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Rhizobial infection triggers systemic transport of endogenous RNAs between shoots and roots in soybean

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Legumes have evolved a symbiotic relationship with rhizobial bacteria and their roots form unique nitrogen-fixing organs called nodules. Studies have shown that abiotic and biotic stresses alter the profile of gene expression and transcript mobility in plants. However, little is known about the systemic transport of RNA between roots and shoots in response to rhizobial infection on a genome-wide scale during the formation of legume-rhizobia symbiosis. In our study, we found that two soybean (*Glycine max*) cultivars, Peking and Williams, show a high frequency of single nucleotide polymorphisms; this allowed us to characterize the origin and mobility of transcripts in hetero-grafts of these two cultivars. We identified 4,552 genes that produce mobile RNAs in soybean, and found that rhizobial infection triggers mass transport of mRNAs between shoots and roots at the early stage of nodulation. The majority of these mRNAs are of relatively low abundance and their transport occurs in a selective manner in soybean plants. Notably, the mRNAs that moved from shoots to roots at the early stage of nodulation were enriched in many nodule-related responsive processes. Moreover, the transcripts of many known symbiosis-related genes that are induced by rhizobial infection can move between shoots and roots. Our findings provide a deeper understanding of endogenous RNA transport in legume-rhizobia symbiotic processes.

symbiosis, nodulation, systemic transport of RNAs, soybean, rhizobia

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INTRODUCTION

Legume plants form unique nitrogen-fixing root nodules containing symbiotic bacteria called rhizobia; these symbionts fix N_2 into organic nitrogen (Oldroyd, 2013; Oldroyd and Downie, 2004; Remigi et al., 2016). This cross-kingdom symbiotic relationship is maintained by extensive signal and material exchange between the two organisms and within the host plants (Geurts and Bisseling, 2002; Oldroyd and Downie, 2004), transferring carbohydrates, ammonium, phytohormones, small peptides, proteins, and RNAs, etc. This long-distance communication network operates within the vascular system of higher plants (Ham and Lucas, 2017). The plant vascular system, comprising xylem and phloem, functions as a pipeline for the delivery of long-distance signaling essential for the physiological function and development of distant tissues or organs (Lucas et al., 2013). Within plants, mRNAs can move to and function in distant

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tissues (Haywood et al., 2005; Kehr and Kragler, 2018); however, information about long-distance mRNA transport on a genome-wide scale is limited in the legume-rhizobia symbiotic process.

Reciprocal grafting experiment between the shoot and the root is frequently applied to explore the movement of systemic transcripts in plants (Thieme et al., 2015; Yang et al., 2015; Zhang Z., et al., 2016). Extensive mRNA exchange was found in two different *Arabidopsis thaliana* ecotypes displaying a high frequency of single nucleotide polymorphisms (SNPs) (Thieme et al., 2015) and mRNA exchange was also observed between scions and rootstocks in grafted grapevines (*Vitis vinifera*), cucumber (*Cucumis sativus*), and watermelon (*Citrullus lanatus*) (Yang et al., 2015; Zhang Z., et al., 2016). Compared with transport of proteins, long-distance RNA transport is more economical and requires less energy (Kehr and Buhtz, 2008; Lucas et al., 2001). For example, it is more efficient to transport one mRNA molecule to be translated numerous times in the target tissue than to traffic hundreds or thousands of copies of a protein. However, the extent of long-distance transport of RNA in the legume-rhizobia symbiosis process is largely unknown. Here, we report that rhizobial infection triggers the transport of a large amount of mRNAs between shoots and roots at the early stage of nodulation in soybean (*Glycine max*), and that different transcripts are transported from shoot to root versus root to shoot. Most importantly, we found that the transcripts of many known symbiosis-related genes that are induced by rhizobial infection can move between shoots and roots.

RESULTS

Grafting of two soybean cultivars reveals a large set of mobile mRNAs

To explore the systemic RNA exchange between the shoot and the root during the legume-rhizobia symbiotic process, we designed a grafting experiment using two distantly related soybean cultivars, Peking and Williams (Figure 1A–C). The difference of single nucleotide variations (SNVs) in RNA molecules between the two cultivars enabled us to characterize their origin and mobility in hetero-grafted plants. First, we performed whole-genome resequencing of these two cultivars. Using the sequenced genome of Williams 82 as a reference, we detected a large number of SNVs in the two cultivars, with only a small proportion of SNVs shared by both cultivars. Peking has 2,316,620 SNPs (2,181,588 unique SNPs) and Williams has 152,454 SNPs (42,962 unique SNPs) (Figure 1D). The different genomic SNPs localized to 25,738 genes in soybean, which account for nearly half of the total genes in soybean (Data S1 in Supporting Information). Reciprocal grafting between these two cultivars was performed at the hypocotyl 7 days after germination. After the joint wound was healed, roots of the grafts were inoculated with *Bradyrhizobium diazoefficiens* USDA110 (Figure 1). Root and shoot materials were harvested from the inoculated grafted plants (3, 7 days post inoculation) and the un-inoculated control. To eliminate individual differences, every sample was a mixture of eight grafted plants growing in the same environment and condition. Then the samples were subjected to next-generation sequencing (NGS). About 80 to 143 million reads were produced for each individual RNA-Seq library (Table S1 in Supporting Information). The obtained RNA-seq data and the SNVs identified based on the genome resequencing data were used to identify heterologous transcripts transported through the grafted tissues.

