A cis Element within Flowering Locus T mRNA Determines Its Mobility and Facilitates Trafficking of Heterologous Viral RNA

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The Arabidopsis flowering locus T (FT) gene encodes the mobile florigen essential for floral induction. While movement of the FT protein has been shown to occur within plants, systemic spread of FT mRNA remains to be unequivocally demonstrated. Utilizing novel RNA mobility assay vectors based on two distinct movement-defective viruses, Potato virus X and Turnip crinkle virus, and an agroinfiltration assay, we demonstrate that nontranslatable FT mRNA, independent of the FT protein, moves throughout Nicotiana benthamiana and mutant Arabidopsis plants and promotes systemic trafficking of viral and green fluorescence protein RNAs. Viral ectopic expression of FT induced flowering in the short-day N. tabacum Maryland Mammoth tobacco under long-day conditions. Recombinant Potato virus X bearing FT RNA spread and established systemic infection more quickly than the parental virus. The cis-acting element essential for RNA movement was mapped to the nucleotides 1 to 102 of the FT mRNA coding sequence. These data demonstrate that a plant self-mobile RNA molecule can mediate long-distance trafficking of heterologous RNAs and raise the possibility that FT RNA, along with the FT protein, may be involved in the spread of the floral stimulus throughout the plant.

RNA trafficking plays an important role in systemic signaling that controls plant development and defense against pathogen infection (25). Hundreds of RNA transcripts have been recently identified in phloem, suggesting phloem-mobile RNAs may act as long-distance signaling molecules in plants (4, 8). Indeed, systemic movement of a homeobox fusion transcript and gibberellic acid-insensitive RNA regulates leaf architecture (13, 19), a non-cell-autonomous mobile RNA represents a long-distance signal that modulates potato tuber formation (3), and small interfering RNAs are components of intercellular and systemic mobile signals for innate RNA silencing defense (9, 12, 14). RNA trafficking is also critical for plant viruses and viroids to establish systemic infection. It has been demonstrated that an RNA motif directs long-distance trafficking of a small naked RNA viroid (33, 44, 46). Moreover, a short RNA sequence is found to be involved in cell-to-cell movement of a plant viral RNA (24), and replication-independent viral RNA can move over long distances in plants (11).

In floral induction, the mobile florigen is encoded by the Arabidopsis flowering locus T (FT) gene. FT transcribes mRNA in the leaf, but its encoded FT protein functions in the shoot apices where flowers develop (1, 2, 40). The Arabidopsis FT protein and its orthologues have been shown to be involved in long-distance signaling in floral induction (7, 18, 22, 23, 29, 30, 37). However, whether FT mRNA is also capable of systemic spread remains to be demonstrated. We describe novel approaches which show that not only does FT RNA move over long distances but, remarkably, also facilitates the systemic spread of heterologous green fluorescent protein (GFP) mRNA and different viral RNAs in plants. The FT RNA movement does not rely on the expression of the FT protein. The FT RNA mobility is determined by a cis-acting element localized within nucleotides 1 to 102 of the FT mRNA coding sequence.

