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REGULAR PAPER

Adaptation of a seedling micro-grafting technique to the study of long-distance signaling in flowering of *Arabidopsis thaliana*

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Abstract Long-distance signaling via phloem tissues is an important mechanism for inter-organ communication. Such communication allows plants to integrate environmental information into physiological and developmental responses. Grafting has provided persuasive evidence of long-distance signaling involved in various processes, including flowering, tuberization, nodulation, shoot branching, post-transcriptional gene silencing, and disease resistance. A micro-grafting technique to generate two-shoot grafts is available for young seedlings of *Arabidopsis thaliana* and was adapted for use in the study of flowering. Histological analysis using transgenic plants expressing β-glucuronidase (GUS) in phloem tissues showed that phloem continuity between a stock and a scion was established between 7 and 10 days after grafting. Experiments using tracer dyes and enhanced green fluorescent protein (EGFP) showed that the phloem connection was functional and capable of effecting macromolecular transmission. Successful grafts can be obtained at high frequency (10–30%) and selected after 2–3 weeks of post-surgery growth. This method was applied successfully to the study of flowering, one of the important events regulated by long-distance signaling. This grafting technique will facilitate the study of the long-distance action of genes involved in various aspects of growth and development, and in transport of signal molecules.

Keywords Arabidopsis · Grafting · Long-distance signaling · Flowering

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Introduction

Grafting is a method in which two or more living plant parts are joined by regenerated tissues to support their growth as a single plant. It has been in use since at least 1000 BC for agricultural and horticultural purposes, including vegetative propagation and modification for disease resistance and hardiness (Hartmann et al. 1990). Grafting, as a convenient surgical method to make genetic, physiological, and inter-specific chimeras, has also proved a useful experimental tool for the study of various aspects of plant biology.

The vascular system serves both as a long-distance communication network and a transport pathway for water and nutrients. It provides an important mechanism for inter-organ communication that allows plants to integrate environmental inputs into physiological and developmental responses (Lough and Lucas 2006). Environmental inputs are sensed by mature organs and the signals generated in the sensing organs are then transported to the meristematic regions where newly formed organs adopt a developmental

fate to better adapt to the environment in which they will develop and function. Grafting experiments have provided persuasive evidence that long-distance signaling is involved in diverse developmental and physiological processes, including photoperiodic flowering (Zeevaart 1976), tuberization (Jackson 1999), nodulation (Oka-Kira and Kawaguchi 2006), leaf development (Haywood et al. 2005; Kim et al. 2001), shoot branching (Beveridge 2006), and defense against pathogens (Palauqui et al. 1997). Recent studies of the phloem sap content have revealed the presence of numerous RNA transcripts and proteins in the phloem translocation stream (Lough and Lucas 2006). These findings have provided an emerging insight into the potential functions of such macromolecules in long-distance signaling through the phloem in various processes (Lough and Lucas 2006).