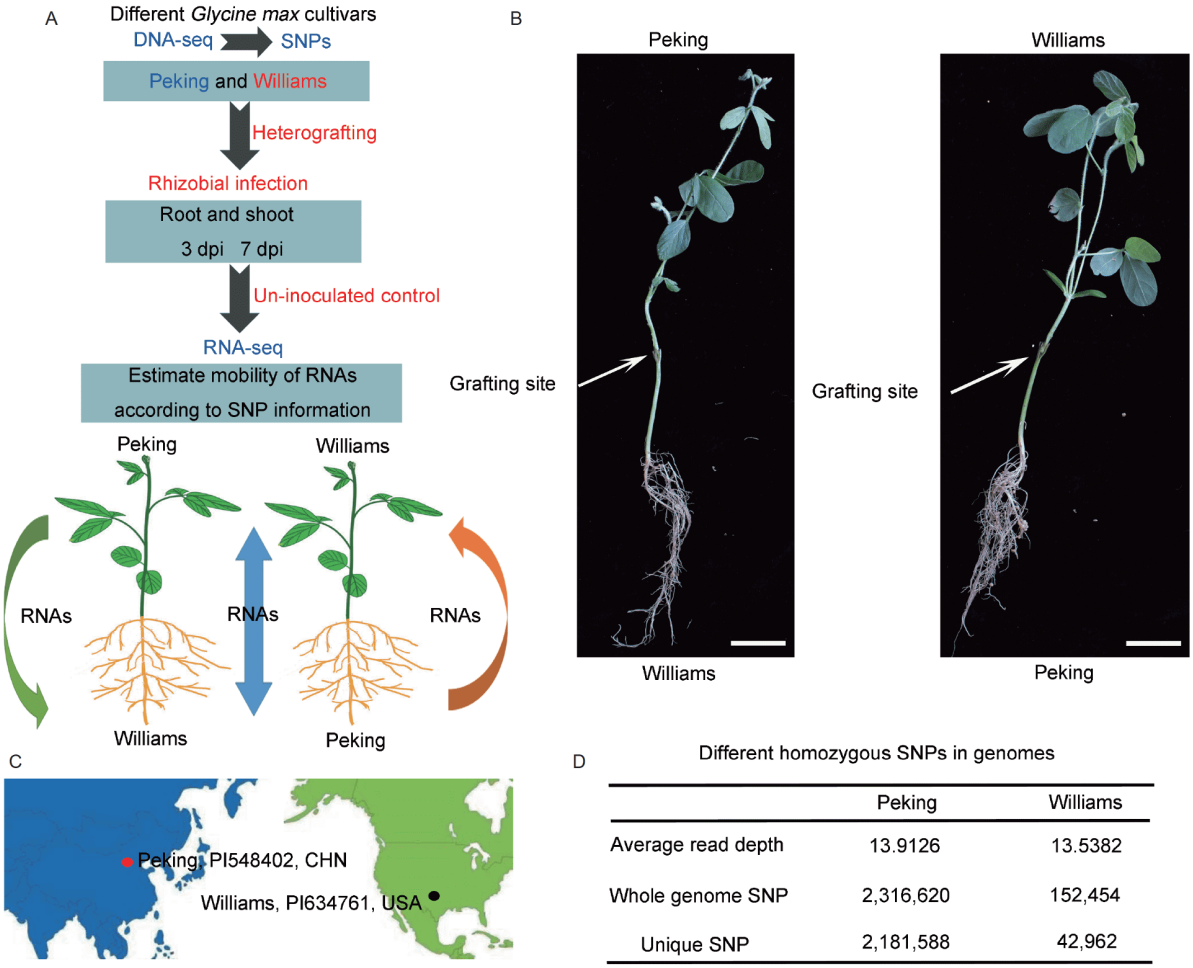
A transcript is defined as mobile when its corresponding RNA-Seq reads from the source (exporting) tissues were detected in the RNA-Seq library of receptor (importing) tissues. A gene that produces transcripts that can be transported between the rootstock and scion is considered to have graft-transmissible transcripts. A mobile transcript had to be detected at least twice within two biological replicates. Based on the SNP information, collectively, 4,552 genes producing graft-transmissible transcripts were identified from these two sets of grafted materials at different stages after rhizobial inoculation (Figure 2; Data S2 in Supporting Information).

Rhizobial infection triggered the transport of masses of mRNAs between shoots and roots

We further examined the mobile mRNAs in detail between Peking and Williams at different periods of rhizobial infection. The number of mobile mRNAs, which were produced and transmitted between Peking and Williams, was different depending on which cultivar served as the rootstock or scion and at different times of infection (Figure 2; Data S2 in Supporting Information).

The un-inoculated soybean grafts were grown under nitrogen-limiting conditions for subsequent rhizobial inoculation. Only a relatively small number of genes produced mobile transcripts (n=108) in the un-inoculated control (Figure 2A), whereas the inoculation of grafts with *B. diazoefficiens* induced the transport of mRNAs of a large number of genes (n=4,524) to distal tissues at 3 dpi (Figure 2B), indicating that rhizobial inoculation rapidly induced long-distance transport of mRNAs. At 7 dpi, mobile mRNAs of only 67 genes were detected (Figure 2C).

When we compared the transcripts that moved from shoots to roots with those that moved from roots to shoots, we found that few genes (about 8%, 377 out of 4,524 genes that produced mobile mRNAs) produced transcripts that moved bidirectionally (Figure 2), suggesting that the directionality of



	Peking	Williams
Average read depth	13.9126	13.5382
Whole genome SNP	2,316,620	152,454
Unique SNP	2,181,588	42,962

Figure 1 Hetero-grafting systems between two soybean cultivars showing a high frequency of SNPs. A, Outline of the experimental strategy and schematic representation of the reciprocal grafts. B, Grafting combinations used between Peking and Williams and inoculation with *B. diazoefficiens* USDA110. Scale bars=2 cm. C, Original collection sites of the Peking (CHN, red dot) and Williams (USA, black dot) cultivars. D, Information of the different genomic SNPs between Peking and Williams.

mRNA movement was highly specific and some selective processes might be involved in promoting or inhibiting the movement of these mRNAs towards a particular tissue or direction. Furthermore, it is also noteworthy that the transcripts seemed to preferably move from tissues of Peking to those of Williams. This might be due to several reasons as follows: (1) the decreased transfer activity in Williams tissues could be induced by the Peking tissue; (2) the source tissues might determine how frequently mobile transcripts are transmitted in a graft; (3) the compatibility between rhizobia and various soybean cultivars could be different. Similar ecotype bias was also reported in *Arabidopsis* (Thieme et al., 2015) and in grafted grapevines (Yang et al., 2015). Although there existed an ecotype effect, these results strongly indicated that the systemic transport of a great number of RNA was triggered by rhizobial inoculation.

To further investigate the tissue-specific delivery of mobile transcripts in response to rhizobial inoculation at different time points, we analyzed the extent and nature of the rhizobia-induced changes of transcript delivery in each tissue (Figure S1 in Supporting Information). At 3 dpi, the rhizobial inoculation resulted in a dramatic shift in the profile of transcripts delivered to the distal shoot or root; however, at 7 dpi, a much smaller number of mobile transcripts were detected (Figure S1 in Supporting Information).

