miR172 Can Move Long Distances in Nicotiana benthamiana

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Abstract: Small interfering RNAs can induce RNA silencing throughout plants owing to their ability to move through the phloem. Although microRNA (miRNA), another noncoding small RNA that regulates protein-coding RNAs through transcript cleavage or translational inhibition, has also been detected in phloem, there are few reports of its long-distance movement via phloem. We investigated whether miR172 molecules can move systemically from source tissues to sink tissues in Nicotiana benthamiana. Results of agro-infiltration and micro-grafting experiments indicate that miR172 can move long distances.

Keywords: miRNA, miR172, Phloem, movement, long distance, grafting.

INTRODUCTION

RNA silencing by small interfering RNAs (siRNAs) in plants can act systemically [1]. After silencing is initiated in a few cells of one tissue, a signal travels along the vascular system and induces silencing in other tissues. Through this mechanism, siRNA has an innate defensive role against invasive nucleic acids such as in viruses [2]. The movement of RNA silencing signal in plants was first shown convincingly by grafting experiments in tobacco [3]. Independent evidence for the involvement of a systemic signal in RNA silencing has come from the induction of systemic silencing in transgenic tobacco plants by the delivery of exogenous DNA homologous to a transgene via Agrobacterium tumefaciens (agro-infiltration) or particle bombardment [1, 4, 5]. As in the grafting experiments, systemic silencing induced by this transient expression can propagate through a graft junction [6].

MicroRNAs (miRNAs) are small noncoding RNAs with important roles in the regulation of gene expression in plants [7]. miRNA genes are transcribed as long pre-miRNA transcripts that form fold-back structures in which mature miRNAs reside in either the 5′ or 3′ arm and are processed by Dicer [8]. miRNAs silence RNAs by targeting cognate miRNAs for degradation or translation repression [9]. Could miRNAs also travel through phloem to carry long-distance signals in plants? Yoo et al. [10] identified populations of miRNAs in the phloem sap of a range of plants. Buhlz et al. [11] found multiple miRNA species in the phloem sap of oilseed rape (Brassica napus). Recently, Lin et al. [12] and Pant et al. [13] suggested that miR399 travels from shoot to root to signal systemic stress in potato [19]. Furthermore, the effect of miR172 on tuberization in potato is transmissible through grafts, suggesting the possibility of miR172 transportability [19]. However, there is no clear evidence so far about its long-distance transport. Here, we characterized the mobility of miR172 from a source of its overproduction to other tissue by agro-infiltration and grafting experiments. The results indicate that miR172 was transported long distances.

MATERIALS AND METHODS

Plant Materials

We grew Nicotiana benthamiana in a plant growth room at 24 °C under a 16-h light / 8-h dark cycle with cool fluorescent light at about 100 μmol m−2 s−1. For the grafting experiment, we used the GFP-overexpressing 16C line (a gift from Prof. David Baulcombe, Gatsby Charitable Foundation, UK) as scion.

Construction of Binary Vectors

Plasmid pIG121 [20] was digested with HindIII and EcoRI to remove the CaMV35S:β-glucuronidase (GUS):nos-ter sequence, and a multi-cloning site sequence, 5′-AAGCTTGCATGCTGCAAGTCTAGAAGATCCCGGGTACCAGCTGAAATTC-3′, was inserted. The resulting plasmid was used as the “Empty” vector. After nos-ter was reinserted into the multi-cloning site with SacI and EcoRI, the companion-cell-specific promoter from pCOI (Matsuda et al. [21]; from Prof. Neil Olszewski, University of Minnesota, St. Paul, MN, USA), amplified by PCR using primers 5′-GGGATCCATGCTTCTGAGCTCTAGAAGATCCCGGGTACCAGCTGAAATTC-3′ and 5′-GGGATCCATGCTTCTGAGCTCTAGAAGATCCCGGGTACCAGCTGAAATTC-3′, was integrated into the SalI/BamHI sites. Finally, the miR172a PCR product of the 35S:mIR172 plasmid (pMAT137/hm from Prof. Xuemei Chen, University of California Riverside, CA, USA), amplified by PCR using primers 5′-AGGATCCATGCTTCTGAGCTCTAGAAGATCCCGGGTACCAGCTGAAATTC-3′ and 5′-ATGGTACCTGAGCTCTAGAAGATCCCGGGTACCAGCTGAAATTC-3′, was integrated into the BamHI/KpnI sites.

