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. Whilst 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 non-translatable *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 1-102 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 raises 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 signalling that controls plant development and defence against pathogen infection (25). Hundreds of RNA transcripts have been recently identified in phloem, suggesting phloem-mobile RNAs may act as long-distance signalling molecules in plants (4, 8). Indeed, systemic movement of a homeobox fusion transcript and *GIBBERELLIC ACID-INSENSITIVE* RNA regulates leaf architecture (14, 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 defence (9, 12, 13). 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 distance 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 signalling 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 it also facilitates systemic spread of heterologous green fluorescence 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 localised within the nucleotides 1-102 of the FT mRNA coding sequence.

MATERIALS AND METHODS

Construction of RNA mobility assay (RMA) vectors. The wild-type and mutant Arabidopsis FT genes were RT-PCR amplified using pfu DNA polymerase and primers PP354 and PP356 or PP356 and PP355, digested with BspEI and SalI, and cloned in-frame fused to the GFP coding sequence in the BspEI/SalI 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 SalI and XhoI 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 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 RMA vectors TCV/mFTΔCP, TCV/GFP-FTΔCP and TCV/GFP-mFTΔCP the mutant Arabidopsis FT gene was RT-PCR amplified using pfu DNA polymerase and 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/trFTΔCP-based RMA vectors carrying truncated (tr) FT for mapping the cis-acting element required for FT RNA trafficking were constructed. All RMA constructs were confirmed by nucleotide sequencing. Primers used for construction of RMA vectors are listed in Table 1.

**RNA mobility assays (RMA).** RNA transcripts from each movement-deficient recombinant PVX and TCV vector were produced by in vitro transcription as previously described (34), pre-treated with RNase-free DNase (Promega) and then mechanically inoculated onto N. benthamiana or A. thaliana ft-10 mutant plants at 5-6 or 15-16 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 RMA, total RNAs (50 ng) extracted from inoculated and newly growing young leaves separately collected at 7 days post-inoculation (DPI) were pre-treated with RNase-free DNase (Promega) and used for RT-PCR (30 cycles) detection (34) with primers PP354 and PP356 for FT, PP371 and PP372 for GFP, PP269 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 Zeiss Axiophot microscope with filters for GFP (excitation, 450 to 490 nm; long-pass emission, 520 nm) or under long-wave length ultraviolet light (Upland UVP Model B 100AP) through a
yellow filter (Kodak No. 58), and photographed with a Nikon Coolpix 995 digital camera (38).

For TCV-based RMAs, inoculated, newly growing young leaves and shoot apices were carefully collected at 7 DPI from mock, and viral inoculated *N. benthamiana* and mutant *ft-10 A. thaliana* plants and used for total RNA extraction. Total RNAs were treated with RNase-free DNase (Promega) and RT-PCR (30 cycles) detection was performed using primers PP354 and PP356 for *FT*, PP371 and PP372 for *GFP*, PP433 and PP434 for TCV RNA, PP267 and PP473a for TCV-*mFT* RNA, PP267 and PP228 for TCV-GFP RNA, PP271 and PP272 for 18S rRNA. The resultant specific TCV-mFT RT-PCR products were purified and verified by direct sequencing.

In two separate mapping experiments, plants were inoculated with recombinant viral RNAs that were produced by *in vitro* transcription from each TCV/trFTΔCP-based RMA vectors and pre-treated with RNase-free DNase (Promega). Total RNAs were extracted at 7DPI from inoculated and non-inoculated young leaves, pre-treated with RNase-free DNase (Promega) and used for RT-PCR (30 cycles) assays using primer sets listed in Table 2.

**Transient agroinfiltration assay of RNA mobility** The *GFP-FT* and *GFP-mFT* fusion genes were isolated from PVX/GFP-FT and PVX/GFP-mFT and inserted into between the *Cauliflower mosaic virus* 35S promoter and terminator-polyA signal sequence of a binary vector in *Agrobacterium tumefaciens* LBA4404 (34) to produce 35S-GFP-FT-polyA and 35S-GFP-mFT-polyA. In two experiments, leaves of of 24-days old *N. benthamiana* plants were syringe-infiltrated with *Agrobacterium* cultures carrying 35S-GFP-polyA (34), 35S-GFP-FT-polyA and 35S-GFP-mFT-polyA. At 4 days post infiltration, agroinfiltrated leaf tissues and non-infiltrated newly developed young leaves were collected for protein and RNA analysis.