Relationship between the abundance and mobility of the transcripts

To explore the function of the graft-transmissible mRNAs, we first evaluated the association between their abundance and mobility induced by rhizobia at the early stage of nodulation (3 dpi). We classified these transcripts into four groups according to their expression patterns in source and receptor tissues at 3 dpi (Figure S2A, Data S3 in Supporting Information). Group I transcripts were relatively highly expressed (RPKM (reads per kilobase million mapped reads) >100) in both source and receptor tissues; and the rest of

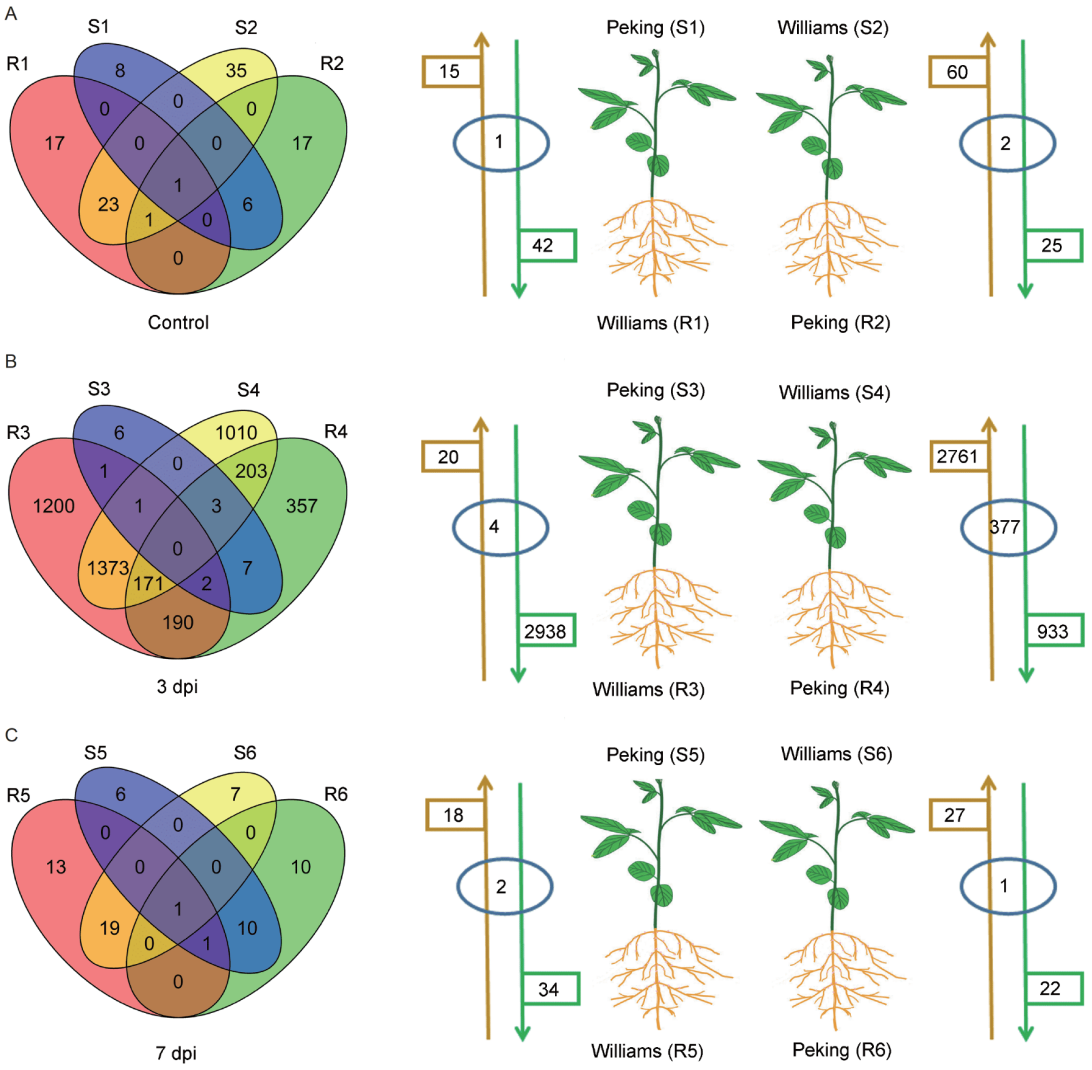


Figure 2 Rhizobial infection triggers the transport of multiple mRNAs between shoots and roots in soybean. Venn diagrams and schematic representation of the reciprocal grafts illustrate the number of mobile mRNAs in detail between Peking and Williams at different periods of rhizobial inoculation. A, Only a relatively small number of mobile mRNAs (n=108) was identified in the un-inoculated control group. B, The inoculation of grafts with *B. diazoefficiens* USDA110 induced the transport of a large number of mRNAs (n=4,524) to the distal tissues at 3 dpi. C, At 7 dpi only 67 mobile mRNAs were found.

mobile transcripts were further classified into three groups (group II, III and IV). Group II transcripts were expressed at similar levels in source and receptor tissues (source RPKM/receptor RPKM >0.5 and <2); group III transcripts were expressed at higher levels in receptor tissues compared to source tissues (source RPKM/receptor RPKM ≤0.5); and group IV transcripts were expressed at higher levels in source tissues compared to receptor tissues (source RPKM/receptor RPKM ≥2). Interestingly, the overwhelming majority of the identified graft-transmissible transcripts belonged to group II in the various hetero-graft combinations (Figure S2A in Supporting Information), and these mobile transcripts exhibited similar expression patterns in source and receptor tissues at 3 dpi (Figure S2B in Supporting Information). These results indicated that selective transport induced by rhizobia was not largely due to differential gene expression between source tissues and receptor tissues and that the gene transcripts that were transported between the tissues could be related to the processes of symbiosis and nodule development.

Classification and analysis of the functional pathway of graft-transmissible mRNAs

To further investigate the functions of these graft-transmissible mRNAs, we performed gene ontology (GO) enrichment analysis in these grafted plants at different times after rhizobial infection (Figure S3–S7, Data S4 in Supporting Information). In the un-inoculated control, we found that the mobile mRNAs from Peking shoots to Williams roots were enriched in processes associated with regulation of translation, responses to nutrient levels, regulation of cellular amide

metabolic process, and protein activity regulation, and the mobile mRNAs from Williams shoot to Peking root were involved in chromatin silencing, negative regulation of gene expression, and macromolecule biosynthetic process, representative of general cellular activities (Figure S3, Data S4 in Supporting Information). Additionally, the mobile mRNAs from Peking roots to Williams shoots were enriched in processes associated with oxidation-reduction process, tricarboxylic acid cycle, citrate metabolic process, and vesicle docking, and the mobile mRNAs from Williams roots to Peking shoots were involved in the regulation of transcription, RNA biosynthetic process, cellular macromolecule biosynthetic process, and gene expression.

Not surprisingly, at 3 dpi, the genes, which produced mobile transcripts from Peking shoots to Williams roots in response to rhizobia, were enriched in nodulation-related responsive processes, including response to cadmium ion, cell division, vesicle-mediated transport, microtubule organization, regulation of actin polymerization or depolymerization, positive regulation of cell cycle, autophagy, and some phytohormone-mediated signaling pathways such as auxin, cytokinin, ethylene, jasmonic acid, and abscisic acid (Figures S4 and S5, Table S2, Data S4 in Supporting Information). And the mobile transcripts from Williams shoots to Peking roots were involved in nodulation-related responsive processes, including responses to chitin, cell cycle process, cell division, lignin metabolic process, and some phytohormone-mediated signaling pathways, such as brassinosteroid, abscisic acid, salicylic acid, ethylene, jasmonic acid, and gibberellin.

