The Cotyledons Produce Sufficient FT Protein to Induce Flowering: Evidence from Cotyledon Micrografting in Arabidopsis

Seong Jeon Yoo, Sung Myun Hong, Hye Seung Jung and Ji Hoon Ahn*

Creative Research Initiatives, Division of Life Sciences, Korea University, Seoul 136-701, Korea
*Corresponding author: E-mail, jahn@korea.ac.kr

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In Arabidopsis, long-distance movement of FLOWERING LOCUS T (FT) protein from the leaf to the shoot apex triggers flower development. In wild-type Arabidopsis plants under long-day conditions, FT is mainly expressed in the cotyledon but is weakly expressed in the first true leaf prior to floral induction. To test the importance of the cotyledon in floral induction, we developed a cotyledon micrografting (Cot-grafting) method that, unlike other grafting methods, allows the FT protein from the graft to be transported via its native route from leaves to the shoot apex. By using Cot-grafting, we found that grafting a single wild-type cotyledon onto an ft-10 mutant strongly suppressed the ft-10 late flowering phenotype. Neither Y-grafting wild-type shoots nor butt-grafting wild-type roots to ft-10 plants resulted in comparably accelerated flowering in the ft-10 recipient plants. ft-10 mutants grafted with a 35S::FT cotyledon flowered as early as wild-type plants. When phloem-specific tracers were applied to a donor cotyledon, the tracers were detected in the vein of the true leaf of recipient plants 6 d after Cot-grafting. Also, macromolecule trafficking of an FT:yellow fluorescent protein:hemagglutinin fusion occurred across the graft junction 6 d after Cot-grafting. These results suggest that Cot-grafting, which allows protein movement in a manner consistent with the natural flow of FT protein from the leaf to the shoot apex, can efficiently suppress the late flowering of ft-10 mutants. Our results further suggest that in Arabidopsis, the cotyledon is an important organ for producing FT protein to induce flowering.

Key Words: Cotyledon • FLOWERING LOCUS T (FT) • Flowering time • Long-distance signaling • Micrografting.

Abbreviations: AP1, APETALA 1; CFDA, 5(6)-carboxyfluorescein diacetate; Cot-grafting, cotyledon micrografting; Ct, threshold cycling; Cv, coefficient of variation; DAG, days after germination; GFP, green fluorescent protein; GUS, β-glucuronidase; HA, hemagglutinin; H33a, heading date 3a; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; MS, Murashige and Skoog; qRT–PCR, quantitative reverse transcription–PCR; YFP, yellow fluorescent protein.

Introduction

The transition from the vegetative phase to the flowering phase, a pivotal event in the life cycle of plants, must be timed correctly to ensure successful sexual reproduction. The optimal timing of this developmental transition is precisely controlled in the plant through the integration of various environmental stimuli, one of which is the inductive photoperiod (Amasino and Michaels 2010, Srikanth and Schmid 2011). Numerous studies have suggested that the leaf is the preferred photoperiod-sensing organ and that it is spatially separated from the site of response, namely the shoot apical meristem, where new floral primordia emerge (Turck et al. 2008). Results from classical grafting experiments indicate the existence of a graft-transmissible substance produced in the leaf following perception of the inductive photoperiodic stimulus in the leaf (Lang et al. 1977).

FLOWERING LOCUS T (FT) encodes a small globular protein (~20 kDa) that acts as a potent floral promoter under long-day conditions (Kardailsky et al. 1999, Kobayashi et al. 1999). FT is expressed in both cotyledons and leaves. Takada and Goto (2003) reported that the expression of pFT::GUS (β-glucuronidase) was seen first in the vascular tissues of the cotyledon and was detected later in the vasculature of the true leaf. FT mRNA is undetectable in the shoot apex, but FT protein interacts with the bZIP transcription factor FD, which is expressed in the shoot apical meristem, resulting in a change in meristem identity to produce floral primordia (Abe et al. 2005, Wigge et al. 2005). Misexpression of FT in the phloem using the SUC2 promoter was able to rescue the late flowering phenotype of ft-10 mutants (Jang et al. 2009). There is a general consensus among plant biologists that FT protein may be the long-sought and elusive florigen—or at least an important component of the florigen (Zeevaart 2008).