MATERIALS AND METHODS

Construction of RMA vectors. The wild-type and mutant Arabidopsis FT genes were reverse transcription-PCR (RT-PCR) amplified using Pfu DNA polymerase and the primers PP354/PP356 or PP356/PP355, digested with BspEI and Sall, and cloned in-frame fused to the GFP coding sequence in the BspEISall sites of PVX and PVX/GFP (38) to produce PVX/FT, PVX/mFT, PVX/GFP-FT, and PVX/GFP-mFT, respectively. Plasmid DNA of PVX/GFP, PVX/GFP-FT, and PVX/GFP-mFT were then digested with Sall and Xhol to remove the coat protein (CP) gene subgenomic RNA promoter and the CP gene and self-ligated to produce PVX/GFPΔCP, PVX/GFP-FTΔCP, and PVX/GFP-mFTΔCP. Expression of the GFP gene from PVX/GFPΔCP and the wild-type and mutated GFP-FT fusion gene from PVX/GFP-FTΔCP and PVX/GFP-mFTΔCP were under the control of an engineered CP subgenomic RNA promoter. For construction of TCV-based RNA mobility assay (RMA) vectors TCV/mFTΔCP, TCV/GFP-FTΔCP, and TCV/GFP-mFTΔCP, the Arabidopsis FT gene was RT-PCR amplified using Pfu DNA polymerase and the primers PP406 or PP407 and PP408, digested with BclI and PmeI, and cloned into the BglII/PmeI sites of TCV/ΔCP (34) or TCV/GFPΔCP (47). Using a similar PCR and cloning strategy, a series of TCV/mFTΔCP-based RMA vectors carrying truncated (tr) FT for mapping the cis-acting element required for FT RNA trafficking were constructed. All RNA constructs were confirmed by nucleotide sequencing. The primers used for the construction of RMA vectors are listed in Table 1.

RMA. RNA transcripts from each movement-deficient recombinant PVX and TCV vector were produced by in vitro transcription as previously described (34), pretreated with RNase-free DNase (Promega), and then mechanically inoculated onto N. benthamiana or A. thaliana fi-10 mutant plants at 5 to 6 or 15 to 16 leaves, respectively, in repeated experiments. N. benthamiana plants aged at only 24 days after sowing seeds and were too young to initiate flowering. For PVX-based RNA, total RNAs (50 ng) extracted from inoculated and newly growing young leaves separately collected at 7 days postinoculation (dpi) were pretreated with RNase-free DNase (Promega) and used for RT-PCR detection (34) and the primers PP354 and PP356 for FT, PP371 and PP372 for GFP, PP268.

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and PP373 for PVX, and PP271 and PP272 for 18S rRNA. Epidermal cells with GFP expression and the movement of GFP-tagged viruses were examined with a Nikon digital CoolPix 995 camera. Total RNAs (50 ng) extracted from systemically infected young leaves collected for protein and RNA analyses.

### RESULTS AND DISCUSSION

**Arabidopsis FT RNA facilitates the movement of heterologous GFP and viral RNAs in N. benthamiana.** To investigate plant RNA trafficking, we developed a GFP-tagged movement-defective *Potato virus X* (PVX/GFP-FT)–based RNA (Fig. 1A). Deletion of viral CP prevented PVX/GFP-ΔCP movement and restricted viral RNAs to single *N. benthamiana* epidermal cells. Thus, unlike the wild-type virus, PVX/GFP-ΔCP was incapable of accessing the phloem to move systemically (10). It should be noted that free GFP, once loaded from the mesophyll into the sieve tube, can move over long distances in the phloem (16, 17, 31, 32). PVX/GFP-ΔCP and PVX/GFP-mFTΔCP had, respectively, the translatable or nontranslatable *Arabidopsis FT* that was fused in-frame to the GFP coding sequence. However, cell-to-cell movement of GFP-FT fusion protein from PVX/GFP-FT was restricted in young leaves, developed after 7 to 14 dpi. All PVX/FT-infected plants started to shoot at ~20 dpi and flowered at ~35 dpi; this was photographically recorded with a Nikon digital CoolPix 995 camera. Total RNAs (50 ng) extracted from systemically infected young leaves collected at 42 dpi were treated with RNase-free DNase (Promega) and used for RT-PCR detection (30 cycles) (34) with the primers PP512 and PP513 for virus-carried FT RNA and the resultant specific RT-PCR products were purified and verified by direct sequencing. The transient FT protein was analyzed by Western blotting detection.

