Mobile FT mRNA contributes to the systemic florigen signalling in floral induction

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In inducing photoperiodic conditions, plants produce a signal dubbed “florigen” in leaves. Florigen moves through the phloem to the shoot apical meristem (SAM) where it induces flowering. In Arabidopsis, the FLOWERING LOCUS T (FT) protein acts as a component of this phloem-mobile signal. However whether the transportable FT mRNA also contributes to systemic florigen signalling remains to be elucidated. Using non-conventional approaches that exploit virus-induced RNA silencing and meristem exclusion of virus infection, we demonstrated that the Arabidopsis FT mRNA, independent of the FT protein, can move into the SAM. Viral ectopic expression of a non-translatable FT mRNA promoted earlier flowering in the short-day (SD) Nicotiana tabacum Maryland Mammoth tobacco in SD. These data suggest a possible role for FT mRNA in systemic floral signalling, and also demonstrate that cis-transportation of cellular mRNA into SAM and meristem exclusion of pathogenic RNAs are two mechanistically distinct processes.

Photoperiodic flowering plants perceive seasonal changes by producing a phloem-mobile signal, known as florigen, which is transported through the phloem translocation stream to the shoot apical meristem (SAM) where it induces flowering. In Arabidopsis the FLOWERING LOCUS T (FT) gene plays a major role in the induction of flowering by photoperiodic5-6. The FT protein7-10 and its orthologs from rice11, cucumber12 and tomato13 have been shown to be a component of the systemic flowering signal. However, using novel RNA mobility assays based on two distinct movement-defective RNA viruses, Potato virus X (PVX) and Turnip crinkle virus (TCV), we have recently demonstrated that the Arabidopsis FT mRNA, independent of the FT protein, moves systemically through the plant and in cis promotes trafficking of heterologous RNAs from one leaf to another14. An RNA ‘zip code’ mapped to the 5' nucleotides 1 to 102 of the FT mRNA coding sequence with predicted stem-loop structures is responsible for its mobility14. These findings provide new insights about the nature of the mobile florigen15, 16. However, whether the FT mRNA is capable of moving into the shoot apex is still unknown. This is an important question that needs to be addressed in order to elucidate the biological significance of the systemic movement of FT mRNA in the context of long-distance florigenic signalling.

In plants, there are three types of mobile RNAs including pathogenic viral and viroid RNAs, small RNAs (siRNA and microRNA), and cellular RNA transcripts17. Indeed, thousands of cellular transcripts have been recently identified in the Arabidopsis phloem, suggesting that these RNAs are capable of long-distance trafficking and may function as systemic signalling molecules18, 19. However not all of the phloem-based transcripts moves into meristematic cells and tissues. Only a few cellular RNAs such as the tomato KNOTTED1-like homeobox fusion transcript and the RNA for GIBBERELIC ACID-INSENSITIVE have so far been shown to move through the phloem and into the meristem in Arabidopsis and tomato20-22. These findings suggest that plants may have evolved a selective mechanism to enable phloem-mobile RNA to access the shoot apex. However, whether common or distinct mechanisms are involved in both the selective meristem entry of cellular RNAs and the meristem exclusion of plant viral RNAs remains to be elucidated.

Using virus-induced gene (RNA) silencing (VIGS) and in situ immune-detection assay, we present evidence that the Arabidopsis FT mRNA can move into the shoot apex of Nicotiana benthamiana and enable recombinant
PVXs carrying the FT mRNA to overcome meristem exclusion and enter the SAM. The FT mRNA-mediated meristem entry does not require the FT protein. The underlying mechanism by which the FT mRNA moves and transports recombinant PVX into the shoot apex does not involve suppression of RNA silencing that is known to mediate meristem exclusion of plant viral RNAs23–27. Furthermore, viral transient expression of a non-translatable FT RNA is able to induce earlier flowering in the short-day (SD) N. tabacum Maryland Mammoth (MM) plants in inducing SD, which demonstrates that the mobile FT RNA plays a role in the induction of flowering.