Many small molecules, such as small RNAs and peptides, can travel in the phloem (Ding et al. 2003, Kehr and Buhtz 2008, Kehr 2009), and various experimental procedures have been used to study the long-distance signaling of FT protein. One approach has been misexpression studies, such as those using
the SUC2 or SULTR2 promoters (Corbesier et al. 2007, Notaguchi et al. 2008), which drive transgene expression in the phloem. The rationale for misexpression studies is that using transgenes to produce FT expression at spatially distinct locations will provide information on signal transmission to a target organ/tissue. However, the results obtained using this strategy should be interpreted with caution due to the fact that the expression level and expression domain of the transgene driven by the chosen promoters may not reflect the endogenous expression of the gene of interest.

The most prominent and powerful tool currently available for such studies is micrografting, which uses a graft donor and a graft recipient that can be of different genotypes. In Arabidopsis studies, two micrografting methods are commonly used (Turnbull et al. 2002): butt-grafting, which is a single-hypocotyl graft configuration; and Y-grafting, which is a two-shoot graft configuration. Indeed, the graft-transmissible action of FT protein has been well documented in studies in which FT protein has been fused with a tag. An apparent graft-transmissible effect has been seen in grafting experiments using plants overexpressing a FT-green fluorescent protein (GFP) fusion (Corbesier et al. 2007) and T7-tagged FT protein (Notaguchi et al. 2008). However, in the case of grafting experiments using plants expressing an endogenous wild-type FT gene, only a marginal effect has been observed (Turnbull and Justin 2004, Notaguchi et al. 2008).

Evidence from misexpression and grafting experiments has combined to show that FT does translocate from leaves to the shoot apical meristem. For example, the analysis of transgenic Arabidopsis plants expressing FT:GFP fusions and transgenic rice plants expressing Heading date 3a (Hd3a):GFP fusions in the vascular tissues revealed that the fusion protein had translocated to the shoot apex or the region closer to the shoot apex (Corbesier et al. 2007, Tamaki et al. 2007). Jaeger and Wigge (2007) obtained comparable results from their analysis of plants expressing Myc-tagged FT proteins in the vasculature. Similarly, mobile and immobile versions of FT proteins localized in the phloem caused a differential effect on floral induction (Mathieu et al. 2007). Finally, FT proteins were detected in the phloem sap of cucurbits (Lin et al. 2007) and Brassica napus (Giavalisco et al. 2006). These results left little doubt that FT protein does move from the leaf to the shoot apex via the phloem.

In this report, we develop and use a cotyledon micrografting (Cot-grafting) method, showing that simply grafting a wild-type cotyledon onto an ft mutant plant should accelerate flowering. We developed a cotyledon micrografting method (hereafter referred to as Cot-grafting) (Fig. 1A, B; see the Materials and Methods for a detailed description of the procedure) to test whether the grafting of a cotyledon from wild-type plants to ft-10 knock-out mutants (Yoo et al. 2005) would be sufficient to rescue the ft-10 late flowering phenotype. The petioles of a cotyledon of a graft donor and a recipient plant were cut (at an angle of 30–45° with respect to the horizontal) as depicted in Fig. 1A. The cotyledon removed from the wild-type plant was grafted to the excised petiole of the ft-10 mutant (Fig. 1C). After cotyledon surgery, the growth of graft recipient plants grown on soil (Supplementary Fig. S5) and on Murashige and Skoog (MS) solid medium (Supplementary Fig. S6A) slowed down substantially for about 5 d.