**Induction of flowering by viral transient expression of the Arabidopsis FT protein.** In three experiments, young short-day *N. tabacum* Maryland Mammoth plants were
mock-inoculated, or infected with PVX/FT, PVX/mFT, PVX/GFP or PVX/GFP-FT and maintained in an insect-free containment glasshouse at 25 °C with a long-day (16-h) photoperiod. Local and systemic symptoms including chlorotic lesions on inoculated leaves and mild chlorosis on young leaves developed after 7-14 days post inoculation (DPI). All PVX/FT-infected plants started to shoot at approximately 20 DPI and flowered at about 35 DPI, were photographically recorded with a Nikon Digital Camera Coolpix 995. 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 (30 cycles) detection (34) with primers PP82 and PP356 for virus-carried mutant FT RNA. The resultant specific RT-PCR products were purified and verified by direct sequencing. The viral transient FT protein was analysed by western blot detection.

**Western blot.** To investigate GFP, FT, GFP-FT fusion protein and PVX CP expression, total proteins were extracted from leaf tissues as described (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 IgG conjugated with alkaline phosphatase (Sigma) and BCIP/NBT substrates (Roche) as described (39).

**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/GFPΔCP)-based RNA mobility assay (RMA) (Fig. 1A). Deletion of viral coat protein (CP) prevented PVX/GFPΔCP movement and restricted viral RNAs to single *N. benthamiana* epidermal cells. Thus, unlike the wild-type virus,
PVX/GFPΔCP 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-FTΔCP and PVX/GFP-mFTΔCP had respectively the translatable or non-translatable *Arabidopsis FT* that was fused in-frame to the GFP coding sequence (Fig. 1A). Individual epidermal cells expressing transient GFP or GFP-FT fusion protein from PVX/GFPΔCP, PVX/GFP-mFTΔCP, or PVX/GFP-FTΔCP showed green fluorescence (Fig. 1A). However, cell-to-cell movement of PVX/GFP-FTΔCP or PVX/GFP-mFTΔCP or intercellular spread of 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. 1, A and B). However, no spread of genomic (g) and subgenomic (sg) RNAs 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 systemically from inoculated leaves to young leaves (Fig. 1C). Furthermore, PVX/GFP-mFTΔCP gRNA and sgRNAs that contained non-sense mutations precluding FT protein synthesis also spread systemically (Fig. 1C), demonstrating that non-translatable 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 neighbouring 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 fluorescent 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 *N. benthamiana* 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. *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* carrying the 35S promoter-controlled *GFP* or *GFP*-tagged *FT* gene expression cassettes, respectively (Fig. 1D). Western blot analysis showed that expression of free GFP occurred in leaves infiltrated with agrobacterium carrying 35S-GFP-polyA and 35S-GFP-mFT-polyA, whilst the larger GFP-FT fusion protein was expressed from 35S-GFP-FT-polyA (Fig. 1E). These data clearly demonstrated that the replacement of the initiation codon with a stop codon in the *FT* open reading frame blocked the translation of FT protein from the 35S-GFP-mFT construct. We also examined the *Arabidopsis FT* sequence and found no internal in-frame start codons, thus, it is unlikely that *mFT* RNA would be translated from internal initiation. Consistent with the results obtained from the viral RMAs, following agroinfiltration, 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 vs cytoplasm) and the means of production of the mobile RNA molecules (35S promoter-controlled transcription vs recombinant viral RNA replication and transcription) in agro-infiltrated 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 short-day (SD) *N. tabacum* Maryland Mammoth tobacco (21) with PVX/FT (Fig. 3A) and PVX/GFP-FT (Fig. 6A). Viral ectopic expression of the GFP-FT fusion protein was unable to induce flowering under non-inducing long-day (LD) conditions (Fig. 3, B and C), however all Maryland Mammoth plants infected with PVX/FT that had the capacity to produce free FT protein flowered in LD, whilst control plants infected with either PVX/mFT carrying a mutated non-translatable FT mRNA or PVX/GFP remained vegetative (Fig. 3, B 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 blot using 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 is not only self-mobile but also it 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 \textit{FT} RNA movement, we used the TCV-based RMA vector TCV/mFT\textDelta CP containing a non-translatable \textit{FT} gene (Fig. 4A). Deletion of the CP gene in TCV\textDelta CP prevents it spreading from infected cells to distal parts of plants (6). However, in contrast to the PVX-based RMA, TCV is able to infect \textit{A. thaliana} (36) and TCV\textDelta CP can establish local infection in this host (unpublished data). We inoculated the \textit{Arabidopsis ft-10} T-DNA insertion mutant (43) with RNA transcripts of TCV/mFT\textDelta CP or TCV/GFP\textDelta CP. In inoculated leaves, accumulation of recombinant viral RNAs from both RMA vectors was readily detected by RT-PCR whilst no specific recombinant viral RNA was detectable in mock-inoculated control plants (Fig. 4B). Not surprisingly, no systemic spread of TCV/GFP\textDelta CP RNA to non-inoculated leaves occurred. However the non-translatable mutant m\textit{FT} RNA in TCV/mFT\textDelta CP moved and facilitated long-distance movement of viral RNAs to young leaves and the shoot apices (Fig. 4B). The non-sense mutation prohibiting FT protein synthesis from TCV/mFT\textDelta CP was maintained in \textit{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 \textit{ft-10} mutant plants, consistent with the mutation in \textit{ft-10} (Fig. 4D). Long-distance trafficking of viral-derived m\textit{FT} RNA from inoculated leaves to non-inoculated leaves and the shoot apices was also detected in \textit{ft-1} mutant plants (X. Liu, C. Li, S. Jackson, Y. Hong. unpublished data). On the other hand, when fusing GFP downstream of FT, the non-sense mutation also eliminates GFP expression (K. Zhang, Y. Hong. unpublished data). Thus, we conclude that the systemic \textit{FT} RNA trafficking does not require the FT protein.