By contrast, the transcripts that were transported from Peking roots to Williams shoot in response to rhizobia were enriched in nitrogen compound metabolic processes, response to nitrogen compounds, amide biosynthetic processes, urea transport, vesicle-mediated transport, cellular response to auxin stimulus, and response to ethylene- and jasmonic acid-mediated signaling pathways (Figure S6, Data S4 and S5 in Supporting Information). In addition, enrichment was observed in processes associated with the regulation of gene expression, shoot or root system development, and signal transduction. And the mobile transcripts from Williams roots to Peking shoots were involved in regulation of RNA biosynthetic process, chromatin silencing, negative regulation of gene expression, and regulation of nitrogen compound metabolic process. Furthermore, genes with mobile transcripts encoding transcription factor (TF) activity were largely enriched in the shoot-to-root direction, but not in the root-to-shoot direction (Data S4 and S5 in Supporting Information). For example, the MADS-box protein (Heard et al., 1997), the ethylene-responsive transcription factor (ERF) (Cerri et al., 2017; Yano et al., 2017), calmodulin-binding transcription activator (Routray et al., 2013; Shimoda et al., 2012; Takeda et al., 2012), NAC domain-containing protein (de Zélicourt et al., 2012), and WRKY, MYB, and bZIP transcription factors (Floss et al., 2017; Imin et al., 2013; Wang et al., 2016) have been reported to be involved in symbiosis (Data S5 in Supporting Information). Interestingly, among these mobile mRNAs, transcripts for RNA-binding protein coding genes were largely enriched in both directions (Data S4 and S5 in Supporting Information) and may participate in the transport of their cargo RNAs (Ham et al., 2009).

At 7 dpi, mobile transcripts from Peking shoots to Williams roots were enriched in photosynthesis, electron transport chain, response to nutrient levels and extracellular stimulus (Figure S7, Data S4 in Supporting Information), and mobile transcripts from Williams shoots to Peking roots were involved in chromatin silencing, negative regulation of gene expression, and cellular macromolecule biosynthetic process. Conversely, transcripts from Peking roots to Williams shoots were enriched in response to starvation, nutrient levels, and extracellular stimulus, and mobile transcripts from Williams roots to Peking shoots were involved in chromatin silencing, negative regulation of gene expression, and regulation of cellular process. The functions of mobile mRNAs at 7 dpi were similar to those in the un-inoculated state. Therefore, the activities and transport of nodule-related mRNAs were substantially reduced at 7 dpi.

Shoots systemically regulate root nodule formation at the transcriptional level

Rhizobial infection induces transient defense responses and persistent Nod factor signaling; meanwhile rhizobia specifically activate hormone biosynthesis and signaling and activation of the cell cycle (Breakspear et al., 2014). Rhizobial infection induces increased expression of cell cycle-related genes (Breakspear et al., 2014). We found that the movement directionality of cell-cycle related gene transcripts was mainly from shoots to roots and this long-distance transport only occurred at the 3 dpi timepoint (Figure S4, Data S4 and S5 in Supporting Information). Several genes encoding cyclin-dependent kinase regulatory subunit 1 (CKS1), cyclin-T1-4 (CCNT), cyclin-H1-1 (CCNH), cyclin-D3-1 (CYCD3), chromatid cohesion factor (MAU2), and E3 ubiquitin-protein ligase (UPL3) are directly involved in regulation of the cell cycle (Data S5 in Supporting Information). The function of these mobile cell-cycle related transcripts was correlated with cell division events during nodule initiation. The period between 3 and 7 dpi is critical for nodule differentiation and development and the mobility of these cell-cycle related mRNAs from shoots to roots could promote nodule formation.

Recent studies have shown that the process of autophagy can regulate legume-rhizobia symbiosis, infection, and nodule development (Nanjareddy et al., 2016) and an autop-

hagy-related kinase, PI3K, is essential for the symbiotic relationship between common bean (*Phaseolus vulgaris*) and rhizobia (Estrada-Navarrete et al., 2016). Here, we identified 15 mobile, autophagy-associated mRNAs that specifically moved downwards from shoots to roots, including the serine/threonine-protein kinase TOR, VPS15, BECN1-regulated autophagy protein 1, and several autophagy-related proteins (Data S5 in Supporting Information).

The cytoskeletal organization plays an important role in nodule organogenesis and functioning (Davidson and Newcomb, 2001; Hardham, 2013; Kitaeva et al., 2016; Vassileva et al., 2005). We found an enrichment of transcripts of genes involved in cortical microtubule organization including mitotic nuclear division, epidermal cell differentiation, cell wall organization, cellulose biosynthetic processes, and regulation of cell shape (Data S5 in Supporting Information), and these biological processes are consistent with a series of changes that occur during the differentiation, elongation, and maturation of root nodules. In addition, the actin cytoskeleton plays a central role in regulating intracellular transport and trafficking, and vesicle-mediated transport is necessary for signal and material exchanges, the formation of the infection thread, and nodule development (Dalla Via et al., 2017; Sinharoy et al., 2013). Here we found that a large number of gene transcripts associated with vesicle-mediated transport (*VAMP7*, *COPB1*, *SEC24*, *SEC26*, *AP2A*, *VPS29*, *Rab1*, *EXOC4*, etc.), microtubule organization (*CESA*, *CSNK1*, *NEK5*, etc.), and actin polymerization or depolymerization (*SCAR1/2*, *ARP2/3*, etc.) are primarily transported from shoots to roots (Figure S4, Data S2 and S5 in Supporting Information), suggesting that the source tissues (shoots) make substantial contributions to the formation of legume-rhizobia symbiosis in the root at the transcriptional level.

Rhizobial infection alters the transport of transcripts involved in hormone biosynthesis and signaling

Nearly all of the phytohormones have been reported to regulate root nodule symbiosis. Recent genetic and physiological evidence point to a crucial role of rhizobia-induced phytohormone changes in the host as a prerequisite for successful nodule formation (Ferguson and Mathesius, 2014). For example, cytokinin and auxin play fundamental roles in the control of nodule cell proliferation and differentiation (Boivin et al., 2016; Miri et al., 2016; Sasaki et al., 2014; Suzaki et al., 2012). Gibberellin, brassinosteroid, and jasmonic acid have positive effects on infection thread formation and nodule development at optimal concentrations, while ethylene and salicylic acid have predominantly negative effects on nodulation (Foo et al., 2014). Abscisic acid can decrease the proportion of curled root hairs and root hairs containing infection threads, and inhibit calcium spiking (Ding et al., 2008). Recent studies about the functions of phytohormones on nodules are limited to examination of hormone levels. Here, we found that rhizobial infection triggers the systemic movement of transcripts involved in the biosynthesis and signal transduction of auxin, cytokinin, gibberellin, ethylene, jasmonic acid, salicylic acid, abscisic acid, and brassinosteroid (Figure S4, Data S6 in Supporting Information). Interestingly, nearly all of the phytohormone-related transcripts were transported from shoots to roots, and only three phytohormone- (auxin, ethylene, and jasmonic acid) related transcripts were found to be transported from roots to shoots.