Regulation of flowering, especially by a photoperiod, is one of the classical processes involving long-distance signaling that has been studied using grafting in a variety of plants (Zeevaart 1976; Suárez-López 2005). Rapid cycling accessions of Arabidopsis (*Arabidopsis thaliana*) and various mutants derived from them have provided a wealth of material on which the current genetic regulatory framework of flowering has been built (Simpson and Dean 2002). In the garden pea (*Pisum sativum*), flowering-related mutants have been extensively used in grafting experiments to show the genetic control of production and perception of graft-transmissible signals in flowering (Weller et al. 1997), although the nature of the respective genes is not yet known in most cases. The combination of a grafting technique and mutants of genes of known molecular identity available in Arabidopsis should provide a powerful tool with which to study long-distance signaling in flowering. However, the small size and rosette growth habit with very short internodes during the short vegetative growth phase in laboratory strains of Arabidopsis have made them unsuitable for the application of grafting to the study of long-distance signaling in flowering. Several grafting methods in Arabidopsis, such as grafting of inflorescence stems (Tsukaya et al. 1993; Rhee and Somerville 1995; Flaishman et al. 2008) or mature vegetative plants at the rosette stage (Ayre and Turgeon 2004; Chen et al. 2006), have been reported. Although these are very useful in some studies, they are not suitable for the study of developmental processes during the seedling stage, such as the floral transition. By contrast, recently reported micrografting techniques for young seedlings in two configurations (one-shoot I-shaped grafting and two-shoot Y-shaped grafting; Turnbull et al. 2002; Bainbridge et al. 2006) can be applied to the study of the earlier developmental processes. In fact, these techniques have recently been used to study diverse phenomena involving long-distance signaling, including branching (Beveridge 2006), flowering (An et al. 2004; Corbesier et al. 2007), nutrient allocation (Rus et al. 2006; Lin et al. 2008; Pant et al. 2008), post-transcriptional gene silencing (Fusaro et al. 2006; Brosnan et al. 2007), and disease resistance (Xia et al. 2004). However, in most cases, only the technique of a so-called I-graft (one shoot scion on a root stock) was used to investigate communication between roots and shoots. In some reports, alternative strategies, such as a local gene-expression system and grafting using other plant species that are more amenable to grafting, were used to investigate shoot-to-shoot communication. This may be due to the difficulties in assembling and establishing two-shoot Y-shaped grafts (two stock and scion shoots on a stock root system), as reflected by the small numbers of plants used in these experiments. Actually, only a small number of successful grafts were obtained using the original method in preliminary trials. In addition, at what point the connection of vasculature is established at a graft junction was not analyzed in any previous report (Turnbull et al. 2002). Knowledge of the timing of functional connection of the phloem is of critical importance for designing experiments to detect long-distance gene action and/or transport of signal molecules.

To investigate long-distance signaling in flowering, the two-shoot Y-shaped grafting technique for Arabidopsis seedlings (Turnbull et al. 2002) was modified, mainly for adaption to more humid growth conditions and to facilitate the handling of the large number of grafts required for biochemical analysis. The timing of establishment of continuity of vascular tissues between hypocotyls of the stock and scion plants was then examined by histological and functional analysis. Finally, the usefulness of this grafting technique, with its minimal effect on flowering, for the study of flowering, specifically the long-distance action of the floral transition gene *FT*, was demonstrated.

Materials and methods

Plant materials and growth conditions

Columbia-0 (Col) accession was used as a wild type. *ft-1* (G171E) introgressed into the Col background was used as an *ft* mutant. *gFT::GUS* (AY#1) and a strong line of *35S::FT* (YK#11-1) were described elsewhere (Notaguchi et al. 2008; Kobayashi et al. 1999, respectively). *rolC::GUS* (YD#7) (a construct originally described in Sugaya et al. 1989 was kindly provided by H. Uchimiya and M. Kawai, The University of Tokyo) and *35S::EGFP* (MK#14-1) in Col background, and *SULTR2;1::FT* (YD#1) (described in Abe et al. 2005) and *SULTR2;1::FT:EGFP* (YD#13) in *ft-1* background are newly generated transgenic lines. *35S::EGFP* (MK#14-1) was generated by

M. Kobayashi in our laboratory. For the expression analyses shown in Fig. 5b (7-day-old *gFT::GUS* plant), S1, and S2, plants were grown on 0.8% (w/v) agar medium containing half-strength Murashige and Skoog salts, 1.0% (w/v) sucrose at 22°C under CL conditions with white fluorescent lights ($\sim$60 μmol m^{-2} s^{-1}).

Plasmid construction and plant transformation

The plasmid *35S::EGFP* was constructed by replacing the *GUS* ORF in pBI121 with an *EGFP* ORF. Plasmid *SULTR2;1::FT:EGFP* was constructed by fusing the *SULTR2;1* promoter with *FT:EGFP* ORF (these materials are described in Abe et al. 2005). These constructs, which are in binary vectors, were introduced into *Agrobacterium tumefaciens* strain pMP90 and transformed into Arabidopsis plants by the floral-dip procedure (Clough and Bent 1998).