\textbf{Mapping of the cis-acting element within \textit{Arabidopsis} FT RNA.} To elucidate what controls long-distance spread of \textit{FT} RNA, we constructed a series of TCV/\textDelta CP-based RMA vectors carrying truncated non-translatable \textit{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 systemic movement of TCV RNA. This was evident by positive RT-PCR detections of TCV and viral-derived *FT* RNAs in the non-inoculated, newly developing and growing young leaves (Fig. 5B). All the truncated *FT* RNAs possess the nucleotides of 1-102 of *FT* RNA (Fig. 5A). In striking contrast, *FT* RNAs generated from TCV/FT103cΔCP, TCV/FT202cΔCP, TCV/FT301cΔCP and TCV/FT400cΔCP that lack the 5'-terminal 102 nucleotides were completely immobile. Consequently, RT-PCRs failed to detect any viral-derived *FT*-related RNA in the young leaves of virus-treated plants although recombinant viral RNAs accumulated in the inoculated leaves (Fig. 5B). Direct sequencing of RT-PCR products confirmed the identities of the mobile and immobile TCV and viral-derived *FT* RNA molecules (Fig. 5, C and D). Thus, the element required for systemic *FT* RNA movement was unequivocally mapped to the 102-nucleotide sequence at the 5'-terminus of *FT* mRNA.

It should be noted that TCV without CP moves readily in *dcl2/dcl4* mutated *Arabidopsis* that is defective in RNA silencing. Thus, the TCV CP is mainly providing a silencing-suppression 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, 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 Potato virus X. The translational and non-translational *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, twenty-four 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 (approx. 10 lesions per inoculated leaf) 3-5 days post-inoculation (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 2-3 extra days. Furthermore, accumulation of free GFP and GFP-FT fusion proteins (Fig. 6F) and viral CP (Fig. 6G) in the inoculated and systemic young leaves following 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 coat protein-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.