The systemic movement of known nodule-related gene transcripts

Next, we explored the movement of known symbiosis-related gene transcripts. The transcripts of *NF-YA1* (Nuclear transcription factor Y), *LYK* (LysM domain receptor-like kinase), *PUB1* (U-box domain-containing protein), *RIP1* (Rhizobium-induced peroxidase 1), *ENOD2* (Early nodulin-like protein 2), and *SUCS1* (Sucrose synthase isoform X1) were found to be transported between shoots and roots in response to rhizobial infection (Table 1; Data S7 in Supporting Information).

Rhizobial Nod factors (NFs) interact with receptors via the extracellular lysin motif (LysM) domains and induce nodulation signaling of legumes (Camp et al., 2011; Liang et al., 2018; Limpens et al., 2003). In *Lotus japonicus*, the CCAAT-box Nuclear Factor-Y (NF-Y) subunit genes, *LjNF-YA1* and *LjNF-YB1*, are expressed in root nodule primordia and act as the transcriptional targets of NODULE INCEPTION (NIN), and root nodule organogenesis was inhibited by knockdown of *LjNF-YA1*, as was seen in loss of function of *NIN* (Soyano et al., 2013). *NF-YA1* controls the progression of rhizobial infection by regulating the formation of infection threads in *M. truncatula* (Combier et al., 2006; Laporte et al., 2014). In *M. truncatula*, the LYK3 symbiotic receptor kinase interacts with an E3 ubiqutin ligase PUB1 and phosphorylates the latter, thus negatively regulates rhizobial infection and nodulation during symbiotic process (Mbengue et al., 2010). Here, we found that the transcripts of LysM domain receptor-like kinase 3 and another LysM domain protein in soybean, *NF-YA1*, and *PUB1* were transported specifically from shoots to roots at 3 dpi (Table 1; Data S7 in Supporting Information).

The early nodulin gene *ENOD2*, a marker for the differentiation of nodule parenchyma, is induced by the lipo-chitin nodulation signals at the early stages of nodule formation (Lauridsen et al., 1993; Minami et al., 1996). And another early nodulin gene *RIP1*, encoding a peroxidase, is induced by NFs, and the expression of *RIP1* is tightly associated with early symbiotic processes (Cook et al., 1995; Peng et al.,

Table 1 Systemic transport of mRNAs that have been previously shown to be involved in the establishment of the legume-rhizobium symbiosis

Genes characterized in soybean				
Gene ID	Annotation	Abbr.	Direction	Ref.
Glyma.09G073600	Sucrose synthase-like isoform X1	SUCS1	Shoot to root	Morell and Copeland, (1985)
Glyma.17G045800	Sucrose synthase-like isoform X1	SUCS1	Bidirection	Morell and Copeland, (1985)
Glyma.05G038100	Iron-sulfur cluster assembly protein 1	ISCU1	Shoot to root	Qin et al. (2015)
Glyma.06G239900	Early nodulin-like protein 2	ENOD2	Root to shoot	Lauridsen et al. (1993)
Glyma.09G263300	Digalactosyldiacylglycerol synthase 2	DGD2	Root to shoot	Gaude et al. (2004)
Glyma.14G201700	Rhizobium-induced Peroxidase 1	RIP1	Root to shoot	Yan et al. (2016)
Glyma.09G173200	Glutamine synthetase	GS	Shoot to root	Bishop et al. (1976)
Glyma.18G041100	Glutamine synthetase	GS	Root to shoot	Bishop et al. (1976)
Homologs to symbiotic genes characterized in other legumes				
Gene ID	Annotation	Abbr.	Direction	Ref.
Glyma.02G195000	Nuclear transcription factor Y subunit A	NF-YA1	Shoot to root	Combier et al. (2006)
Glyma.13G225900	U-box domain-containing protein 17-like isoform 1	PUB1	Shoot to root	Mbengue et al. (2010)
Glyma.13G199800	Annexin-like protein	ANN1	Bidirection	Niebel et al. (1998)
Glyma.05G241600	Histidine kinase 4-like isoform X1	CRE1	Bidirection	Gonzalez et al.(2006)
Glyma.13G324000	LysM domain receptor-like kinase 3	LYK3	Shoot to root	Limpens et al. (2003)
Glyma.16G213400	LysM domain protein	LysM	Shoot to root	Limpens et al. (2003)
Glyma.20G210700	Putative ion channel POLLUX-like 2	POLLUX2	Shoot to root	Charpentier et al. (2008)
Glyma.10G179700	Putative ion channel POLLUX-like 2-like isoform X2	POLLUX2.2	Shoot to root	Charpentier et al. (2008)

1996). *RIP1* is also correlated with Nod factor induction of reactive oxygen species production (Ramu et al., 2002; Yan et al., 2016). Unexpectedly, the mRNA movement of *ENOD2* and *RIP1* was induced upwards from roots to shoots in response to rhizobia infection. The sucrose synthase gene *SUCS1* is expressed in nodules and is required for nitrogen fixation (Baier et al., 2007; Morell and Copeland, 1985). We found that transcripts of the soybean homolog of *SUCS1* were transported bidirectionally between shoots and roots at 3 dpi with relatively high expression levels (RPKM >100) both in source and receptor tissues (Data S7 in Supporting Information). In addition to the known nodule-related genes mentioned above, transcripts of several other symbiosis-related genes were found to be transported between shoots and roots (Table 1; Data S7 in Supporting Information), for example, the cytokinin receptor *CRE1* (Gonzalez-Rizzo et al., 2006; Yin et al., 2019), annexin-like protein *ANN1* (Niebel et al., 1998), and cation channel protein *POLLUX* (Charpentier et al., 2008; Hayashi et al., 2014), which are involved in the symbiotic interaction with rhizobia in legume plants. We also found that transcripts of several genes (*ISCU1*, *GS*, *DGD2*) involved in nitrogen fixation were transported between shoots and roots (Data S7 in Supporting Information) (Bishop et al., 1976; Gaude et al., 2004; Qin et al., 2015).

