig. S2**, the amplification of target sequences was confirmed by the cloning of the PCR products into a blunt TOPO vector (Life Technologies) followed by a subsequent sequencing analysis.

qPCR

The cDNA templates prepared for RT–PCR from Arabidopsis rosette leaf, intact *N. benthamiana* and grafted *N. benthamiana* scion samples were used in 50 cycles of qPCR with a 60°C annealing temperature. The target transcripts were determined by PCR using SYBR Premix Ex Taq (TAKARA) and gene-specific primers (**Supplementary Table S6**). Purified DNA fragments were serially diluted from 100 to 10^{-7} pg μl^{-1} for standard curve preparations. Data were collected using the StepOne Real-Time PCR System (Life Technologies) in accordance with the manufacturer's protocol. The *N. benthamiana elongation factor-1α* (*EF-1α*) gene was used as an internal control for intact *N. benthamiana* and grafted *N. benthamiana* scion samples (Yang et al. 2004).

ddPCR

The cDNA templates prepared for RT–PCR from Arabidopsis rosette leaf, intact *N. benthamiana* and grafted *N. benthamiana* scion samples were used in ddPCR assays performed with a QX100 droplet digital PCR system (Bio-Rad Laboratories). The ddPCR mixture for *eIF5A2* contained 2 μl of cDNA templates in a final volume of 20 μl. The ddPCR mixture for *RBCS1A* and *ERECT* contained 1 μl of 10× diluted Arabidopsis rosette leaf cDNA sample, or 4.5 μl of of intact *N. benthamiana* or grafted *N. benthamiana* scion cDNA sample, as the template. TaqMan assay primers and probes were present at final concentrations of 900 and 250 nM, respectively. The PCR mixture was processed by Droplet Generator, and PCR amplification was carried out. The PCRs were run under the following standard cycling conditions: 95°C for 10 min, 40 cycles of 94°C for 30 s; 55°C for 60 s, 98°C for 10 min, followed by a 4°C hold. Each cDNA sample had two technical replicates for *eIF5A2* and three for *RBCS1A* and *ERECTA*. After amplification, the fluorescence signals were detected by Droplet Reader. The quantification of the target molecules was presented originally as the number of copies per microliter of PCR mixture, which was recalculated to the number of copies per cDNA, which was derived from 10 ng of total RNA. Primers and probes are provided in **Supplementary Table S7**.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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