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Agro-Infiltration

Agrobacterium tumefaciens EH105 containing the binary vector (Fig. 1a) was grown overnight in Luria-Bertani (LB) medium with the appropriate antibiotics and 10 μM acetosyringone. The bacteria were briefly spun down (5000 g, 15 min, RT) and resuspended in suspension buffer (10 mM MES-KOH, pH 5.2, 10 mM MgCl₂, 100 μM acetosyringone) to an OD₆₀₀ of 0.1 and left for at least 3 h at room temperature. One ml of the suspension was infiltrated into the leaves of N. benthamiana via a needle-less 1-mL syringe (Fig. 1b). To check transient gene expression, we used 35S:VlmybA [22] (kindly provided by Prof. Shozo Kobayashi, National Institute of Fruit Tree Science, Hiroshima, Japan), the transient activity of which was confirmed via anthocyanin pigmentation in the infiltrated area.

Micrografting

Seeds were germinated on MS [23] agar (0.7%) for 1 week. Hypocotyl-hypocotyl grafts were performed between 7-d-old seedlings of N. benthamiana. The hypocotyl of the rootstock donor was cut horizontally at approximately 5 mm below the cotyledon and the root part was inserted into a silicone tube (2 mm length, 0.4 mm internal diameter). The scion plant was cut in the same way, and the cut surface of the shoot part was pressed against that of the rootstock donor and the root part was inserted into a silicone tube (Fig. 1c). All grafting procedures were performed under a stereomicroscope on a clean bench. The shoot part was pressed against that of the scion plant was cut in the same way, and the cut surface of the scion plant was cut in the same way, and the cut surface of the shoot part was pressed against that of the root stock was cut in the same way, and the cut surface of the shoot part was pressed against that of the root part midway along the tube (Fig. 1c). All grafting procedures were performed under a stereomicroscope on a clean bench. The grafts were grown on MS agar in Petri dishes. One week after grafting, the tube was cut from the graft interface, and the plants were then grown for another 2 weeks on MS agar in a culture bottle. Scions and rootstocks were collected separately for RNA isolation (Fig. 1, experiment 2). They were then transferred to autoclaved potting compost and grown for 3 weeks. The shoot part above the 3rd leaf, lateral leaves from the 1st to the 3rd nodes, and the 1st and 2nd lateral buds were cut off, leaving only the 3rd lateral bud. Two weeks later, emerged lateral leaves were harvested for RNA isolation (Fig. 1c).

RNA Isolation and RT-PCR

Total RNA was isolated with a miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. Residual genomic DNA was eliminated with a TURBO DNA-free Kit (Ambion). The cDNAs used for quantitative RT-PCR of mature miR172 were synthesized from 10 ng total RNA with a Taq-Man MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Mature miR172 was quantified by use of Taq-Man Gene Expression Master Mix combined with Taq-Man MicroRNA Assay (Applied Biosystems). NbUbi (N. benthamiana ubiquitin, accession no. EU862550) as an internal control mRNA was quantified from 10 ng total RNA isolated with a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Real-time PCR used primers 5'-CAGGACAAAGGAGGTATC-3' and 5'-CACGTCACCAC-AACAGA-3'. PCR product melting curves confirmed the specificity of single-target amplification. RT-PCR of precur-