Two-shoot Y-graft

The outline of the procedures is summarized in Fig. 1a. Seeds were surface-sterilized and imbibed at 4°C for 3 days to synchronize germination, and then sown with a regular spacing of 3–5 mm on a cellulose nitrate filter (HAWP09000, Millipore, Bedford, MA) over a single layer of Whatman No.1 filter paper (Whatman, Maidstone, UK) containing 0.04 ml cm^{-2} distilled water in Petri dishes. The dishes were sealed and placed at an angle of 60–75° in a growth cabinet. The seedlings were grown for 4 days under continuous light conditions (CL, $\sim$60 μmol m^{-2} s^{-1}) at 22°C.

Four-day-old seedlings, which had started greening and had opened cotyledons, were used for graft surgery. The surgery and assembling of the grafts were performed on the filter in the Petri dish under a dissecting stereomicroscope (MZ16, Leica, Solms, Germany) using 26G × 1/2″ needles (NN-2613S, Terumo, Japan) as scalpels and a pin. Stock and scion, in that order, were prepared as follows (see Fig. 1b, c). Stock: the whole part of one cotyledon was removed and a sharp downward slit was made at a shallow angle nearly halfway into the hypocotyl on the side of the removed cotyledon. Scion: one cotyledon was removed as in the stock, and the hypocotyl was cut in the middle obliquely to maximize the cut surface. The oblique cut surface should be on the side of the removed cotyledon. A graft was then assembled by inserting the hypocotyl of the scion deeply into the slit on the hypocotyl of the stock such that the oblique cut surface of the hypocotyl of the scion is in contact with the upper side of the slit (Fig. 1c, inset in d). In this configuration, the remaining cotyledons of the stock and scion were on opposite sides and did not interfere with the tight assembly of the graft (Fig. 1d). Care was taken to avoid injuring the seedlings during surgery. To avoid exposure of the graft interface to the water of the wet membrane surface, the grafted plant was kept standing by lifting the basal part of the hypocotyl of the stock using a pin and placing some supporting material (e.g. an empty seed coat) below the stock hypocotyl (Fig. 1b, d). This is important, because water prevents adhesion of the graft interface and, in some cases, the surface tension of water dissembles the graft by pulling the inserted scion away from the stock. After assemblage, a small amount of water was applied to part of the filter paper some distance from the grafted plant. The whole procedure of preparing stock and scion and assembling one graft takes less than 2 min. To prevent drying, opening of the Petri dishes for serial surgeries was kept to under 15 min. To maintain appropriate humidity, the finished dishes were placed on a layer of wet paper towels in a large plastic tray, and the tray was covered with Saran wrap. The trays were then transferred to a growth room at 27°C under CL ($\sim$30 μmol m^{-2} s^{-1}) conditions. The grafted plants were grown under these conditions for the next 5 days to facilitate graft adhesion and to suppress adventitious root formation. A lower number of successful grafts is obtained if the grafts are maintained at a lower temperature of 22°C. It was also important to reduce light intensity during the period of growth at 27°C.

After 5 days at 27°C, most of the grafted plants showed adhesion at the graft interface. These grafted plants were transferred to 6-cm diameter pots containing medium-grain vermiculite (one plant per pot). A small amount of Perlite powder (UBE Kosan K. K., Ube, Japan) was placed around the roots to facilitate root growth (Fig. 1e). The entire grafted plant was covered with a plastic cap to maintain humidity for the next 5–9 days and was grown at 22°C under CL ($\sim$40 μmol m^{-2} s^{-1}) conditions. In some cases, the scion detached from the stock during planting due to weak adhesion. These grafts were discarded.