In this report, we provide compelling evidence that Arabidopsis FT RNA is capable of systemic movement and 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′-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 signalling in plants. It is possible that the cis-RNA sequence may bind host proteins to form an RNA-protein 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 viral RNA movement by the Arabidopsis FT RNA raises the possibility that viruses and plants might have evolved similar mechanisms 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 (SAM) (7, 18, 22, 23, 29, 30, 37). Elegant experiments where the inter-cellular movement of FT protein is prevented by either a large C-terminal fusion and/or a nuclear localisation 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 cross a graft union from a donor scion and reach the apex of the recipient plant (7, 37), and FT-derived peptides have been identified in phloem exudates from Cucurbita (23), suggesting that the protein is transported through the phloem. Once the FT protein enters the apical meristem it interacts with the bZIP transcription factor FD to activate floral identity genes such as APETELA 1 (1, 40). However, these studies do not definitely rule out a role for FT mRNA as part of the mobile florigenic signal. In this context, it is worthwhile noting that mRNA of the rice FT ortholog Hd3a has been detected at extremely low levels in shoot apices of rice even though
the HD3a promoter is not active in the SAM (37). The evidence presented here that the Arabidopsis FT RNA is able to move systemically is 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 immobilised viruses in single cells, could increase the levels of systemic FT RNA over the detection limit. Our data raises the possibility that systemic movement of the FT RNA may also contribute to the long-distance florigen signalling although the FT protein is still required to trigger flowering as shown by the non-flowering of PVX/mFT-inoculated plants.

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CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of *Arabidopsis*. Development 131:3615-3526.


Figure Legend

Fig. 1. (A) PVX-based RNA mobility assay (RMA) vectors. Viral 7.0-kb genomic gRNA, 2.6-kb subgenomic sgRNA1, and 1.4-kb GFP-FT sgRNA are indicated. The coat protein (CP) gene was deleted. The positions of a stop codon (*) replacing FT start codon in PVX/GFP-mFTΔCP, and three sets of RT-PCR primers for detections of FT (▶◄, PP354/PP356), GFP (▶◄, PP371/PP372) or PVX (▶◄, PP269/PP373) RNAs are indicated. Individual epidermal cells expressing free GFP or GFP-FT fusion protein showed green fluorescence. (B, 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. RNAs were extracted from leaves of plants mock-inoculated (mock), or inoculated with PVX/GFPΔCP (GFP), PVX/GFP-FTΔCP (GFP-FT), or PVX/GFP-mFTΔCP (GFP-mFT). The 1-kb DNA ladder and sizes of RT-PCR products are indicated. (D) Construction of 35S promoter-controlled FT gene expression cassettes for a transient agro-infiltration assay of RNA mobility. The position of an introduced stop codon (*) replacing the FT gene start codon in 35S-GFP-mFT-polyA is indicated. (E) Expression of free GFP or GFP-FT fusion protein. Total proteins were extracted from N. benthamiana leaves infiltrated with agrobacteria carrying the 35S-controlled gene expression cassette and were analysed by Western blot using a GFP-specific antibody. The positions of free GFP and GFP-FT fusion protein are indicated. (F) FT and GFP mRNAs in non-agroinfiltrated newly developed systemic young leaves were analysed by RT-PCR. Samples are from plants with mock infiltration (mock), or infiltrated with agrobacteria carrying 35S-GFP-polyA (GFP), 35S-GFP-FT-polyA (GFP-FT), or 35S-GFP-mFT-polyA (GFP-mFT). The sizes of RT-PCR products, free and GFP-FT fusion proteins and the pre-stained protein markers or 1-kb DNA ladder are indicated.
Fig. 2. The *Arabidopsis* FT RNA supports TCV RNA movement. (A) GFP-tagged movement-deficient *Turnip crinkle virus* (TCV)-based RNA mobility assay (RMA) vectors. The position of an introduced stop codon (*) replacing the *FT* gene start codon in TCV/GFP-mFTΔCP and three sets of RT-PCR primers for detections of *FT* (►◄, PP354/PP356), *GFP* (►◄, PP371/PP372) or TCV (►◄, PP433/PP434) are indicated. (B, C) RT-PCR analysis of *FT*, *GFP* and TCV RNA and 18S rRNA (18S) in inoculated (B) and newly formed young leaves and shoot apices (C) of *N. benthamiana* plants. Total RNAs used as RT-PCR templates were extracted from inoculated or young leaves of plants with mock inoculation (mock), or inoculated with RNA transcripts from TCV/GFPΔCP (GFP), TCV/GFP-FTΔCP (GFP-FT), or TCV/GFP-mFTΔCP (GFP-mFT). The 1-kb DNA ladder and the sizes of RT-PCR products are indicated.