The current rate of successful Cot-grafts is very low (<2%), with only a few successfully Cot-grafted plants per day (Supplementary Fig. S2)—despite substantial efforts of a grafting expert in our laboratory, who can generate up to 40 successfully established Y-grafted plants/day (note that Y-grafting is more difficult than butt-grafting). One of the main reasons for this low success rate is that the grafted cotyledon often fails to remain attached to the petiole of the recipient plant because

## Results

### FT expression in the cotyledon before floral commitment under long-day conditions

The spatial expression pattern of FT was examined by using pFT::GUS plants (Takada and Goto 2003) to determine the main expression domain of FT in wild-type plants under long-day conditions before flowering. Under our growth conditions, Arabidopsis seedlings remained in the vegetative phase until 9 days after germination (DAG), based on the morphology of the shoot apex (Supplementary Fig. S1A) and AP1 expression, a molecular marker of flowering (Supplementary Fig. S1C). At 6 DAG, weak pFT::GUS expression was observed in the vascular tissues of the cotyledon (mainly in the distal part) (Supplementary Fig. S1B). With continuing plantlet development, pFT::GUS expression expanded to all of the vascularature of the cotyledon, and its intensity gradually increased (Yamaguchi et al. 2005, Adrian et al. 2010). However, pFT::GUS staining in the true leaves was absent until 7 DAG. FT expression in the true leaves became clearly visible 11 DAG, but it was still weaker than that in the cotyledon. Quantitative reverse transcription–PCR (qRT–PCR) analysis also showed that there was a high level of FT mRNA in the cotyledon at 8 DAG (Supplementary Fig. S1D). Taken together, these results indicated that FT was mainly expressed in the cotyledon before flowering.

### Grafting of a wild-type cotyledon accelerated the flowering of ft-10 mutants

If the cotyledon acts as a source of FT protein prior to flowering, the grafting of a wild-type cotyledon onto an ft mutant plant should accelerate flowering. We developed a cotyledon micrografting method (hereafter referred to as Cot-grafting) (Fig. 1A, B; see the Materials and Methods for a detailed description of the procedure) to test whether the grafting of a cotyledon from wild-type plants to ft-10 knock-out mutants (Yoo et al. 2005) would be sufficient to rescue the ft-10 late flowering phenotype. The petioles of a cotyledon of a graft donor and a recipient plant were cut (at an angle of 30–45° with respect to the horizontal) as depicted in Fig. 1A. The cotyledon removed from the wild-type plant was grafted to the excised petiole of the ft-10 mutant (Fig. 1C). After cotyledon surgery, the growth of graft recipient plants grown on soil (Supplementary Fig. S5) and on Murashige and Skoog (MS) solid medium (Supplementary Fig. S6A) slowed down substantially for about 5 d.

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the cut off petiole of the recipient ft-10 plant still retains its circadian rhythmic oscillation, even without the cotyledon (Supplementary Fig. S3).

The flowering time of Cot-grafted ft-10 mutants was measured to examine the effect of grafting a wild-type cotyledon. As a control, ft-10 mutant cotyledons were grafted to ft-10 mutant plants for comparison of flowering time. We found that the grafting of a wild-type cotyledon resulted in accelerated flowering in the recipient ft-10 mutants (Fig. 2). The ft-10 recipient plants with grafted wild-type cotyledons [this Cot-grafting combination was designated as ‘wild-type > ft-10’ (cotyledon donor > recipient)] flowered with 21.5 ± 2.4 (average ± SD) leaves, whereas control ft-10 > ft-10 plants flowered with 37.3 ± 2.3 leaves. However, neither Y-grafting wild-type shoots nor butt-grafting wild-type roots resulted in a comparable degree of accelerated flowering in the ft-10 recipient plants. This result demonstrated that grafting only the wild-type cotyledon onto recipient ft-10 mutants suppressed the late flowering phenotype of ft-10 mutants.