About 70% of the grafts developed adventitious roots on the scion from 5 days after grafting. During the 2–3 week period of growth after grafting, adventitious roots were removed with dissecting scissors or tweezers as soon as they were noticed, through frequent inspection. After 2–3 weeks of post-graft growth, good grafts in which stock and scion shoots were of nearly equal size and vigor (Fig. 1f), were selected for further growth. Unbalanced or poor grafts, in which growth of either the scion or the stock or both was retarded, were discarded. A majority of the selected grafts grew healthily and developed a rigid connection at the graft interface between the hypocotyls (Fig. 1g, h). These grafts were defined as "successful grafts". By 4 weeks after grafting, it was possible to select successful grafts. In the experiments reported in the present work and published elsewhere (Notaguchi et al. 2008), no further selection was

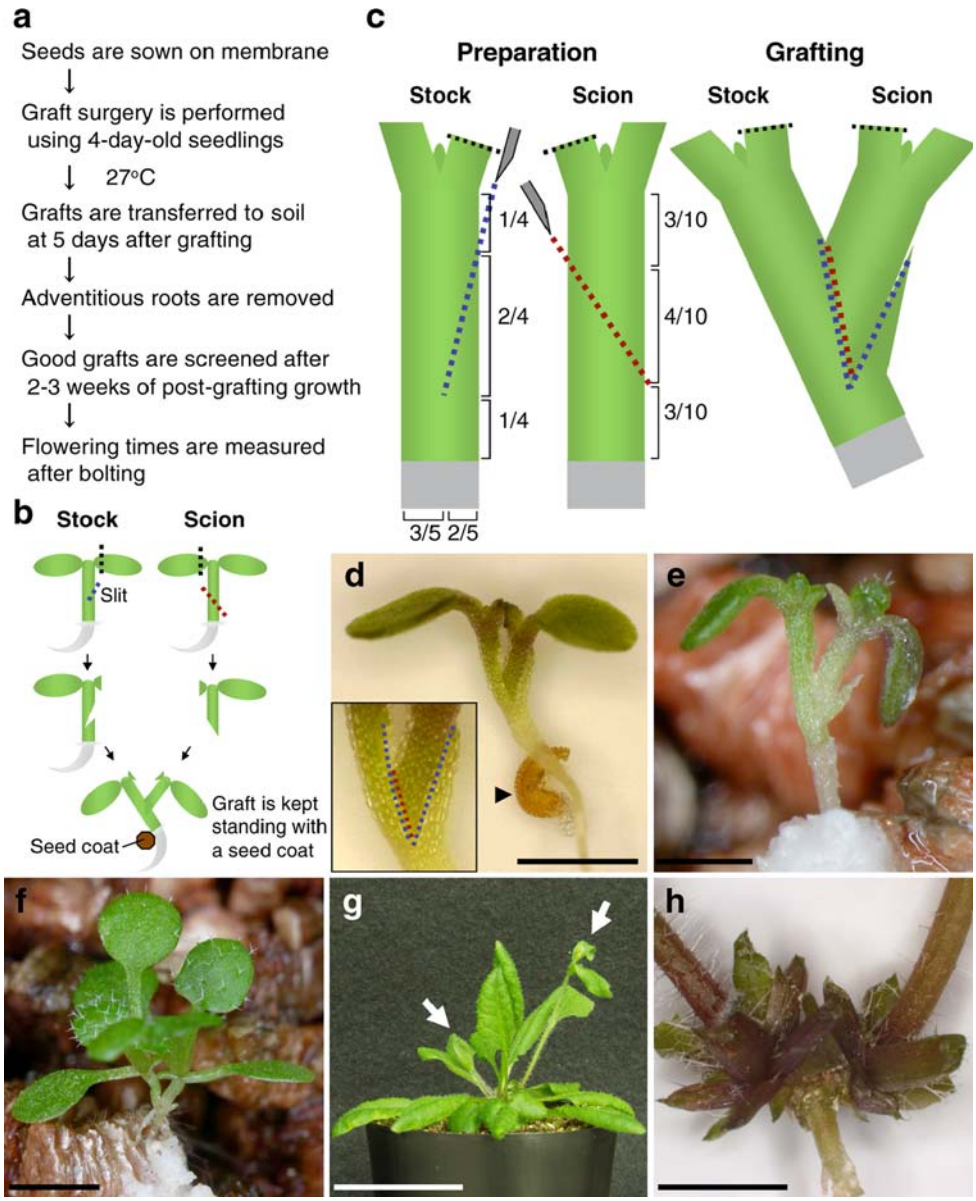

Fig. 1 Two-shoot Y-graft in Arabidopsis seedlings. **a** Outline of two-shoot Y-shaped grafting procedure. **b, c** Preparation of a stock and a scion and assembly of a graft; *blue* and *red dotted lines* represent a slit on the stock and a cut on the scion (and resulting surfaces), respectively. **d–h** Self-grafts of wild-type plants at various stages of growth. A stock is on the left and a scion is on the right side of each photograph. **d** Grafted plants soon after surgery; *arrowhead* empty seed coat placed below the hypocotyls of the stock to prevent the graft from making contact with the wet surface of the filter membrane. *Inset* Magnified (2×) image of the graft interface; *blue* and *red dotted lines* indicate the slit on the stock hypocotyl and the cut surface of the scion hypocotyl, respectively. **e–g** Plants at 5 (**e**), 14 (**f**), and 37 (**g**) days after grafting; *arrows* in **g** indicate the primary inflorescence stems of stock (*left*) and scion (*right*). **h** Graft junction of plant shown in **g**. Rosette leaves of stock and scion were removed. *Bars* **d**, **e** 1 mm; **f** 2 mm; **g** 3 cm; **h** 3 mm

done at the time of physiological assessment (counting of leaves for determination of flowering time) to avoid biased data collection.

Histological analysis of GUS staining

For GUS staining, tissues were fixed with 90% acetone for 10–30 min on ice, rinsed three times with 50 mM sodium phosphate buffer, infiltrated for 10–20 min with staining solution (1.0 mg ml^{-1} X-Gluc, 50 mM sodium phosphate buffer, pH 7.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.2% Triton X-100) under vacuum, and incubated for 15 min to 1 h at 37°C in the dark. For sectioning, samples were dehydrated through an ethanol series, embedded in Technovit 7100 (Heraeus Kulzer, Germany) and sectioned at a thickness of 5 μm with a microtome.