**Results**

*Arabidopsis FT RNA promotes VIGS in SAM*. Most plant cells and tissues are vulnerable to virus infection. However, the apical meristem is generally free of viral invasion24. Recent studies have demonstrated that RNA silencing plays an essential role in meristem exclusion of PVX and other viruses, and that VIGS can be effectively triggered by viruses in leaves, stems and roots, but not in the apices23–27. Exploiting these specific properties associated with virus infection and VIGS, we utilised a series of recombinant PVX constructs (Fig. 1a) and a non-conventional approach involving VIGS, to investigate whether FT mRNA could move into the shoot apex. In mock-inoculated GFP transgenic *N. benthamiana* line 16c plants, GFP green fluorescence was readily visible in leaves and stems of the whole plant under long-wavelength ultraviolet light (Fig. 1b). However inoculating transgenic plants with the PVX/GFP construct expressing GFP induced VIGS of GFP mRNA, and GFP-silenced tissues showed red chlorophyll auto-fluorescence in leaves and stems (Fig. 1c). Similarly, PVX/FT-GFP and PVX/mFT-GFP constructs, expressing a translatable and non-translatable *Arabidopsis FT* mRNA (the entire coding sequence of 525 nucleotides without any 5’ or 3’ UTR) fused in frame to the GFP coding sequence respectively (Fig. 1a), were also able to trigger local and systemic GFP RNA silencing (Fig. 1d, e). PVX/GFP-mediated VIGS could not spread into the shoot apex and the surrounding emerging leaves, in which the endogenous transgenic GFP expression remained strong (Fig. 1f).

In striking contrast, both PVX/FT-GFP and PVX/mFT-GFP were able to promote GFP mRNA silencing in these tissues demonstrated by the remarkably weaker GFP fluorescence (Fig. 1f). Further examination by confocal microscopy confirmed GFP fluorescence in the shoot apices of line 16c plants which were either mock-inoculated (Fig. 1g) or infected with PVX/GFP (Fig. 1h), indicating that no VIGS of GFP mRNA occurred. However the PVX/FT-GFP and PVX/mFT-GFP constructs were able to enter the shoot apex and effectively induce VIGS of GFP expression (Fig. 1i, j). Moreover, PVX/GFP-FTn102 carrying the 102-nt FT RNA movement domain fused to GFP was also found to be capable of moving into and promoting VIGS of GFP expression in the shoot apex (Fig. S1). These phenomena were observed in all 6–9 plants treated with different recombinant viruses, suggesting that the *Arabidopsis FT* mRNA, independent of the FT protein, can move into the shoot apex and is also able to promote entry of associated plant viral RNAs into the shoot apex and induce VIGS there.

*Arabidopsis FT RNA overcomes meristem exclusion of PVX*. PVX/GFP, like wild-type PVX, infects *N. benthamiana* effectively. Systemic infection of plants appeared at 7 – 9 days post-inoculation (dpi). Extensive spread of PVX/GFP throughout infected plants at 12 dpi was readily detectable by GFP green fluorescence. However, in the shoot apex no GFP fluorescence was observed (Fig. S2). Exclusion of PVX/GFP from the SAM was further demonstrated by *in situ* immunocytochemical assays using an antibody specifically raised against the PVX coat protein (CP). CP-antibody probing of sections of 4 *N. benthamiana* plants with severe systemic symptoms following PVX/GFP infection at 12 dpi showed that PVX/GFP was transported via the phloem to the base of the shoot apex where pink staining of the antibody was evident. PVX/GFP was absent, and no virus was detected, in the SAM of the same infected plants (Fig 2a). However, insertion of the *Arabidopsis FT* mRNA coding sequence (528 nucleotides including the stop codon) into the viral positive sense RNA