Fig. (1). Illustrations of plasmids used and materials sampled in agro-infiltration and grafting experiments. (a) Agrobacterium plasmids. 35S:VlmybA was used for reporting transient expression efficiency through anthocyanin pigmentation. Doubled-35S and companion cell-specific CoYMV promoters were linked to AtMiR172a (accession No. At2g28056). The precursor miR172 region is indicated in gray with the PCR primer sites. (b) Four-week-old plants were pruned above the 7th lateral branch (L7), and L1, L2, and L7 were also cut off to encourage the establishing source and sink leaves. At 4 days after agro-infiltration into L3-L5, L5 and the lateral leaves that emerged from L7 (arrowheads) were sampled to isolate total RNA. L6 was also agro-infiltrated with 35S:VlmybA to check the infiltration efficiency. (c) Five-day-old seedlings were grafted. After 1 week on MS agar, the scion and stock were sampled to check the levels of transgene expression. Grafted plants grown until L7 stage were pruned above L3, and only the L3 lateral bud was left. After 1 week, the leaves that emerged from L3 were sampled. Arrowheads show the materials sampled to measure the amount of miR172.
Furthermore, the expression of miR172 was determined by quantitative RT-PCR with the specific Taq-Man probe, which can accurately quantify miRNA [26]. miR172 was detected in even Empty-vector-infiltrated leaves on account of conservation of the miR172 sequence between Arabidopsis and N. benthamiana [27]. The leaves infiltrated with 35S:MIR172 accumulated 80-fold and 1100-fold as much miR172 as those infiltrated with the Empty vector (Fig. 2). This large difference in range seems to indicate that susceptibility to Agrobacterium differed between leaves. In the newly emerged leaves, the amount of miR172 was 4.7-fold that of the leaves infiltrated with the Empty vector, and about 10-fold that of the leaves infiltrated with 35S:MIR172. These results suggest that some of the miR172 produced in source leaves traveled to sink leaves.

**RESULTS**

**Agrobacterium Infiltration Experiments**

An advantage of agro-infiltration is that it is possible to obtain results within a week. Thus, first, we used agro-infiltration to see whether miR172 produced transiently in leaves moved to new leaves freshly emerged from the top axial bud (Fig. 1b). Anthocyanin pigmentation in leaves infiltrated by the 35S:VlmybA vector proved the effectiveness of agro-infiltration [25]. Then we used the 35S:MIR172 plasmid (Fig. 1a), which encodes Arabidopsis MIR172A [24] under the control of the doubled 35S promoter of cauliflower mosaic virus (CaMV). The amount of mature miR172 was confirmed by quantitative RT-PCR with the specific primers that were designed across the miR172 target sequence [27]. The leaves infiltrated with 35S:MIR172 accumulated 80-fold and 1100-fold as much miR172 as those infiltrated with the Empty vector (Fig. 2). This large difference in range seems to indicate that susceptibility to Agrobacterium differed between leaves. In the newly emerged leaves, the amount of miR172 was 4.7-fold that of the leaves infiltrated with the Empty vector, and about 10-fold that of the leaves infiltrated with 35S:MIR172. These results suggest that some of the miR172 produced in source leaves traveled to sink leaves.

**COYMV:MIR172 Transgenic Plants**

The Commelina yellow mottle virus (CoYMV) promoter was used to express the MIR172 gene in the companion cells of transgenic N. benthamiana. We generated several T2 plants. Some of them produced a few flowers with partial sepal-to-petal transformations (data not shown), as reported by Mlotshwa et al. [27], indicating that the CoYMV:MIR172 transgene could produce enough mature miR172 to induce floral homeotic transformation. Since the CoYMV promoter functions exclusively in the companion cells of all organs [21], the transgenic plants at this stage showed almost same level of miR172 in both roots and shoots (Fig. 3). The line indicated by an arrow in Fig. 3 produced the highest amount of miR172 in roots and was selected for use in the following grafting experiment. Furthermore, the expression levels of the target gene NbAP2L1 (GenBank sequence CK287095) were analyzed by RT-PCR using the gene-specific primers that were designed across the miR172 target sequence. However, no differences in the expression of the NbAP2L1 mRNA were detected between the wild-type and transgenic plants (data not shown), as reported by Mlotshwa et al. [27]. Thus, we didn’t analyze the expression levels of the target gene in the following grafting experiments.

![Fig. (2)](image)

**Fig. (2).** miR172 levels in agro-infiltrated leaves and in leaves that emerged from L7 lateral bud (experiment 1 in Fig. (1)). The relative quantitative RT-PCR amounts are shown as mean ± SD of three replicates. The actual data (scion/stock) of respective points are indicated.

![Fig. (3)](image)

**Fig. (3).** miR172 levels in roots and shoots of one CoYMV:Empty and four CoYMV:MIR172 transgenic plants. Two-week plants after germination were used (Fig. 1c, experiment 2). Quantitative RT-PCR data are shown as means ± SD of three replicates. The actual data (scion/stock) of respective points are indicated.