Dye loading and detection

A 0.1–0.3 μl drop of 5(6)-carboxyfluorescein diacetate (CFDA) (C8166, Sigma, St. Louis, MO; stock solution 50 mg ml^{-1} in acetone) or 8-acetoxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS-acetate) (A-3332, Sigma) was applied to the cut edge of a single cotyledon at a concentration of 0.1 and 50 mg ml^{-1} in distilled water, respectively. The fluorescence of the dyes was visualized and recorded 5–30 min after the application using a fluorescence stereomicroscope (SMZ-U, Nikon, Japan) and a CCD camera (DXM1200, Nikon, Japan).

Detection of GFP fluorescence

Tissues were embedded in 5% agar and sectioned at a thickness of 100 μm with a vibratome. GFP fluorescence was visualized using a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan) with an argon laser.

RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and was treated with RNase-free DNaseI (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized in a 20 μl reaction mixture with 0.5 μg RNA as a template using a Superscript III (Invitrogen). After the reaction, the mixture was diluted with 30 μl water, and 1 μl aliquots were used for PCR; primers and PCR conditions are shown in Table S2. PCR products were resolved by electrophoresis on agarose or polyacrylamide gels, and visualized by ethidium bromide staining.

Measurement of flowering time of plants

To avoid biased selection at the time of flowering time measurement, all grafted plants with a firm graft junction and a scion shoot of nearly equal size and strength as the stock shoot after 2–3 weeks of post-grafting growth (Fig. 1f) were subject to measurement without further selection. The flowering time of the grafted plants was measured by counting the number of rosette and cauline leaves on the primary axis (''total leaf number'') after inflorescence stem elongation (usually 50–100 days after grafting, depending on the graft combination).

Results

Two-shoot Y-shaped grafting procedure

To investigate long-distance signaling in flowering, we modified a micro-grafting technique for Arabidopsis seedlings (Turnbull et al. 2002), mainly to adapt to more humid growth conditions and to enable handling of the large number of grafts required for biochemical analysis. The resulting procedure (summarized in Fig. 1a) is described in detail in Materials and methods.