**Western blotting.** To investigate GFP, FT, GFP-FT fusion protein, and PVX CP expression, total proteins were extracted from leaf tissues as described previously (15). Western blot analyses of protein aliquots (10 μg) were performed with polyclonal antibodies specifically raised against GFP, the *Arabidopsis* FT and PVX CP, and detected using a goat anti-rabbit immunoglobulin G conjugate with alkaline phosphatase (Sigma) and BCIP/NBT substrates (Roche) as described previously (39).
GFP or GFP-FT among epidermal cells was not detected by epifluorescence microscopy (Fig. 1A). We also examined the upper leaves for systemic movement of GFP or GFP-FT protein and these recombinant viruses but failed to observe any GFP in any type of cells.

We then used a more sensitive RT-PCR assay to test for RNA movement. Accumulation of recombinant viral RNAs from each vector was readily detected in inoculated leaves by RT-PCR using FT-, GFP-, and PVX-specific primers (Fig. 1A and B). However, no spread of genomic RNA (gRNA) and subgenomic RNA (sgRNA) of PVX/GFPΔCP to newly formed young leaves occurred (Fig. 1C). In striking contrast, inclusion of FT RNA in PVX/GFP-FTΔCP enabled GFP-FT sgRNA and PVX/GFP-FTΔCP gRNA to move systematically from inoculated leaves to young leaves (Fig. 1C). Furthermore, PVX/GFP-mFTΔCP gRNA and sgRNAs that contained nonsense mutations precluding FT protein synthesis also spread systematically (Fig. 1C), demonstrating that nontranslatable mFT RNA was able to move and promote long-distance trafficking of heterologous GFP and PVX RNAs. FT and mFT possessed similar abilities to facilitate the spread of heterologous RNAs, although the levels of mobile RNAs detected in the systemic leaves varied between plants for the FT and mFT constructs. The absence of GFP signal in neighboring epidermal cells and upper leaves after FT RNA-mediated movement from lower parts into younger tissues suggests that CP may be required for vascular exit, which could be a novel role for CP in viral movement, although we cannot exclude the possibility that it is due to the limited sensitivity of fluorescence detection of free GFP and GFP-FT fusion proteins.

FT and mFT RNA movement and FT-mediated heterologous RNA trafficking were also demonstrated in a distinct Turnip crinkle virus (TCV)-based RMA (Fig. 2). In a similar experimental design, we used a GFP-tagged movement-deficient TCVΔCP-based RMA vector (Fig. 2A) and also showed that the Arabidopsis FT RNA promoted systemic movement of TCV RNA. RT-PCR detection of FT, GFP, and TCV RNA and 18S rRNA (18S) in inoculated leaves (Fig. 2B) and particularly in the newly formed young leaves and shoot apices (Fig. 2C) of Arabidopsis plants infected with TCV/GFPΔCP, TCV/GFP-FTΔCP, or TCV/GFP-mFTΔCP mirrored the results obtained with the PVX-based RMA.

Mobility of Arabidopsis FT RNA is independent of viral RNA sequences. The self-mobility of FT RNA and the associated heterologous RNAs was further confirmed by an agroinfiltration assay of RNA mobility. The position of an introduced stop codon (*) replacing the FT gene start codon in 35S-GFP-mFT-poly(A) is indicated. The CP gene was deleted. The positions of a stop codon (*) replacing the FT gene start codon in PVX/GFP-mFTΔCP, and three sets of RT-PCR primers for the detection of FT (red), PP354/PP356), GFP (green), PP371/PP372), or PVX (blue) RNAs are indicated. Individual epidermal cells expressing free GFP or GFP-FT fusion protein showed green fluorescence. (B and C) RT-PCR analysis of FT, GFP, and PVX RNA and 18S rRNA in inoculated and newly growing young leaves, including shoot apices of N. benthamiana leaves infiltrated with agrobacteria carrying the 35S-controlled gene expression cassette and were analyzed by Western blotting with a GFP-specific antibody. The positions of free GFP and GFP-FT fusion protein are indicated. (D) Construction of 35S promoter-controlled FT gene expression cassettes for a transient agroinfiltration assay.
both FT (mFT) and FT (mFT)-GFP RNAs spread systemically to young leaves that had developed on the plant after the agroinfiltration (Fig. 1F). It should be pointed out that systemic movement of FT (mFT) and FT (mFT)-GFP RNA seems to be stronger in virus-based RMAs than in the agroinfiltration assays. This may be due to differences in the site (nucleus versus cytoplasm) and the means of production of the mobile RNA molecules (35S promoter-controlled transcription versus recombinant viral RNA replication and transcription) in agroinfiltrated cells compared to viral inoculated cells. Nevertheless, our data indicate that no viral sequences and proteins are necessary for systemic spread of FT RNA.