We also performed butt-, Y- and Cot-grafting using p35S::FT plants as donors to compare the effect of using different organs in grafting experiments. The 35S::FT > ft-10 plants flowered with 15.2 ± 2.0 leaves (Fig. 3), which was as early as wild-type plants. In contrast, ft-10 > ft-10 plants flowered with 39.7 ± 3.3 leaves. In the case of Y-grafting, ft-10 recipient plants (ft-10/35S::FT) flowered with 26.2 ± 3.5 leaves, whereas the control ft-10/ft-10 plants flowered with 38.2 ± 2.4 leaves, consistent with previous reports (Turnbull and Justin 2004, Notaguchi et al. 2008). To examine the effect of Cot-grafting surgery on its own on flowering time, we also compared the flowering time of wild-type > wild-type grafted and intact wild-type plants. The wild-type > wild-type plants flowered with 14.5 ± 1.6 leaves, which is almost identical to the flowering time of intact wild-type plants (13.6 ± 0.9 leaves) (Fig. 3). These results indicated that Cot-grafting itself does not affect flowering time, but grafting a 35S::FT cotyledon led to a stronger acceleration of flowering than Y-grafting 35S::FT shoots.

**Functional phloem re-connection occurred earlier in Cot-grafting than in Y-grafting**

We next studied the establishment of functional phloem re-connection at the graft–graft junction in Cot-grafting by using the phloem-specific tracers 5(6)-carboxyfluorescein diacetate (CFDA) and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) (Notaguchi et al. 2008). The tracers were applied to the cut edge of a donor cotyledon and the fluorescence of the tracers was examined in the true leaves of Cot-graft recipient plants at specific time points after grafting. In the veins of true leaves of recipient plants, CFDA and HPTS fluorescence was not detected at 4 d but became visible 6 d after Cot-grafting (Fig. 4). These results suggested that in Cot-grafting, the establishment of functional phloem re-connection at the graft junction took about 6 d. In contrast, in the case of Y-grafting, fluorescence in the recipient plants
was absent until 12 d after grafting. At 14 d after Y-grafting, HPTS and CFDA fluorescence was detected in the veins of the true leaves of recipient plants, which is consistent with a previous report (Notaguchi et al. 2008). When the tracers were applied to wild-type control plants, the dyes were detected in the other cotyledon about 30–60 min after application on a cotyledon (data not shown). These results indicated that the establishment of functional phloem re-connection at the graft junction occurred earlier in Cot-grafting than in Y-grafting.

**FT:YFP:HA protein moves across the graft junction 6 d after Cot-grafting**

To address the question of whether a macromolecule can move across the graft junction after Cot-grafting surgery, we examined the movement of FT:YFP:HA protein. We generated transgenic plants overexpressing FT fused with YFP:HA and confirmed that they showed early flowering (Fig. 5A). We then grafted cotyledons of 35S::FT:YFP:HA plants onto ft-10 mutants and examined fluorescence in the recipient plants. Using confocal microscopy, we detected YFP fluorescence in the vascular tissue of the petiole of recipient plants at 6 d after Cot-grafting surgery (Fig. 5B, top left). However, we could not observe any fluorescence in ft-10 > ft-10 plants, which were used as a negative control (Fig. 5B, bottom left). These results suggested that macromolecule (FT:YFP:HA protein) trafficking occurred from the donor cotyledon to the recipient plants at the graft junction 6 d after Cot-grafting.

**Removal of both cotyledons from wild-type plants did not dramatically change flowering time**

If the cotyledon is a major source of FT protein, then, in the absence of compensation by other leaves, removal of the cotyledons may delay flowering time. To test whether removal of the cotyledon delays the flowering time of wild-type plants, we removed both cotyledons from 5-day-old wild-type seedlings and measured their flowering time under long-day conditions. We observed no dramatic delay (very minor) in flowering time of the wild-type seedlings with the cotyledons removed (Fig. 6A), which was consistent with the results from earlier studies (Haupt 1952, Paton and Barber 1955). We also analyzed...
FT expression in the remaining tissues of plants with cotyledons removed. Removal of the cotyledons causes growth to slow down, confounding direct comparison of FT expression levels with plants with intact cotyledons. Therefore, we compared FT expression levels in plants that were at an identical growth stage (1.02) (Boyes et al. 2001, Lee et al. 2007) (Supplementary Fig. S4). Interestingly, FT expression in the true leaves was higher (approximately 5-fold) in plants with cotyledons removed than in plants with intact cotyledons, whereas FT expression in whole seedlings without cotyledons was only slightly higher than in seedlings with intact cotyledons (Fig. 6B). We also compared FT expression levels between plants at the same chronological age (day 10, which was 5 d after cotyledon removal). At day 10, plants without cotyledons contained small true leaves due to the overall slowing of growth, whereas plants with intact cotyledons had generated four true leaves. We found that although FT transcript levels were very low in plants with cotyledons removed, high levels of FT transcript were detected in the emerging young true leaves (Supplementary Fig. S6A), which was consistent with the results obtained from plants in an identical growth stage (Fig. 6).