There are three small but critically important technical tips to ensure successful grafting. The first and most important is to keep the assembled graft standing away from the membrane surface with the aid of supporting materials, so that the graft interface is not in contact with water on the wet membrane (Fig. 1b, d). Second, it is important to prepare a stock and a scion and to assemble the graft quickly while causing little damage. Standardized procedures (Fig. 1a–e) enable preparation and assembly within a few minutes in skilled hands. The third point is the length of time for which the plants are kept on the membrane at 27°C. This should be kept as short as possible. Under our conditions, a period of 5 days gave best results. Through the use of standard procedures, a frequency of successful grafts (Fig. 1f–h) as high as 10–30% of total grafts was consistently achieved (Tables 1, 2).

With the relative ease of the whole procedure, and the high frequency of successful grafts, this technique can be applied to both quantitative physiological analysis of long-distance signaling and detection of the transport of signal molecules.

Table 1 Timing of establishment of phloem continuity at the graft junction between stock and scion

Days after grafting	Grafted[a]	Examined[b]	Phloem continuity (*P*)[c]	Successful (*S*)[d]	P/S × 100[e]
Experiment 1					
4	65	57	0		0.0
7	65	45	3		15.0
10	65	44	18		90.0
14	65	31	19		95.0
At flowering	65			20	
Experiment 2					
14	166	38	21		91.3
21	166	32	21		91.3
28	166	23	23		100.0
33	166			23	

[a] Number of assembled grafts

[b] Number of grafts examined for phloem continuity at indicated dates

[c] Number of grafts in which phloem continuity was established

[d] Number of successful grafts at the time of flowering (in Experiment 1) or at 33 days after grafting (in Experiment 2)

[e] The percentage of grafts in which phloem continuity was established (*P*) among expected successful grafts (*S*)

Establishment of phloem continuity at the graft junction between the stock and scion

When using grafts to investigate long-distance signaling via phloem, it is important to know the timing of the establishment of the phloem connection between the stock and the scion plants. To examine when the phloem tissues of the stock and scion plants became connected under our grafting conditions, histological observations of the graft junction were made. Functional continuity of the phloem was also verified by examining the transport of phloem-specific tracer dyes from scion to stock.

Table 2 Frequency of successful grafts

Graft combination (scion/stock)[a]	Grafted	Potted (%)[b]	Successful (%)[c]
Experiment 1 (shown in Fig. 6)			
ft-1/ft-1	311	172 (55.3)	40 (12.9)
35S::FT (YK#11-1)/*ft-1*	119	90 (75.6)	15 (12.6)
SULTR2;1::FT (YD#1-1); *ft-1/ft-1*	356	204 (57.3)	36 (10.1)
SULTR2;1::FT:EGFP (YD#13); *ft-1/ft-1*	401	248 (61.8)	44 (11.0)
Experiment 2[d]			
ft-1/ft-1	415	160 (38.6)	52 (12.5)
35S::FT (YK#11-1)/*ft-1*	410	253 (61.7)	66 (16.1)
35S::FT:EGFP (YD#2-2); *ft-1/ft-1*	467	325 (69.6)	43 (9.2)
Wild type/*ft-1*	448	226 (50.4)	78 (17.4)
35S::FT:EGFP (YD#2-2)/*ft-1*	452	269 (59.5)	43 (9.5)
Experiment 3[d]			
ft-1/ft-1	422	238 (56.4)	56 (13.3)
35S::FT:EGFP (YD#2-2); *ft-1/ft-1*	499	325 (65.1)	33 (6.6)
Wild type/*ft-1*	458	263 (57.4)	40 (8.7)
35S::FT:EGFP (YD#2-2)/*ft-1*	504	327 (64.9)	28 (5.6)
Experiment 4			
ft-1/ft-1	600	323 (53.8)	51 (8.5)
35S::FT (YK#11-1)/*ft-1*	600	379 (63.2)	42 (7.0)
Experiment 5			
ft-1/ft-1	300	150 (50.0)	36 (12.0)
Wild type/*ft-1*	600	400 (66.7)	53 (8.8)
Experiment 6			
ft-1/ft-1	311	218 (70.1)	31 (10.0)
Wild type/*ft-1*	150	116 (77.3)	29 (19.3)
Experiment 7			
ft-1/ft-1	150	90 (60.0)	19 (12.7)
SULTR2;1::FT (YD#1-1); *ft-3* (L*er*)/*ft-1*	1,000	546 (54.6)	13 (1.3)
PDF1::FT (MA#8-1); *ft-1/ft-1*	200	172 (86.0)	58 (29.0)

