# *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 mRNAs 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. Buhtz *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 that the shoot is under low inorganic phosphate stress. These results indicate a role of miRNAs in long-distance systemic information transfer.

*miR172* promotes flowering primarily by repressing a set of transcription factors [14-18] at downstream of miR156. *miR172* was detected in the phloem sap of oilseed rape [11], and in the epidermis and vascular cells 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.

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## 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 *Hin*dIII and *Eco*RI to remove the *CaMV35S:β-glucuronidase* (GUS):*nos-ter* sequence, and a multi-cloning site sequence, 5′-AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCGGGGTACCGAGCTCGAATTC-3′, was inserted. The resulting plasmid was used as the "Empty" vector. After *nos-ter* was reinserted into the multi-cloning site with *Sac*I and *Eco*RI, 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′-GCGTCGACGGTATCGATTTCTTAGG-3′ and 5′-GCGGATCCTTGTTGTGTTGGGTTTTC-3′, was integrated into the *Sal*I/*Bam*HI sites. Finally, the *miR172a* PCR product of the 35S:MIR172 plasmid (pMAT137hm from Prof. Xuemei Chen, University of California Riverside, CA, USA), amplified by PCR using primers 5′-GAGGATCCATCCCACCAGGTCTTTCTCTGG-3′ and 5′-ATGGTACCGTTAACGAGCTCTAGAAGCTTTGC-3′, was integrated into the *Bam*HI/*Kpn*I sites.

## 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 $MgCl_2$, 100 µM acetosyringone) to an $OD_{600}$ 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 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′-CAGGACAAGGAGGGTATC-3′ and 5′-CACGTCAAC-AACAGA-3′. PCR product melting curves confirmed the specificity of single-target amplification. RT-PCR of precur-

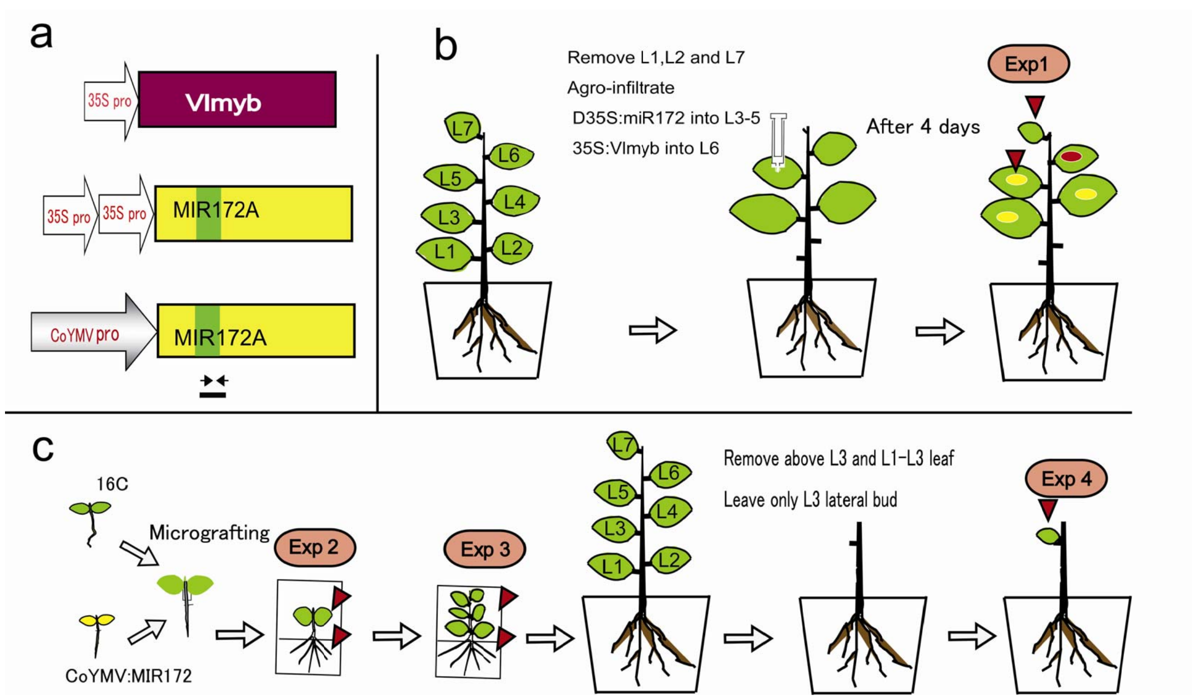


**Fig. (1).** Illustrations of plasmids used and materials sampled in agro-infiltration and grafting experiments. (**a**) *Agrobacterium* plasmids. *35S:VlmybA* was used for reporting transient expression efficiency through anthocyanin pigmentation. Doubled-35S and companion cell-specific *CoYMV* promoters 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*.

sor *AtmiR172A* [24] was performed with primers 5′-GGTA-CGAGTTTCTAGTGTCTAT-3′ and 5′-CACATGAAAC-AAGATACCCAC-3′. All PCR products were sequenced, confirming specific amplification by the primers.