Discussion

In this study, we describe our cotyledon micrografting method and show that the grafting of a wild-type cotyledon onto recipient ft-10 mutants is sufficient to accelerate the flowering of these mutants.

The cotyledon as a main producer of FT protein

FT expression was mainly detected in the cotyledon before flowering. However, weak FT expression could still be seen in the first pair of true leaves prior to floral induction, thereby raising the possibility that FT expressed in the true leaves may also provide FT protein to induce flowering. However, we do not believe that this is the case because at the time of floral transition under long-day conditions, the first pair of true leaves
is actively growing and probably acts as a sink. As a consequence, the weak FT signal produced from this first pair of true leaves is unlikely to be exported efficiently to the shoot apical meristem. Thus, it is unlikely that FT protein produced in the true leaves before flowering plays a major role in inducing flowering.

Cot-grafting can be a useful method to test long-distance signaling to the shoot apex

The need for the Cot-grafting method is based on the observation that butt-grafting and Y-grafting (Turnbull et al. 2002) are inadequate for testing the functionality of the cotyledon, since they are primarily suitable for testing root- and shoot-generated signals, respectively (Turnbull et al. 2002, Turnbull and Justin 2004). Especially in Y-grafted plants, shoot-generated signals should move downward along the hypocotyl axis of the graft donor plants, which is largely inconsistent with natural FT protein movement from the leaf along the petiole to the shoot apex (Turnbull 2011). Although our Cot-grafting protocol had a low efficiency, we were able to demonstrate that the presence of a single wild-type cotyledon was sufficient to accelerate flowering in an ft-10 mutant (Fig. 2). Since the failure to maintain the attached cotyledon was a primary reason for the low success rate, our Cot-grafting could be improved by using organic substances, such as pectin or gelatin, which would act as adhesives. Despite the clear need for further optimization of this technique, we propose that Cot-grafting can be a useful method to test long-distance signaling from the cotyledon to the shoot apex.

Fig. 4 Functional phloem re-connection in Cot-grafted plants. (A) Schematic drawing of application of phloem-specific dyes. The tracer dyes were applied on a donor cotyledon (arrows). Transferred dyes were examined in the recipient plants by confocal microscopy (arrowheads). (B) Time-course detection of phloem-specific dyes (HPTS and CFDA) in Cot-grafted and Y-grafted recipient plants. N.D., not determined. Scale bar = 0.1 mm.
Acceleration of flowering time of ft-10 mutants by grafting a wild-type cotyledon

We observed stronger acceleration of flowering time of ft recipient plants by grafting a wild-type cotyledon than was observed for previously reported grafting experiments (42.4% reduction in Cot-grafting (37.3 to 21.5 leaves) vs. 10.0% reduction in leaf numbers of ft-1 plants (Notaguchi et al. 2008) and about 10% reduction in the number of days to flower emergence in ft-7 recipient plants (Turnbull and Justin 2004)). There are several possible explanations for why Cot-grafting led to a stronger effect in accelerating flowering. First, the length of time needed to establish a functional phloem connection may be a reason. In Cot-grafted plants, it took 6 d (Fig. 4), whereas in Y-grafted plants it took 14 d, which is consistent with findings of a previous report (Notaguchi et al. 2009b). The difference in time required for reconnection may be due to the different forms of adhesion that occur at the graft interface. In Cot-grafting, section-to-section adhesion takes place, whereas in Y-grafting, wedge-to-cleft adhesion occurs. Secondly, the growth of recipient plants seemed to be arrested for about 5 d after cotyledon removal (Supplementary Fig. S5); during this time, leaf production is likely to be repressed in the recipient plants. In contrast, in Y-grafting, recipient plants still produce leaves without a supply of FT protein from a donor shoot due to the delayed establishment of phloem continuity. Finally, the distance that FT protein moves from a donor part to the shoot apex of a recipient plant is shorter in Cot-grafting than in Y-grafting. Thus, a hypothetical threshold level of FT protein that can induce flowering can be reached more rapidly in Cot-grafted recipient plants.