**Grafting of COYMV:MIR172 Plants**

As the scion, GFP-expressing line 16C (Fig. 1c) was used to test the graft union. At 2 weeks after grafting, we observed the 16C/CoYMV:Empty and 16C/CoYMV:MIR172 grafted plants for the GFP signal under UV light, and discarded plants exhibiting incomplete graft unions or GFP in roots. The remaining 1/3 of plants were analyzed for the miR172 precursor fragment in the scion and stock by RT-PCR with primers for the MIR172 precursor sequence (Fig. 1a). The results showed no precursor in the scion of 16C/CoYMV:MIR172 plants (Fig. 4), confirming that the MIR172 transgene was transcribed only in the stock of grafted plants. Three weeks later, we collected the whole scion from 0.5 cm above the graft union and the whole stock from 0.3 cm below the union (Fig. 5a). The data points of the 16C/WT plants are all located at 1.0/1.0, but those of the 16C/miR172 plants are scattered (Fig. 5b). Although the stock and scion data did not show a parallel relationship like the results of Fig. 3, three of the eight samples showed a higher scion miR172 amount than the 16C/Empty.
miR172 traveled from source to sink tissues when it was overexpressed in the source. Agro-infiltration experiments showed that more than 1000 times the control amount of miRNA could accumulate in source leaves, suggesting that at least transiently produced miR172 could avoid degradation such as by small RNA degrading nucleases [31]. On the other hand, the amount of miR172 in the sink tissue was not as marked. In a grafting experiment using miR399-overexpressor and wild-type plants, the level of miR399 in receiver wild-type roots was only 0.38% of the overexpressor scion level in Arabidopsis and 4.3% in N. benthamiana [12]. Our miR172 results were very similar: 0.9% by agro-infiltration (10.9/1120 in Fig. (2)) and 6.6% by grafting (2.8/21.4 in Fig. (5)). The higher value may be due to our use of the companion-cell-specific promoter. RNA molecules are thought to move in the form of ribonucleoprotein complex through pore-plasmodesmata units (PPU) from companion cells to sieve elements [32]. The PPU corridor passage of the complex is considered to be controlled by specific proteins [33]. The entrance to sieve tubes of the phloem is also enabled by interaction of miRNAs with proteins. Yoo et al. [10] reported that pumpkin (Cucurbita maxima) phloem small RNA binding protein (CmpSRP1) mediates cell-to-cell trafficking of small single-strand RNA, but not of double-strand RNA. In miRNA, protein components such as PSRP1 may control the amount of transport into the sieve tubes.

miR172 molecules were not transmitted in approximately 75% of grafts (5/8 in Fig. (5), 12/15 in Fig. (6)). Similar low efficiencies of graft transmission have been reported for RNA silencing signal [1, 24, 34, 35]. Long-distance transport of RNA in sieve tubes appears to be mediated by RNA-binding proteins [32, 36, 37]. Ham et al. [38] identified RNA-binding proteins involved in mRNA transport, and proposed a model in which a ribonucleoprotein complex moves in the phloem. It is clear that miRNA also binds to chaperone proteins for stability and delivery to target tissues. As a matter of course, this large complex must pass the graft union, where vascular bundles are developed in the callus at the union [39]. The de novo sieve tube passage is prone to be unorthodox, showing features such as a winding path, disrupting the passage of the large ribonucleoprotein complex. In an extreme case, the complex would become clogged. Since the conductance of a vessel is proportional to the fourth power of the vessel radius (Hagen-Poiseuille law), a slightly reduced diameter would pose an obstacle to passage. On the other hand, grafting of many horticultural crops is a well-developed technology; these plants may experience less of a problem.

We show here that a miR172 can move from the stock to the scion over a long distance. The miRNA-directed regulation of development and stress responses has been revealed [40], and the use of engineered miRNA to alter agronomically relevant traits has been advanced [41, 42]. Merging the long-distance miRNA transfer and grafting could allow us to create innovative technologies for horticultural crop improvement.

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Fig. (6). miR172 levels in lateral leaves that emerged from grafted scions (experiment 4 in Fig. (1e)). (a) Grafted plant when lateral leaves were sampled. The graft union is arrowed. Bar indicates 0.5 cm. (b) miR172 levels of lateral leaves. Quantitative RT-PCR data from 13 16C/Empty and 15 16C/CoYMV:miR172 grafted plants are shown as means ± SD of three replicates.

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REFERENCES