To confirm the mobility of these transcripts, we transformed the roots with *pro35S:ANN1-GFP*/*pro35S:ENOD2-GFP*/*pro35S:SUCS1-GFP* and empty vector (*pro35S:GFP*) using the hairy root transformation method in soybean (Figure 3A). Then RT-PCR assays on RNA samples from transgenic roots and shoots were performed. *GFP* (green fluorescent protein) is a non-plant mRNA and we fused the mobile transcripts with *GFP* in roots to detect the transcript of *GFP* in shoots. We detected the *GFP* mRNAs fused to *ANN1*, *ENOD2*, and *SUCS1* in the shoot when the transgenic plants were inoculated with *B. diazoefficiens* USDA110 three days later, while *GFP* mRNA was not detected in the un-inoculated control or at 7 dpi (Figure 3B). Thus, the mobile transcript sequence could trigger *GFP* RNA transport from the root to the shoot. These results were consistent with our RNA-Seq data and also provided important evidence to connect mRNA movement with rhizobial infection.

DISCUSSION

Signal and material exchanges between shoots and roots of the host plant are necessary for the formation of the legume-rhizobia symbiotic relationship. Previous studies have reported extensive movement of RNA across graft junctions in several model plant species (Kim et al., 2014; Yang et al., 2015; Zhang Z., et al., 2016) and the presence of mRNA was found in phloem sap (Aoki et al., 2005; Zhang et al., 2009). In our study, we found that two soybean cultivars, Peking and Williams, show a high frequency of SNPs, which allowed us to study the origin and mobility of transcripts in hetero-grafted plants.

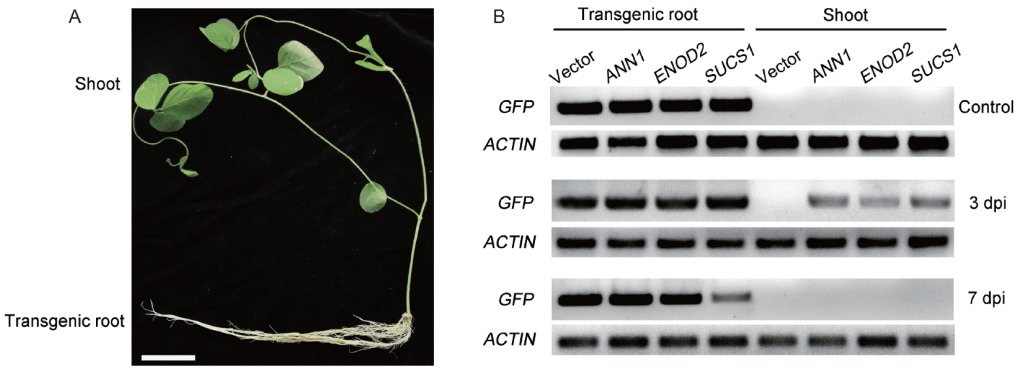


Figure 3 (Color online) RT-PCR analysis of *GFP* fused to mobile transcripts in transgenic soybeans. A, Image of transgenic soybean using the hairy root transformation method. Bar=2 cm. B, At 3 dpi, *GFP* mRNAs fused to *ANN1*, *ENOD2*, and *SUCS1* were detected in the shoots (n=12). In the un-inoculated control or at 7 dpi, *GFP* mRNA was not detected in the shoots (n=12). These experiments were repeated three times. *ACTIN* was used as the internal control.

A total of 4,552 annotated genes, which accounted for about 8.2% of the total protein-coding genes (56,044) in soybean, were found to produce mobile mRNAs that could cross the graft junctions (Figure 2; Data S2 in Supporting Information). The extent of mRNA exchange between Peking and Williams was extensive, at a similar scale as reported in *Arabidopsis* (about 6%, 2006 out of 33,602 genes that produced mobile mRNAs) (Thieme et al., 2015). In addition, the previous study (Thieme et al., 2015) also showed that only 264 mobile RNAs were detected in grafted *Arabidopsis* plants grown under nitrogen-limiting conditions. Similarly, our study revealed that only 108 genes produced mobile transcripts in the un-inoculated control under nitrogen-limiting conditions. This slight difference between *Arabidopsis* and soybean could be due to the time of plant growth, environment temperatures or illumination conditions.

Several studies have shown that mRNA transport occurs in both a selective and nonselective manner (Kim et al., 2014; Notaguchi et al., 2015; Yang et al., 2015; Zhang Z., et al., 2016). Within the nonselective model, the mRNA species are mobile as a consequence of high abundance (Calderwood et al., 2016). However, active long-distance mRNA trafficking had been reported for *StBEL5* in potato (*Solanum tuberosum*) and *GAI* in Arabidopsis (Banerjee et al., 2006; Hannapel, 2010; Huang and Yu, 2009) and selective movement of mRNAs across graft junctions was recently shown in grafted Arabidopsis plants (Paultre et al., 2016; Thieme et al., 2015; Zhang W., et al., 2016). In our study, we observed that a large number of gene transcripts (n=4,524) are transported to distant tissues at the early stages (3 dpi) of soybean-rhizobia symbiosis, while only a small number of mobile transcripts (n=67) were found at 7 dpi (Figure 2). This result indicated that selective processes could be involved in regulation of transcripts mobility during root nodule symbiosis. Most importantly, our analysis revealed that the majority of mobile transcripts had relatively low abundance (Data S2 in Supporting Information) and were expressed at similar levels in source tissues and receptor tissues. The mobility of specific transcripts across the graft union did not show correlation to their abundance, suggesting that a special shuttling mechanism is required for long-distance trafficking of mRNA molecules. Therefore, it is possible that some intrinsic elements contained in the sequence of mobile genes facilitate their long-distance transport. For example, RNA can be transmissible due to the tRNA-derived sequences with predicted stem-bulge-stem-loop structures (Zhang W., et al., 2016). Future work will need to explain this proposed selective mRNA delivery model.

The mobile transcripts detected at different time points of rhizobial infection or in different directions of movement were from genes involved in many different biological processes. The mobile mRNAs in the un-inoculated control group or at 7 dpi were enriched in processes associated with chromatin and gene silencing, transcription and translation regulation, response to nutrient stress, and protein activity regulation, representative of general cellular activities and epigenetic processes (Figures S3 and S7, Data S4 in Supporting Information). Additionally, there was no significant difference in the movement of transcripts from roots to shoots or shoots to roots in the un-inoculated control and at 7 dpi. However, at 3 dpi, genes whose mRNAs were transported from shoots to roots in response to rhizobia were enriched in some nodulation-related responsive processes, including response to cadmium ion, cell division, vesicle-mediated transport, cytoskeleton organization, cell cycle, autophagy, and some phytohormone- (auxin, cytokinin, gibberellin, ethylene, jasmonic acid, salicylic acid, abscisic acid, and brassinosteroid) mediated signaling pathways (Figure S4, Data S4 in Supporting Information). By contrast, the genes whose transcripts were transported from roots to shoots were enriched in nitrogen compound metabolic processes, amide biosynthetic processes, urea transport, and ammonium transmembrane transport (Figure S6 in Supporting Information), which potentially serve to prepare the

plant for nitrogen fixation at the transcriptional level. When mature nodules begin to fix nitrogen, the transported RNAs could be translated into proteins to perform specific functions.