**No dramatic effect of cotyledon removal on flowering time**

If the cotyledon is the main source of FT protein to induce flowering, it is expected that cotyledon removal would severely delay flowering. However, no dramatic change in flowering was observed (Fig. 6). This suggested that there is a compensating part(s) that redundantly produces FT protein when the cotyledon is absent. Indeed, we observed high FT expression in the true leaves in plants whose cotyledons were removed. It is thus possible that the increase in FT expression in the true leaves compensated for the loss of the cotyledon, thereby potentially explaining why the flowering time of plants with cotyledons removed was not dramatically delayed. The question of...
whether FT up-regulation in the true leaves is part of a possible compensation mechanism remains to be answered. Considering that classical studies suggested the cotyledon and the leaf as the sources of florigen (Lang 1952, Zeevaart 1976), a more likely scenario is that both cotyledons and leaves are required for the production of sufficient amount of FT protein to induce flowering.

In summary, the results of our study demonstrate that grafting of a wild-type cotyledon with the endogenous FT::GUS line has been described previously (Takada and Goto 2003). Seeds used in this study were sown under sterile conditions onto solid MS medium (Murashige and Skoog 1962) (1/2× MS salt, 0.7% phyto-agar and 1.5% sucrose), and the plates were placed at 4°C in the dark for 2 d. Germinated seedlings were then grown in vertical plates in a growth chamber (23°C) under long-day conditions (16:8 h, light:dark). Butt- and Y-grafting experiments were performed according to Notaguchi et al. (2009b) and Turnbull et al. (2002), with the exception that MS medium was used for grafting surgery instead of filter paper.

**Cot-grafting experiments**

For the Cot-grafting experiments, 5-day-old wild-type Arabidopsis plants grown on MS medium under long-day conditions were used as the graft donors. The Cot-grafting surgery was carried out on solid MS medium under a Nikon SMZ1000 dissection microscope. Throughout the Cot-grafting surgery, it was important not to apply excessive pressure in order to prevent damage to the cotyledon when picking the cotyledon. We used ring forceps (#11101-09; Fine Science Tools) for the purpose. A petiole of a cotyledon of graft donor plants was cut using a razor blade (Fig. 1). Sectioning to produce a larger cross-sectional area was key to successful adhesion at the graft interface during the Cot-grafting experiments. The dissected cotyledon was placed on solid MS medium to prevent desiccation during the preparation of the graft recipient plants. For the graft recipient plants, a cotyledon was removed at the same angle as described for the graft donor plant. Recipient plants were positioned upright on MS medium. The cotyledon removed from the donor plants was handled with care to avoid any wounding because the cotyledon is very susceptible to

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**Fig. 6** The effect of cotyledon removal on flowering time in wild-type plants. (A) Comparison of the flowering time of wild-type plants with intact cotyledons (n = 29) and plant with cotyledons removed (n = 39) under long-day conditions. A plus (+) sign indicates plants with the cotyledons present, whereas a minus (−) sign indicates plants with the cotyledons removed. Error bars indicate the standard deviation. (B) An increase in FT expression levels in the true leaves in wild-type plants with the cotyledons removed. A plus (+) sign indicates plants with the cotyledons present, whereas a minus (−) sign indicates plants with the cotyledons removed. Error bars indicate the standard deviation.
damage. The donor cotyledon was placed on the tips of the forceps and carefully transferred to the section of the petiole of graft recipient plants (ft-10) (Columbia background) (Yoo et al. 2005). Caution was used when placing a cotyledon onto the petiole of a recipient plant because if the donor cotyledon was misaligned it was very hard to fix the misalignment. Incorrect alignment seemed to lead to a delay in functional phloem re-connection. In addition, extra care was taken not to allow grafted cotyledons to contact the surface of the solid MS medium, as this could also lead to the detachment of the grafted cotyledon.