### Figure 1 | *Arabidopsis FT* RNA enables virus-induced RNA silencing (VIGS) in shoot apex. (a) A diagrammatic representation of recombinant PVX vectors. The introduced stop codon (*) that replaces the FT start codon in the FT mutant *mFT* to prevent translation is indicated. Gene of interest (GOI) indicates the position of individual gene insertion in the PVX genome. (b–d) Systemic VIGS of GFP expression in leaves and stems of the GFP transgenic *Nicotiana benthamiana* line 16c plants. Plants were mock-inoculated (b), or inoculated with PVX/GFP (c), PVX/FT-GFP (d) or PVX/mFT-GFP (e). (f) VIGS in stem tips of line 16c plants mock-inoculated (mock), or infected with PVX/GFP (GFP), PVX/FT-GFP (FT-GFP), or PVX/mFT-GFP (mFT-GFP). (g–h) VIGS in shoot apices and surrounding young leaves of line 16c plants mock-inoculated (g) or infected with PVX/GFP (h), PVX/FT-GFP (i), or PVX/mFT-GFP (j). Photographs were taken at 21 days post-inoculation under long-wavelength UV illumination through a yellow Kodak No. 58 filter (b–f), and using a Zeiss LSM710 Laser Scanning Microscope (g–j) through transmitted light (TM) to show the outlines of the shoot apices and their surrounding tissues, or through lasers to show green (GFP) and red (Chlorophyll) fluorescence. The merged images of green and red fluorescence (GFP + Chlorophyll) are displayed (g–h). GFP-expressing tissues showed green fluorescence and tissues with silencing of GFP mRNA by VIGS appeared red due to chlorophyll fluorescence. Shoot apices and surrounding tissues are indicated by arrows. Bar = 1 mm.
genome enabled PVX/FT to invade the shoot apex. This recombinant virus was constantly found to be present in different types of cells and tissues (pink staining), including the meristematic layers of the apical dome and leaf primordia (Fig. 2b). Moreover, a non-translatable FT mRNA also enabled the entry of PVX/mFT into the shoot apex (Fig. 2c) of all infected plants.

Western analysis using specific antibodies raised against PVX CP, Arabidopsis FT or GFP, was used to confirm the presence/absence of these proteins in virus-infected plants. PVX CP was readily detected in all infected plants, but not in the mock-inoculated control (Fig. 3a), consistent with systemic viral symptom development observed in these plants. Whilst viral expression of GFP was only observed in plants infected with PVX/GFP (Fig. 3b), expression of the Arabidopsis 19.8 kDa FT protein, however, was only detectable in PVX/FT but not PVX/mFT or PVX/GFP infected plants (Fig. 3c). Considering the equal loading of total soluble cellular proteins in each lane (Fig. 3d), these data support the conclusion that the acquired mobility of PVX/FT and PVX/mFT into the shoot apex is directly associated with FT mRNA and is independent of FT protein.

Taken together, our data demonstrate that whilst PVX is normally excluded from the shoot apex, FT RNA, no matter whether it possesses the capability to translate the FT protein or not, functions in cis to overcome the viral meristem exclusion mechanism.

Ectopic expression of a non-translatable Arabidopsis FT mRNA triggers early flowering. To address the potential significance of FT RNA trafficking in floral induction, we exploited a virus-induced flowering assay in SD N. tabacum MM tobacco14. In SD inducing conditions, mock-inoculated MM plants bolted, produced floral buds and eventually open flowers (Fig. 4a). Remarkably, PVX/mFT-treated MM plants started to bolt approx 1–2 weeks earlier than mock inoculated control MM plants. Viral expression of the non-translatable Arabidopsis FT mRNA also resulted in early budding and flowering (Fig. 4a, Fig. 5a). RT-PCR analyses confirmed that the non-translatable FT mRNA was present in PVX/mFT-treated, but not mock-inoculated MM plants (Fig. 4b). The double mutations that preclude translation of the FT protein were maintained in the recombinant viral RNA, as shown by direct sequencing of the RT-PCR product (Fig. 4c). Consistent with these data, western blotting detected no Arabidopsis FT protein in these MM plants although the FT protein was readily detectable in MM plants infected with PVX/FT (Fig. 4d–f). On the other hand, in long-day (LD) non-inducing conditions, all MM plants that had been mock-inoculated or infected with PVX/mFT neither bolted nor flowered, and remained vegetative (Fig. 4g, h), although expression of wild-type FT mRNA and the free FT protein from PVX/FT was able to stimulate early flowering in both LD (Fig. 4i) and SD (Fig. 4j–l, Fig. 5b). These data suggest that the non-translatable Arabidopsis FT mRNA alone is not a direct inducer or gene expression regulator that is essential for MM plants to flower as it does not induce flowering in non-inducing LD conditions. Instead, the non-translatable FT mRNA promotes earlier flowering, perhaps by acting as a transporter that facilitates the long-distance trafficking of the endogenous tobacco FT protein from the leaf to the SAM where it triggers flowering in SD.