Viral transient expression of Arabidopsis FT protein promotes floral induction. Arabidopsis FT is required to induce flowering. However, it did not stimulate early flowering in day neutral N. benthamiana expressing GFP-FT, probably due to interference by the GFP fusion. To test this, we infected S. tabacum Maryland Mammoth tobacco (21) with PVX/FT (Fig. 3A) and PVX/GFP-FT (see Fig. 6A). Viral ectopic expression of the GFP-FT fusion protein was unable to induce flowering under noninducing LD conditions (Fig. 3B and C); however, all Maryland Mammoth plants infected with PVX/FT that had the capacity to produce free FT protein flowered in LD, while control plants infected with either PVX/mFT carrying a mutated nontranslatable FT mRNA or PVX/GFP remained vegetative (Fig. 3B and C). Viral delivery of wild-type and mutated FT RNA was readily detected in systemically infected leaves by RT-PCR and further confirmed by direct sequencing of the specific RT-PCR products (Fig. 3D). Free FT protein expressed from PVX/FT but not from PVX/mFT was also detected by Western blotting with an antiserum specifically raised against FT (Fig. 4D). The fact that PVX/FT could induce flowering but PVX/mFT could not shows that the Arabidopsis FT RNA alone is not sufficient to initiate flowering but that its protein product expressed from PVX/FT is necessary for floral induction.

Systemic movement of FT RNA does not require FT protein. The virus-based RMAs, together with the agroinfiltration assay, demonstrate that Arabidopsis FT RNA not only is self-mobile but also can mediate systemic trafficking of heterologous RNAs in N. benthamiana. To eliminate the possibility that endogenous plant-derived FT protein might be involved in facilitation of the FT RNA movement, we used the TCV-based RNA vector TCV/mFTACP containing a nontranslatable FT RNA. Systemic movement of TCV/mFTACP RNA seems to occur. However, the nontranslatable mutant mFT RNA in TCV/mFTACP moved and facilitated long-distance movement of viral RNAs to young leaves and the shoot apices (Fig. 4B). The nonsense mutation prohibiting FT protein synthesis from...
TCV/mFTΔCP was maintained in the Arabidopsis mutant (Fig. 4C). Moreover, no viral transiently expressed or endogenous FT protein was detected in different leaf tissues of mock-inoculated and virus-treated ft-10 mutant plants, a finding consistent with the mutation in ft-10 (Fig. 4D). Long-distance trafficking of virus-derived mFT RNA from inoculated leaves to noninoculated leaves and the shoot apices was also detected in ft-1 mutant plants (X. Liu, C. Li, S. Jackson, and Y. Hong, unpublished data). On the other hand, when fusing GFP downstream of FT, the nonsense mutation also eliminates GFP expression (K. Zhang and Y. Hong, unpublished data). Thus, we conclude that the systemic FT RNA trafficking does not require the FT protein.

Mapping of the cis-acting element within Arabidopsis FT RNA. To elucidate what controls long-distance spread of FT RNA, we constructed a series of TCV/ΔCP-based RNA vectors carrying truncated nontranslatable FT fragments (Fig. 5A). We found that viral-derived FT RNAs from TCV/FTn102ΔCP, TCV/FTn201ΔCP TCV/FTn300ΔCP and TCV/FTn399ΔCP were capable of movement and facilitating sys-
The translational and nontranslational *Arabidopsis* FT RNA mobile function is independent of the FT protein. Consistent with this idea, an engineered plant RNA virus 