## 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 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.

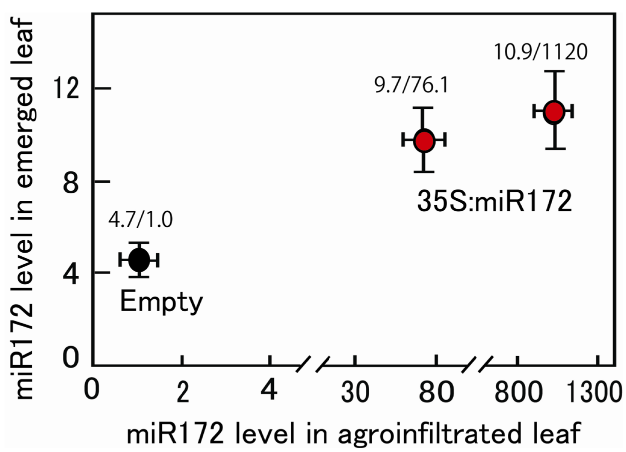


**Fig. (2).** *miR172* levels in agro-infiltrated leaves and in leaves that emerged from L7 lateral bud (experiment 1 in Fig. (**1**)). The relative quantitative RT-PCR amounts are shown as mean ± SD of three replicates. The actual data (scion/stock) of respective points are indicated.

### *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 $T_2$ 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 site. However, no differences in the expression of the *NbAP2L1* mRNA were detected between the wild-type and transgenic plants (date 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.

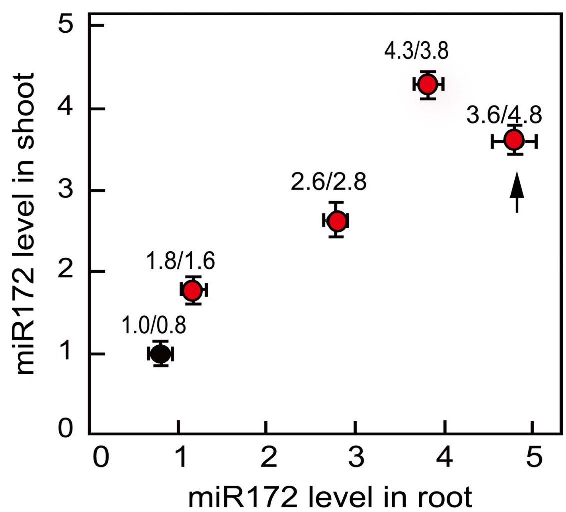


**Fig. (3).** *miR172* levels in roots and shoots of one CoYMV:Empty and four CoYMV:MIR172 transgenic plants. Two-week plants after germination were used (Fig. **1c**, experiment 2). Quantitative RT-PCR data are shown as means ± SD of three replicates. The actual data (scion/stock) of respective points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

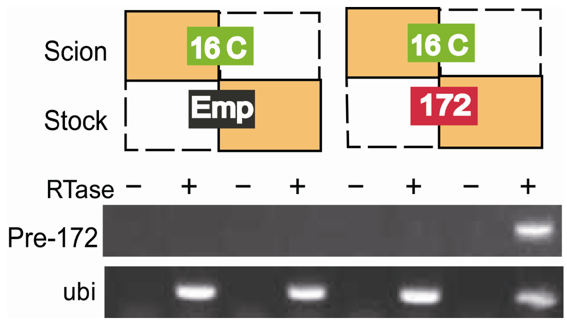


**Fig. (4).** Absence of *miR172* precursor fragments in scion of 16C/CoYMV:MIR172. The *miR172* precursor product was obtained only from *CoYMV:MIR172* stock. *Ubiquitin* (EU862550) mRNA was used as a positive transcript. RTase, reverse transcriptase; ubi, *ubiquitin*.