Discussion

Arabidopsis FT mRNA, independent of the FT protein, is capable of long distance movement and can act as a cis transportation carrier for heterologous RNAs35. In this report, we further demonstrate that FT mRNA can move into the SAM, and is also able to promote entry into the SAM of recombinant viruses that carry translatable (wild-type) or non-translatable FT mRNA sequences (Figs. 1–3). However, the
Figure 4 | Viral transient expression of a non-translatable mFT RNA triggers early flowering. (a) Virus-based flowering assay. MM plants were either mock inoculated (mock) or treated with PVX/mFT (mFT) and grown under SD with a 12-hr photoperiod of light at 25 °C. An arrow indicates a flower bud at the shoot tip of a mock-inoculated plant whilst a grown under SD with a 12-hr photoperiod of light at 25 °C. An arrow indicates a flower bud at the shoot tip of a mock-inoculated plant whilst a.

Photographs were taken at 10 weeks post-inoculation (wpi). (b) RT-PCR detection of viral transient mFT RNA in MM plant treated with PVX/mFT (mFT), but not mock inoculation (mock). The 1-Kb DNA marker (M) (1500, 1000, 750, 500 and 400-bp from top to bottom) is included to show the expected size (684 bp) of the RT-PCR product. (c) Direct sequence of the RT-PCR product verified the viral transient mFT RNA, showing the double mutations. Mutation of the native FT start codon ATG with TAG (asterisked) and a nucleotide deletion (Δ) are indicated. The codon triplets are underlined. (d–f) Western blot detection of the Arabidopsis FT protein (d) in MM plants treated with PVX/FT (FT), but not with mock inoculation (mock) or PVX/mFT (mFT); of the PVX coat protein (e) in plants treated with PVX/mFT or PVX/FT, but not in mock-inoculated plant. Coomassie blue-stained gel indicates the equal loading of protein samples. (f). The positions and sizes of the protein marker (M) are indicated. (g–i) MM plants mock-inoculated (g), or treated with PVX/mFT (h) or PVX/FT (i) were grown under a non-inducing LD 16-hr photoperiod and only plants treated with PVX/FT flowered. Photographs were taken at 10 wpi. (j–l) MM plants mock-inoculated (j), or treated with PVX/mFT (k) or PVX/FT (l) were grown in SD. Plants with PVX/FT treatment developed buds at 4 wpi and flowered at 6 wpi whilst plants mock-inoculated or inoculated with PVX/mFT were only bolting during these periods. Photographs were taken at 6 wpi.

underlying mechanism by which the Arabidopsis FT mRNA transports PVX into the shoot apex remains to be elucidated. Phloem-mobile cellular RNA is known to be selectively taken up into the shoot apex whilst most viruses and viral RNAs are excluded from this region. There is evidence that RNA silencing, a cellular antiviral surveillance system, is involved in viral meristem exclusion. Prevention of meristem invasion by PVX requires RDR6 that is involved in the biogenesis of siRNA which is an immediate response that restricts the systemic spread of viruses into the shoot meristem26, 27. Moreover, RNA viruses are able to invade the shoot apex in plants expressing viral suppressors of silencing23, 24. This raises the possibility that the Arabidopsis FT RNA may suppress silencing to enable entry of PVX/FT or PVX/mFT into the shoot apex. However because both PVX/GFP-FT and PVX/GFP-mFT were able to trigger RNA silencing in leaves, stems and shoot apices (Fig. 1), an silencing suppression mechanism is unlikely to be involved in the movement of these recombinant viruses into the SAM. This idea was further confirmed by a local RNA silencing assay (Supplementary Text; Fig. S3). Therefore, Arabidopsis FT RNA meristem entry occurs independently of RNA silencing suppression.