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**FIG. 5.** Functional mapping of the cis-acting element that controls the FT RNA movement. (A) TCV/trFTΔCP-based RNA vectors carrying truncated (tr) nontranslatable *Arabidopsis* FTs. (B) Detection of virus-derived FT RNA (top panel) by RT-PCR in systemic young leaves (Y) from plants inoculated with TCV/FTn102ΔCP (n102), TCV/FTn201ΔCP (n201), TCV/FTn300ΔCP (n300), or TCV/FTn399ΔCP (n399); but not from plants inoculated with TCV/FT103cΔCP (103c), TCV/FT202cΔCP (202c), TCV/FT301cΔCP (301c) or TCV/FT400cΔCP (400c). Recombinant viral RNA of each TCV/trFTΔCP was readily detectible in inoculated leaves (I). No specific virus-derived FT RNA was detected in mock-inoculated plants. RT-PCR analysis of 18S rRNA (bottom panel) is included as an RNA control. (C and D) Direct sequencing of RT-PCR products verified the presence of virus-derived truncated FT RNA. Example sequence panels are shown for truncated FT RNA expressed in the young leaves of plants inoculated by TCV/FTn102ΔCP (C) or in the TCV/FT103cΔCP-inoculated leaves (D). The TAG stop codon is underlined and TCV and virus-derived FT sequences are indicated.

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It should be noted that TCV without CP moves readily in *Arabidopsis* that is defective in RNA silencing. Thus, the CP is mainly providing a silencing-suppressor function which allows virus movement, although it may also be offering some movement support (28). This is consistent with the idea that the CP silencing-suppressor possesses a differential role in viral intercellular spread (35). However, the *Arabidopsis* FT RNA and indeed FT protein have no effect on RNA silencing (K. Zhang, C. Li, and Y. Hong, unpublished data). Therefore, FT RNA is only providing movement function and the FT RNA-mediated systemic spread of TCV seems to operate with a distinct mechanism.

**The *Arabidopsis* FT RNA enhances systemic spread of PVX.** The translational and nontranslational *Arabidopsis* FT coding sequences were fused in-frame with the GFP coding sequence of PVX/GFP to produce PVX/GFP-FT and PVX/GFP-mFT (Fig. 6A), respectively. In repeated experiments, 24-day-old *Nicotiana benthamiana* plants were mock inoculated (Fig. 6B) or infected with RNA transcripts produced by in vitro transcription from PVX/GFP (Fig. 6C), PVX/GFP-FT (Fig. 6D), or PVX/GFP-mFT (Fig. 6E). Local infection of the inoculated leaves induced GFP-expressing green lesions (ca. 10 lesions per inoculated leaf) 3 to 5 dpi. However, only plants challenged with PVX/GFP-FT or PVX/GFP-mFT quickly established systemic infection at 7 dpi, showing GFP green fluorescence in newly developing young leaves. Development of systemic symptoms in plants infected by PVX/GFP took 2 to 3 extra days. Furthermore, the accumulations of free GFP and GFP-FT fusion proteins (Fig. 6F) and viral CP (Fig. 6G) in the inoculated and systemic young leaves after mock inoculation (mock) or infection of PVX/GFP (GFP), PVX/GFP-FT (GFP-FT), or PVX/GFP-mFT (GFP-mFT) were consistent with the development of viral symptoms. Western blot assays of total proteins extracted from inoculated and systemic young leaves using GFP- and PVX CP-antiserum indicated that only a trace amount of free GFP and PVX CP was detected in the inoculated leaves but not in the systemic young leaves of PVX/GFP-infected plants. However, viral CP as well as the GFP-FT fusion protein or free GFP expressed from PVX/GFP-FT or PVX/GFP-mFT, respectively, could be readily detected in both inoculated and systemic leaves.