Fig. 3. Ectopic expression of *FT* induces flowering. (A) The translatable and mutated (*) non-translatable *Arabidopsis* FT coding sequences were cloned into wild-type PVX vector to produce PVX/FT and PVX/mFT, respectively. (B, C) Floral induction caused by viral expression of FT protein. Young short-day *N. tabacum* Maryland Mammoth plants were mock-inoculated, or infected with PVX/FT or PVX/mFT and grown under non-inducing long-day photoperiod. Twelve plants infected by PVX/FT in three separate experiments started bolting at approximately 20 days post-inoculation (DPI), and flowered at about 35 DPI, and were photographed at 42 DPI (B and inset). Tobacco mock-inoculated, or infected with PVX/mFT, PVX/GFP or PVX/GFP-FT did not flower (B, C). (D) Detection of viral transient *FT* RNA. Viral transient *FT* RNA was detected by RT-PCR using primers PP82 (►) and PP356 (◄) in systemic young leaves from two separate plants infected with PVX/mFT (mFT) or PVX/FT (FT), but not in mock (mock). The position and the sizes of 1-kb DNA ladder (lane M) are indicated. Direct sequencing of RT-PCR products (648 bp) verified the presence
of viral-expressed wild-type and mutant *FT* RNA in flowering and non-flowering plants respectively. The native *FT* ATG (underlined) in PVX/FT and its TAG replacement (underlined) together with a nucleotide deletion (double-arrow) in PVX/mFT are indicated.

**Fig. 4.** (A) Movement-deficient *Turnip crinkle virus* (TCV)-based RNA mobility assay (RMA) vectors. The parental TCV vector and long-distance movement-deficient TCV/GFPΔCP were previously constructed (34). The position of the introduced stop codon (*) replacing ATG in the *FT* gene in TCV/mFTΔCP and two sets of RT-PCR primers are indicated. (B, C) RT-PCR analysis of *FT-TCV* and *GFP-TCV* RNA (top panel) using primers PP267/PP473a (►◄) or PP267/PP228 (►◄), and 18S rRNA (bottom panel) in inoculated (In) and newly formed young leaves (M1 and M2) and shoot apices (Ap) of ft-10 A. thaliana mutant plants. RNAs were extracted from tissues of plants mock-inoculated (mock) or inoculated with TCV/GFPΔCP (GFP) or TCV/mFTΔCP (mFT). The positions and sizes of the 1-kb DNA ladder are indicated. (C) Direct sequencing of mFT RT-PCR products (372 bp) generated from total RNA extracted from young leaves of *Arabidopsis* mutant plants inoculated with TCV/mFTΔCP. (D) Western blot analysis indicated no endogenous or viral transient FT protein was expressed in the mock-inoculated (mock) or different leaves (In, M1, M2) and shoot apices (AP) of ft-10 plants inoculated with TCV/mFTΔCP. Viral ectopic expression of FT protein (red-asterisk) was detected in *N. tabacum* Maryland Mammoth plants infected with PVX/FT (FT) but not with PVX/mFT (mFT), consistent with that the FT protein is required for floral induction (Fig. 3B). Coomassie blue stained gel indicates the equal loading of protein samples. The positions and sizes of the protein markers are indicated.

**Fig. 5.** Functional mapping of the *cis*-acting element that controls the *FT* RNA movement. (A) TCV/trFTΔCP-based RMA vectors carrying truncated (tr) non-translatable *Arabidopsis*
FTs. (B) Detection of viral-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 viral-derived FT RNA was detected in mock-inoculated plants. RT-PCR analysis of 18S rRNA (bottom panel) is included as RNA control. (C, D) Direct sequencing of RT-PCR products verified the presence of viral-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 viral-derived FT sequences are indicated.