Considering the limitations of systemic regulatory mechanisms on nodulation between the legume host and rhizobia, we could not definitively determine which metabolic pathway is functional in the symbiotic process. However, we found that a large number of transcripts associated with the cell cycle, autophagy, vesicle-mediated transport, microtubule organization, and actin polymerization or depolymerization are primarily transported from shoots to roots at the early stage (3 dpi) of nodulation, suggesting that the shoots may play a role in the formation of legume-rhizobia symbiosis in the root. These biological processes correspond to a series of changes that occur in the differentiation, elongation, and maturation of root nodules. More importantly, we found that transcripts of nearly all of the phytohormone-encoding genes are transported downwards to the root, while only three phytohormone- (auxin, ethylene, jasmonic acid) related transcripts were transported upwards to the shoot (Figures S4 and S6 in Supporting Information), indicating that these mobile hormone-related transcripts presumably provide additional levels of regulation of root nodule growth and development. Therefore, we found that rhizobial infection alters the mobility of gene transcripts involved in hormone biosynthesis and signaling, presumably functioning in nodule formation.

Studies on legume-rhizobia symbiosis have focused on the key components of the nodulation signaling pathway and a great many genes involved in the regulation of symbiosis and nodulation-related proteins have been discovered. However, little is known about the systemic regulation of symbiosis at the transcriptional level and its role in nodule formation. We found that rhizobial infection induces the transportation of a series of known symbiosis-related transcripts between shoots and roots. Intriguingly, the transcripts of LysM domain receptor-like kinase 3, *NF-YA1*, and *PUB1* were transported specifically from shoots to roots at the early stage (3 dpi) of nodulation (Data S7 in Supporting Information). Further investigation is required to determine why rhizobia infection triggers the movement of nodulation-related gene transcripts in the shoot and whether preventing this kind of mobility could affect nodulation events.

It is generally assumed that mobile transcripts act as signals or regulatory molecules and even produce functional proteins in the receptor tissues. The movement of a large set of protein-encoding gene transcripts suggests that gene expression and transcript function can be separated both in space and in time, which could allow the precise control of their expression in target cells. Therefore, gene function can extend beyond gene expression and even separate from gene expression (Thieme et al., 2015). In addition, mobile transcripts could function as a signal to distant tissues and cells, inducing the systemic adaptation to nutritional changes and response to stresses. Our findings shed light on a deeper understanding of systemic regulation in the legume-rhizobia symbiotic processes.

MATERIALS AND METHODS

Plant materials and rhizobia growth conditions

Soybean seeds (*Glycine max* cv. Peking [PI548402] and cv. Williams [PI548631]) were surface sterilized with 70% ethanol and germinated in filter paper soaked with distilled water for 3 days. *B. diazoefficiens* USDA110 was grown in TY liquid medium containing 5 g Tryptone, 3 g yeast extract, and 0.7 g L^{-1} $CaCl_2 \cdot 2H_2O$ at 28°C for 4–5 days. The seedlings were grown in nitrogen-limiting sterile vermiculite with 10,000 lux light at 27°C and a 16 h light/8 h dark cycle before inoculation with rhizobia as previously described (Wang et al., 2009). The plants were inoculated with a suspension of *B. diazoefficiens* strain USDA110 (OD_{600}=0.08). Un-inoculated seedlings grown under the same conditions and on sterilized vermiculite were used as controls. For RNA extraction, seedling roots and shoots were rinsed briefly in PBS buffer, pH 7.5, to remove vermiculite particles. Harvested tissues were frozen immediately in liquid nitrogen and stored at −80°C until they were used for RNA extraction.

Soybean grafting analysis

Soybean seedlings were cut transversely using a sharp razor leaving 3–5 cm of stem above the root system. The shoot as the scion was connected with stocks and wrapped tightly using medical adhesive tape. After grafting, plants were grown under high humidity at 27°C with a photoperiod of 16 h light/8 h dark for two weeks. After joint wound healing, the grafts were inoculated with *B. diazoefficiens* USDA110. Root and shoot materials were harvested from the inoculated grafted plants (3 and 7 dpi) and the un-inoculated control for two biological replicates. To eliminate individual differences, every sample was a mixture of eight grafted plants growing in the same environment and condition. Then the shoot samples or root samples were subjected to NGS.

Library preparation for DNA and RNA sequencing

Total DNA was extracted from 100 mg of each sample by using a cetyltrimethylammonium bromide (CTAB)-based method (Abdel-Latif and Osman, 2017). The genomic DNA concentration was determined using a NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, USA). DNA libraries for Illumina sequencing were constructed for each sample according to the manufacturer's specifications (Illu-

mina, USA). After DNA library construction, sequencing was performed by a commercial Illumina HiSeq2000, with 150 bp read length. Raw reads were filtered based on the following criteria: pair-end reads with >10% "N" bases; average base-quality less than 20 (Phred-like score); quality score of 3' bases ≤40 (Shen et al., 2019).

Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific, USA) and used as the initial material for the strand-specific RNA library preparations following previously published protocols (Zhong et al., 2011). RNA integrity and concentration were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, USA). Ribosomal RNA was removed using Epicentre Ribo-Zero Gold Kits (Epicentre, USA). Subsequently, the sequencing libraries were constructed using the TruSeq RNA Library Prep Kit v2 (Illumina, Annoroad Co. Ltd, Beijing). Libraries were sequenced on the HiSeq2000 (Illumina, USA) to 150 bp paired-end reads and sequenced. To avoid potential contamination, each library was sequenced on a separate lane. The quality of RNA-seq reads was evaluated using FastQC.

RNA-seq data processing

Illumina adapter sequences and low-quality regions were clipped from raw read sequences using Trimmomatic v0.30. Bowtie2 was used for building the genome index, and the cleaned data was mapped to the *Glycine max* genome reference assembly using TopHat v2.0.12. The Integrative Genomics Viewer was used to analyze the mapping results by the heatmap, histogram, scatter plot, or other styles. Reads count for each gene in each sample was counted by HTSeq v0.6.0, and RPKM was then calculated to estimate the expression level of genes in each sample. DESeq (v1.16) was used for differential gene expression analysis between two samples. *P*-value was assigned to each gene and adjusted by the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR).

Detection of informative SNPs to identify mobile transcripts

Paired-end Illumina DNA-Seq reads were mapped to the *Glycine max* genome reference assembly v2.0 using bwa with default parameters (Shen et al., 2018). Then the lower reads were filtered out (mapping quality <20) and duplicated reads were removed. SNPs in DNA-Seq were called by samtools mpileup and bcftools (depth ≥3 and genotype=="homozygous"). Then the SNPs that were identical in Peking and Williams were removed. In the end, the informative SNPs for identifying mobile transcripts were confirmed in two cultivars (read depth ≥3 and without heterozygote in all two cultivars). Based on these rules, 186,212 informative SNPs in 25,738 genes were identified.