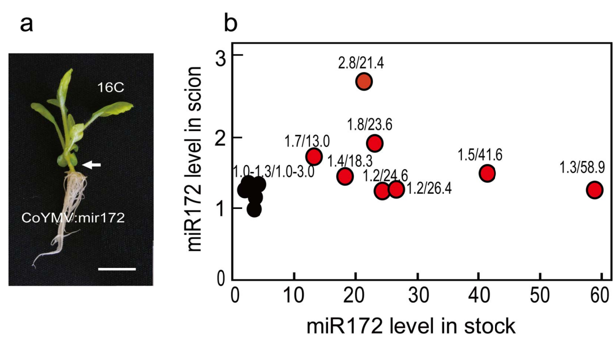


**Fig. (5).** 16C/CoYMV:MIR172 plants and *miR172* levels of stock and scion. (a) Three-week grafted seedlings (experiment 3 in Fig. **1c**). The graft union is arrowed. Bar indicates 0.5 cm. (b) Quantitative RT-PCR data of five 16C/Empty and eight 16C/CoYMV: MIR172 seedlings are shown as means ± SD of three replicates. The actual data (scion/stock) of respective points are indicated.

### *miR172* Amount in Sink Leaves of Grafted Plants

The siRNA phloem flow showed a strict correlation between the establishment of phloem sink-source relations and transport [28]. Therefore, we removed all developed leaves from the scion (Fig. **1c**, experiment 3) to make the lateral bud of the scion a strong sink tissue. Ten days after the removal of all leaves, we sampled the newly emerged leaves of the scion (Fig. **6a**). Although 12 out of 15 samples did not show clear differences from the control (16C/Empty), the other 3 accumulated up 4.7-fold as much *miR172* (Fig. **6b**). These results show that mature *miR172* travels from the stock to the scion through the graft union.

## DISCUSSION

Grafting experiments using *miR399*-overexpressing *Arabidopsis* suggested that *miR399* is involved in phloem-mediated signaling [13]. Similar experiments using *N. benthamiana* also suggested the long-distance movement of *miR399* through phloem [12]. In addition, localized expression of the *miR399* promoter in the vascular cylinder [29] supported the direct involvement of this miRNA in information transfer over long distances in plants. Several other miRNAs were also detected in phloem [30], but direct evidence for their long-distance transport is still lacking.

*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 high latter 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 transportation 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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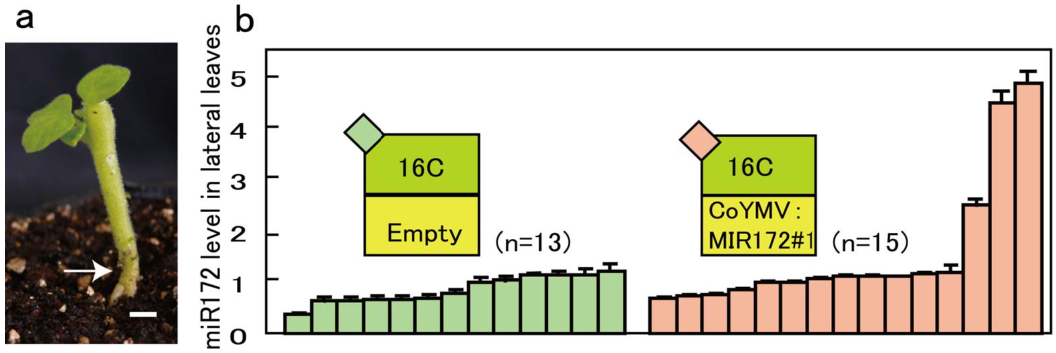


**Fig. (6).** *miR172* levels in lateral leaves that emerged from grafted scions (experiment 4 in Fig. (**1c**)). (**a**) Grafted plant when lateral leaves were sampled. The graft union is arrowed. Bar indicates 0.5 cm. (**b**) *miR172* levels of lateral leaves. Quantitative RT-PCR data from 13 16C/Empty and 15 16C/CoYMV:MIR172 grafted plants are shown as means ± SD of three replicates.

GFP 16C plants; to Xuemei Chen, University of California Riverside, for Ti-plasmid 35S::MIR172a-1; to Neil Olszewski, University of Minnesota St. Paul, for the pCOI binary vector containing the *CoYMV* promoter; and to Shozo Kobayashi, National Institute of Fruit Tree Science, Japan, for the *VlmybA* plasmid. We also thank S. Oozeki and S. Kida for their expert assistance in tissue culture. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN).

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