On the other hand, plants have evolved selective mechanisms that govern entry of RNA into meristems where germ cells arise after vegetative development has ceased27. Protection of the shoot apex from pathogenic RNA invasion and from random endogenous RNAs is essential for plants to ensure the integrity of the germline. Our findings show that FT RNA overcomes selective meristem exclusion and suggest that the mechanism controlling the selective entry of endogenous plant RNAs into the SAM may be different from the silencing-mediated meristem exclusion of viral RNA (Fig. 1; Fig. S3). Such selective entry may require specific host proteins that bind to the endogenous RNA28-30. Thus, the FT mRNA may bind to an escort/gateway protein that moves it across.

There are numerous examples in animal and plant systems for intra- and inter-cellular mobile RNAs31. In animals, mRNAs are transported within the cell in a tightly regulated process to facilitate their function. For instance, the Staufen protein of Drosophila and the bicoid and oskar mRNAs involves RNA-protein, RNA-RNA and protein-protein interactions to determine their transportation, localization and repression of translation of the mRNAs in the egg cell32-34. In plants, intra- and intercellular RNA trafficking also plays an important role in growth, development and responses to stresses34. It has been well documented that specific RNA motifs are required for viroid RNA movement23. Another example includes that the Arabidopsis GA-INSENSITIVE (GAI) RNA constitutes motifs that are essential for its own long distance movement and transportation of heterologous RNA35. Moreover, an RNA-protein complex with six mRNAs and approx. 16 proteins that transports the pumpkin GAI RNA has been identified36 and the key RNA-binding protein has recently been found to be a polypyrimidine tract binding protein, designated CmRBPs30. Nevertheless, the precise nature of the mobile FT mRNA zip code and potential FT RNA-interacting cellular factors that may be required for the systemic florigenic signalling remains to be elucidated.

The long-distance transport of wild-type and mutant non-translatable FT mRNA within plants31 and their ability to enter the meristem raises the question of the biological significance of FT mRNA movement in systemic floral signalling. Both wild-type FT mRNA and FT protein have been detected in the shoot apex of rice at low levels, even though FT is not expressed there31. It has already been established that Arabidopsis FT and its tomato SFT, rice Hd3a and Cucurbit Cm-FTL1/2 protein orthologs7-12 act as a component of the systemic flowering signal. FT-derived peptides have been identified in phloem exudates37-41, suggesting that the protein is transported through the phloem into the shoot apical meristem. Once FT is present in the shoot apex, it interacts with a basic region/leucine zipper transcription factor FD encoded by FLOWERING LOCUS D
to activate floral identity genes such as APETALA 1 and SUPPRESSOR OF OVEREXPRESSION OF CO1, the latter activates LEAFY and induces flowering⁴. These data do not rule out a possible role for FT mRNA as part of, or in promoting movement of, the florigenic signal that moves from the leaf to the shoot apex to induce flowering. However, only half of mock-inoculated plants started to produce floral buds at 9 wpi; and less than 18% of floral buds converted into flowers at 11 wpi. The average numbers of combining floral buds and flowers per plant [25 ± 7 (n = 7) vs 10 ± 9 (n = 9), p = 0.002; 30 ± 5 vs 18 ± 9, p = 0.005] were significantly higher (Student’s t-Test) in PVX/mFT- than mock-treated plants at (before) 10 and 11 wpi, but showed no significant difference (33 ± 3 vs 24 ± 11, p = 0.094) at 13 wpi. These data suggest that viral transient expression of the non-translatable Arabidopsis FT mRNA induced earlier budding and flowering in MM plants in SD. This important baseline information indicates that the FT mRNA and its protein product had a synergetic effect on systemic florigenic signalling.
Methods
Details of methods and materials are provided in Supplementary Information.

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Author contributions
C.L. and M.G. designed and performed experiments; N.S., H.Z. and X.Y. performed researches; T.O., Y.L., H.W. M.V. and S.J. contributed through discussion and revised paper. Y.H. initiated the project, designed and performed experiments and wrote the paper.

Additional information
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