Detection of mRNAs that can move between the scion and rootstock of grafted plants

The mobility of a transcript was determined by comparison of corresponding genomic and RNA-seq reads of rootstocks and scions. Bamtools 2.4.1 was used to count the depth of the informative SNPs of RNA-Seq data. A transcript was considered mobile if there were more than three reads supporting the alternative allele coming from another ecotype. We defined a transcript as mobile if it satisfied the following conditions: RPKM of the receptor tissue is greater than or equal to 3; RPKM of the source tissue is greater than or equal to 3; ratio of mobile SNP reads (p in the formula below) is between 0.05 and 1; ratio of valid SNPs is greater than or equal to 0.5; average binominal probability ($mean_{SNPs}(B(n, p))$ in the formula below) of valid informative SNPs is greater than or equal to 0.004. In total, 4,552 genes in all samples were categorized as having mobile transcripts. The number of genes producing graft-transmissible transcripts in two biological replicates was shown in Table S3 in Supporting Information. The mobility score was calculated for ranking as

$$mobilityscore(transcript) = \frac{\#SNPs_{mobile}}{\#SNPs} \times p \times mean_{SNPs}(B(n,p))$$

$$p = \frac{\#reads(SNPs_{mobile})}{\#reads(SNPs)}$$

Where SNPs means all informative positions supported by DNA-Seq. $SNPs_{mobile}$ refers to all SNP sites that indicated transcript mobility. The binomial probability $B(n,p)$ represents the mean of probability that the observed read distribution at a particular SNP site is consistent with the background probability (p) estimated from the read counts for the mobile and reference allele.

GO enrichment analysis of mobile transcripts

Gene Ontology terms were searched using agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php), and tested statistically for set-specific GO-term enrichment by the Fisher exact test. The significance of GO (Gene Ontology, http://geneontology.org/) term enrichment was calculated based on a hypergeometric test, and the P value was further adjusted based on the q-value. Total genes in the whole genome were used as background for calculating GO category frequency. GO terms with FDR corrected p-values of less than 0.01 were considered to be significantly enriched.

Classification of mobile mRNAs

The expression levels of the mobile mRNAs in source tissues and receptor tissues at 3 dpi were ranked by mean RPKM values derived from two biological replicates. The mobile mRNAs having high expression levels (RPKM >100) in both source tissues and receptor tissues were placed in group I. The remaining mobile mRNAs were then screened to identify those having similar expression levels in source and receptor tissues (ratio of source/receptor >0.5 and <2, group II). Next, the mRNAs having higher expression levels in receptor tissues than source tissues (ratio of source/receptor ≤0.5) were placed into group III. Finally, mobile mRNAs having higher expression levels in source tissues than sink tissues (ratio of source/receptor ≥2) were placed into group IV. The mobile transcripts were excluded when we calculated the RPKM in each sample. And we classified these transcripts into four groups according to the ratio between the total RPKM corresponding to Peking transcripts and the total RPKM corresponding to Williams transcripts.

Vector information

The transcripts of *ANN1*, *ENDO2* and *SUCS1* were cloned to the Gateway donor vector pEntry-topo-SD and then recombined into the pEarleyGate103 vector under the control of the *Cauliflower mosaic virus* 35S promoter by LR reactions (Invitrogen, USA) to generate the constructs *pro35S:ANN1-GFP*/*pro35S:ENOD2-GFP*/*pro35S:SUCS1-GFP*. All primers used in plasmid construction are described in Table S4 in Supporting Information.

Soybean hairy root transformation

Soybean hairy root transformation was performed using *Agrobacterium rhizogenes* K599 as previously described (Kereszt et al., 2007). Soybean seeds (Williams 82) were surface sterilized and germinated and after 5 days, the hypocotyl close to the cotyledonary node was stabbed. The square signs which part of the seedling must be infected for successful transformation. Transgenic composite plants were transplanted to pots (10 cm×10 cm) containing a 3:1 mixture of vermiculite and perlite and grown for 1 week (16 h of light, 25°C, and 50% relative humidity (RH)) to allow recovery. When the hairy root formed, the axial root was cut off.

Semi-quantitative RT-PCR analysis

We isolated total RNA from transgenic roots and shoots of soybean at 3 and 7 dpi and in the un-inoculated control using Trizol reagent (Invitrogen, USA). cDNA was prepared from 2 μg of total RNA with Superscript III reverse transcriptase (Invitrogen, USA). We used 0.5% of the total reverse transcriptase reaction per 20 μL PCR reaction performed. The PCR was performed as follows: 95°C for 1 min, 32 cycles (denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 1 min), and termination at 72°C for 5 min. Equal volumes of the RT-PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. *ACTIN* transcripts were used as a control to confirm constant levels of amplified fragments for all samples.

Accession numbers

All sequencing Data Sets are available at the NCBI Small Read Archive (SRA), ID PRJNA509520. The sequenced reference genome Williams 82 are available in the Phytozome database (http://www.phytozome.net/).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 The tissue-specific delivery of mobile mRNAs in response to rhizobial infection at different time points.

Figure S2 The association between the abundance and mobility of graft-transmissible transcripts induced by rhizobia infection.

Figure S3 GO enrichment analyses of the total mobile transcripts in the un-inoculated control group.

Figure S4 Shoots systemically regulate root nodule formation at the transcriptional level.

Figure S5 The ‘‘Interactive graph’’ view of GO enrichment of common mobile transcripts from shoots to roots in the two different grafted plants at 3 dpi.

Figure S6 GO enrichment analyses of the mobile transcripts from roots to shoots at 3 dpi.

Figure S7 GO enrichment analyses of the total mobile transcripts at 7 dpi.

Table S1 Number of reads produced for each individual RNA-Seq library

Table S2 Gene ontology analysis of graft-transmissible transcripts from shoots to roots in both grafted plants at 3 dpi

Table S3 Primers used in this study

Table S4 Number of mobile genes in two biological replicates

Data S1 SNP information in all transcripts

Data S2 Catalog of Graft-transmissible transcripts responsive to rhizobial infection

Data S3 Classification of graft-transmissible mRNAs at 3 dpi

Data S4 Gene ontology analysis of graft-transmissible mRNAs at different infection periods

Data S5 Representative function of graft-transmissible transcripts responsive to rhizobial infection

Data S6 Hormone-related graft-transmissible gene transcripts from shoots to roots responsive to rhizobial infection

Data S7 Annotation and direction of movement of known symbiosis-related genes

The supporting information is available online at http://life.scichina.com and https://link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.