Fig. 6. The Arabidopsis FT RNA enhances systemic spread of potato virus X (PVX). PVX-based FT expression cassettes are schematically represented (A). The translatable and non-translatable Arabidopsis FT coding sequences were fused in-frame with the GFP coding sequence of PVX/GFP by using the BspEl and SalI sites. The unique XhoI site used to produce CP-defective RMA vectors (Fig. 1A) is indicated. Twenty-four day old Nicotiana benthamiana plants were mocked inoculated (B), or infected with PVX/GFP (C), PVX/GFP-FT (D), or PVX/GFP-mFT (E). Local infection of inoculated leaves (IL) induced GFP-expressing lesions (C-E). However, only plants challenged with PVX/GFP-FT or PVX/GFP-mFT quickly established systemic infection at 7 days post-inoculation, showing GFP green fluorescence in newly developed young leaves (YL) (D, E). Moreover, protein were extracted from inoculated and systemic young leaves of plants with mock (mock) or viral infection, resolved by SDS-PAGE, and detected by Western blot using antiserum raised against GFP (F)
and PVX CP (G). The positions of free GFP, GFP-FT fusion protein and the PVX CP are indicated. Productions 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) were consistent with symptom development. These data indicate that the Arabidopsis FT RNA with or without its translatable capacity enhances systemic spread of PVX.
Table 1 Primers used in this study

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<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Origin and modification</th>
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<td>PP228</td>
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<td>PP267</td>
<td>AGAAAAAGTCATGAAGGTTCTGCTAGCCACGG</td>
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<td>GAGCTGGAATTACCAGGGGCTG</td>
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a Introduced restriction endonuclease sites are in boldface, and the start and mutated non sense stop codons are underlined.
Table 2 Mapping cis-acting element for FT RNA movement

<table>
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<tr>
<th>RMA vectors</th>
<th>FT RNA 5'-3' coordinates</th>
<th>Primers for PCR/cloning</th>
<th>Primers for RT-PCR detection</th>
<th>Predicted sizes of RT-PCR products[^c]</th>
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<td>PP519/PP515</td>
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[^a]: A stop codon was introduced to replace the native FT start codon.

[^b]: A stop codon was placed at the immediate upstream of the truncated FT RNA. Thus, all truncated FT RNAs produced from the eight RMA vectors are non-translatable.

[^c]: The predicted size of each RT-PCR product includes TCV and cloning sequences (273 bp) plus the corresponding truncated FT sequence.
Fig. 1

A

PVX/GFP-mFTΔCP

PVX/GFP-FTΔCP

PVX/GFPΔCP

B

Viral inoculated leaf

C

Young leaf

D

E

Agro-infiltrated leaf

F

Non-infiltrated young leaf
Fig. 2

A

TCV

\[ \text{p28} \quad \text{p88} \quad \text{p8} \quad \text{p9} \quad \text{CP} \]

TCV/GFP\( ^\Delta \)CP

\[ \text{p28} \quad \text{p88} \quad \text{p8} \quad \text{p9} \quad \text{GFP} \]

TCV/GFP-FT\( ^\Delta \)CP

\[ \text{p28} \quad \text{p88} \quad \text{p8} \quad \text{p9} \quad \text{GFP} \quad \text{FT} \]

TCV/GFP-mFT\( ^\Delta \)CP

\[ \text{p28} \quad \text{p88} \quad \text{p8} \quad \text{p9} \quad \text{GFP} \quad \text{mFT} \]

B

Viral inoculated leaf

C

Young leaf

\( \text{FT} \)

\( \text{GFP} \)

\( \text{TCV} \)

\( \text{18S} \text{ rRNA} \)

\( \text{530bp} \)

\( \text{420bp} \)

\( \text{502bp} \)

\( \text{185bp} \)
### Figure 4

#### A

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### Notes

- **TCV/GFPΔCP** and **TCV/mFTΔCP** diagrams illustrate the gene expression of TCV variants.
- **B** shows the results of RT-PCR for different samples.
- **C** presents a gel electrophoresis of DNA markers and mFT PRimers.
- **D** compares the protein markers from Maryland and Mammoth with the ft-10 mutant and TCV/mFTΔCP samples.
Fig. 6

(A) PVX/GFP-mFT

PVX/GFP-FT

PVX/GFP

BopEI SalI XhoI

RDRP

25K 12K

GFP CP

mock Protein GFP GFP-FT GFP-mFT

(B) IL YL

(C) IL YL

(D) IL YL

(E) IL YL

(F) GFP-FT GFP mock

(G) PVX CP