After the grafting surgery, Petri dishes containing grafted plants on solid MS medium were sealed with micro-pore tape (3M Healthcare) to maintain humidity. The Petri dishes were returned to the growth chamber (long days) and allowed to recover for 7 d. During the recovery period, most of the donor cotyledons became detached a few days after the surgery due to a circadian oscillation of the petiole of the cotyledon of the graft recipient plants. Grafted plants with the graft donor cotyledons attached were selected for further studies and transferred onto soil covered with a plastic dome for 7 d to maintain humidity in a growth chamber (23°C) under long-day conditions (16:8 h, light:dark).

qRT–PCR analysis
The qRT–PCR protocol has been published in detail elsewhere (Hong et al. 2010). The threshold cycle (Ct) and PCR efficiency of the primers used were calculated using LinRegPCR (Ramakers et al. 2003). The relative abundance of the transcripts was calculated using the statistical formula from geNorm. From three technical replicates, the coefficient of variation (Cv) was calculated according to the following formula: 

\[ Cv = 100 \times \frac{SD \text{ of } Ct}{\text{average of } Ct} \]

where Cv is the coefficient of variation, SD is the standard deviation, and average is the arithmetic mean of Ct values. The Ct and Cv values of each sample were then examined. If a Cv value in a sample was >2.0%, which indicated that there was a reaction that significantly deviated from the mean in three technical replicates, this sample was considered an outlier and was thus excluded from further analyses.

Total RNA was isolated from whole seedlings and individual tissues using Plant RNA Purification Reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA treated with DNase I. The qRT–PCR analysis was carried out in 384-well plates with LightCycler 480 (Roche Applied Science) using SYBR green to monitor the PCR amplification. Two reference genes (AT1G13320/AT2G28390) that are stably expressed (Hong et al. 2010) were used for normalization. The JH6350q (5’-AGCCACTCT CCCTCTGACAA-3’) and JH6351q (5’-AGGAGCAGTGGGATCAGCAG-3’) primers were used to detect FT mRNA. The JH6323 (5’-AGAGACGATGGGATCAGCAG-3’) and JH6324 (5’-AGGAGCAGTGGGATCAGCAG-3’) primers were used to measure transcript levels of AP1. All qRT–PCR experiments were carried out in two or three biological replicates with three technical triplicates each, with similar results.

Loading and detection of phloem-specific tracers
Two phloem-specific tracer dyes, CFDA (C8166; Sigma) and HPTS (H1529; Sigma), were used to determine how long it took for functional phloem re-connection in Y-grafting and Cot-grafting experiments. Concentrations of the dyes and their application onto the donor cotyledon were performed as previously described (Notaguchi et al. 2008). After application of tracer dyes, the fluorescence of the dyes in the true leaves of graft recipient plants was examined by fluorescence microscopy.

YFP fluorescence analysis and microscopy
For the detection of trafficking of a macromolecule, plants expressing the 35S::FT::YFP:HA construct (pSJY0052) were generated. Cot-grafting was performed with 35S::FT::YFP:HA plants as cotyledon donors and ft-10 plants as recipients. YFP signal in the petiole of the recipient plants was examined under a confocal microscope (CLSM 510 Meta, Carl Zeiss). Whole plants were photographed using a Nikon SMZ1000 dissection microscope coupled with a Canon EOS Rebel T1i camera.

For longitudinal sections of the shoot apical meristem, the shoot apex region was fixed and embedded in paraffin. Paraffin-embedded tissue was sectioned using a Leica RM2145 rotary microtome (8 μm). After deparaffinization, tissue sections of the shoot apex region were examined using an Axioscope 2 plus microscope and Axi Imager M1 (Carl Zeiss).

Supplementary data
Supplementary data are available at PCP online.

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