We provide here compelling evidence that *Arabidopsis* FT RNA is capable of systemic movement and that FT RNA can also act as a cis transportation carrier for heterologous RNAs. The FT RNA mobile function is independent of the FT protein. Consistent with this idea, an engineered plant RNA virus...
carrying *Arabidopsis* FT RNA spread more quickly than its parental virus to establish infection of young tissues. It should be noted that the CP genes of PVX and TCV participate in virus-plant interactions and contribute to symptom development (5, 10, 20, 45); thus, it was not surprising that the CP-deleted recombinant viruses that were transported through the plant via FT RNA in *cis* could still not start a proper infection.

Moreover, we demonstrate that the systemic mobility of FT RNA is determined by a *cis*-acting element of 102 nucleotides at the 5′/H11032 terminus of FT RNA. The positive identification of *cis*-acting element for FT RNA trafficking provides a unique opportunity to dissect the molecular mechanism that governs cellular RNA signaling in plants. It is possible that the *cis*-RNA sequence may bind host proteins to form an RNA-protein complex.

FIG. 6. *Arabidopsis* FT RNA enhances the systemic spread of PVX. PVX-based FT expression cassettes are schematically represented (A). The translatable and nontranslatable *Arabidopsis* FT coding sequences were fused in-frame with the GFP coding sequence of PVX/GFP by using the BspEI and SalI sites. The unique Xhol site used to produce CP-defective RNA vectors (Fig. 1A) is indicated. Twenty-four-day-old *N. benthamiana* plants were mock inoculated (B) or infected with PVX/GFP (C), PVX/GFP-FT (D), or PVX/GFP-mFT (E). (C to E) Local infection of inoculated leaves (IL) induced GFP-expressing lesions. However, only plants challenged with PVX/GFP-FT or PVX/GFP-mFT quickly established systemic infection at 7 dpi, showing GFP green fluorescence in newly developed young leaves (YL) (D and E). Moreover, protein were extracted from inoculated and systemic young leaves of plants with mock (mock) or viral infection, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and detected by Western blotting with antiserum raised against GFP (F) and PVX CP (G). The positions of free GFP, GFP-FT fusion protein, and the PVX CP are indicated. The production of viral CP, free GFP, and GFP-FT fusion proteins associated with PVX/GFP (GFP), PVX/GFP-FT (GFP-FT) or PVX/GFP-mFT (GFP-mFT) was consistent with symptom development. These data indicate that the *Arabidopsis* FT RNA with or without its translatable capacity enhances systemic spread of PVX.
complex for FT RNA spread. Indeed, some plant and viral proteins can bind RNAs and facilitate their intercellular and long-distance trafficking (26, 27, 41, 42). The discovery that a host RNA molecule can mediate systemic trafficking of heterologous RNAs is also significant. In particular, facilitation of host RNA molecule can mediate systemic trafficking of heterologous RNA (26, 27, 41, 42). The discovery that a method for systemic RNA trafficking.

There is now collective evidence that the Arabidopsis FT protein and its tomato SFT and rice Hd3a orthologs may act as a non-cell-autonomous flower-inducing signal that can move from the end of the vasculature into the meristematic tissue in the shoot apical meristem (7, 18, 22, 23, 29, 30, 37). Elegant experiments wherein the intercellular movement of FT protein is prevented by either a large C-terminal fusion and/or a nuclear localization signal have demonstrated that FT protein needs to move, and is sufficient on its own, to induce flowering (18, 29). FT- and Hd3a-GFP fusion proteins were found to needs to move, and is sufficient on its own, to induce flowering.

The Arabidopsis FT RNA is able to move systemically in contrast to these recent reports where FT RNA movement could not be detected (7, 18, 22, 23, 29, 30), possibly due to the different host plants and experimental systems used. For example, high levels of viral FT expression, even from immobilized viruses in single cells, could increase the levels of systemic FT RNA over the detection limit. Our data raise the possibility that systemic movement of the FT RNA may also contribute to the long-distance florogenic signaling, although the FT protein is still required to trigger flowering, as shown by the nonflowering of PVX/mFT-inoculated plants.

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