[a] Genetic background of stock and scion is in Columbia in all experiments, except for *SULTR2;1::FT* (YD#1-1); *ft-3* scion in Experiment 7 which is in a Landsberg *erecta* (L*er*) background

[b] Number of plants transferred to soil 5 days after graft surgery (percentage to grafted plants)

[c] Number of successful grafts (percentage to grafted plants)

[d] Measurement of flowering time in these experiments was published in Notaguchi et al. (2008)

For histological analysis, *rolC::GUS* transgenic plants were used as both stock and scion plants. Since *rolC* promoter activity is strong in phloem tissues (Booker et al. 2003), *rolC::GUS* can be used to visualize phloem by staining for GUS activity. Strong GUS expression in the phloem tissues of newly generated *rolC::GUS* transgenic Arabidopsis was confirmed (Fig. S1). In two sets of experiments, 325 (Table 1, Experiment 1) and 664 (Table 1, Experiment 2) grafts were assembled and then divided into five and four equal-sized groups, respectively. For each experiment, one group of grafts was maintained all the way to the end of the experiments to evaluate the percentage of "successful grafts". The grafts in the other groups were sampled at 4, 7, 10, and 14 days (Table 1, Experiment 1), or at 14, 21, and 28 days (Table 1, Experiment 2), after graft surgery and stained for GUS activity. Phloem continuity was examined in longitudinal sections. In Experiment 1, the group that was maintained to the end of the experiments yielded 20 successful grafts (out of 65 grafts). Based on the assumption that the frequency of successful grafts would be the same (i.e. 20 successful grafts per group) in the other four groups, the proportion of grafts with established phloem continuity between the stock and scion to the expected successful grafts (20 grafts) was calculated for a given sampling date. A similar analysis was applied to Experiment 2, in which the frequency of successful grafts happened to be lower (23 out of 166 grafts).

Four days after grafting, no grafted plants showed continuous GUS staining between the stock and scion (Fig. 2d, Table 1). Continuous GUS staining was first observed at 7 days after grafting in 15% of the presumptive successful grafts (Fig. 2e, Table 1). At this time, continuity of xylem was also observed in these grafts (Fig. 2e, inset). Continuity of phloem and xylem tissues became substantial at 10 days after grafting (Fig. 2f, inset). From 10 days after grafting onward, over 90% of the presumptive successful grafts showed continuous GUS staining between the stock and scion (Fig. 2f, j–l, Table 1). These results agree closely with the observation that vigorous growth was notably reestablished at 10 days after grafting in a fraction of the grafted plants (Fig. 2c). These results suggest that histological phloem continuity between hypocotyls of the stock and the scion is established between 7 and 10 days after surgery in most of the successful grafts that will probably survive to maturity.

To confirm that the phloem tissues of the stock and the scion plants are functionally connected in the graft, we tested whether molecules are actually transported between the stock and the scion through the phloem. The tests were done 14 days after surgery, when the histological continuity of the phloem was established. Phloem-specific tracers, 5(6)-carboxyfluorescein diacetate (CFDA) and 8-acetoxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS-acetate) were used for the transport tests. These dyes share the common property that they cross the plasma membrane in the hydrophobic acetate form and their acetyl radicals are cleaved by cytosolic enzymes producing the membrane-impermeable xenobiotics, carboxyfluorescein (CF) and 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS), respectively. These dyes have been used extensively as markers for symplasmic transport (Oparka et al. 1994; Wright and Oparka 1996).

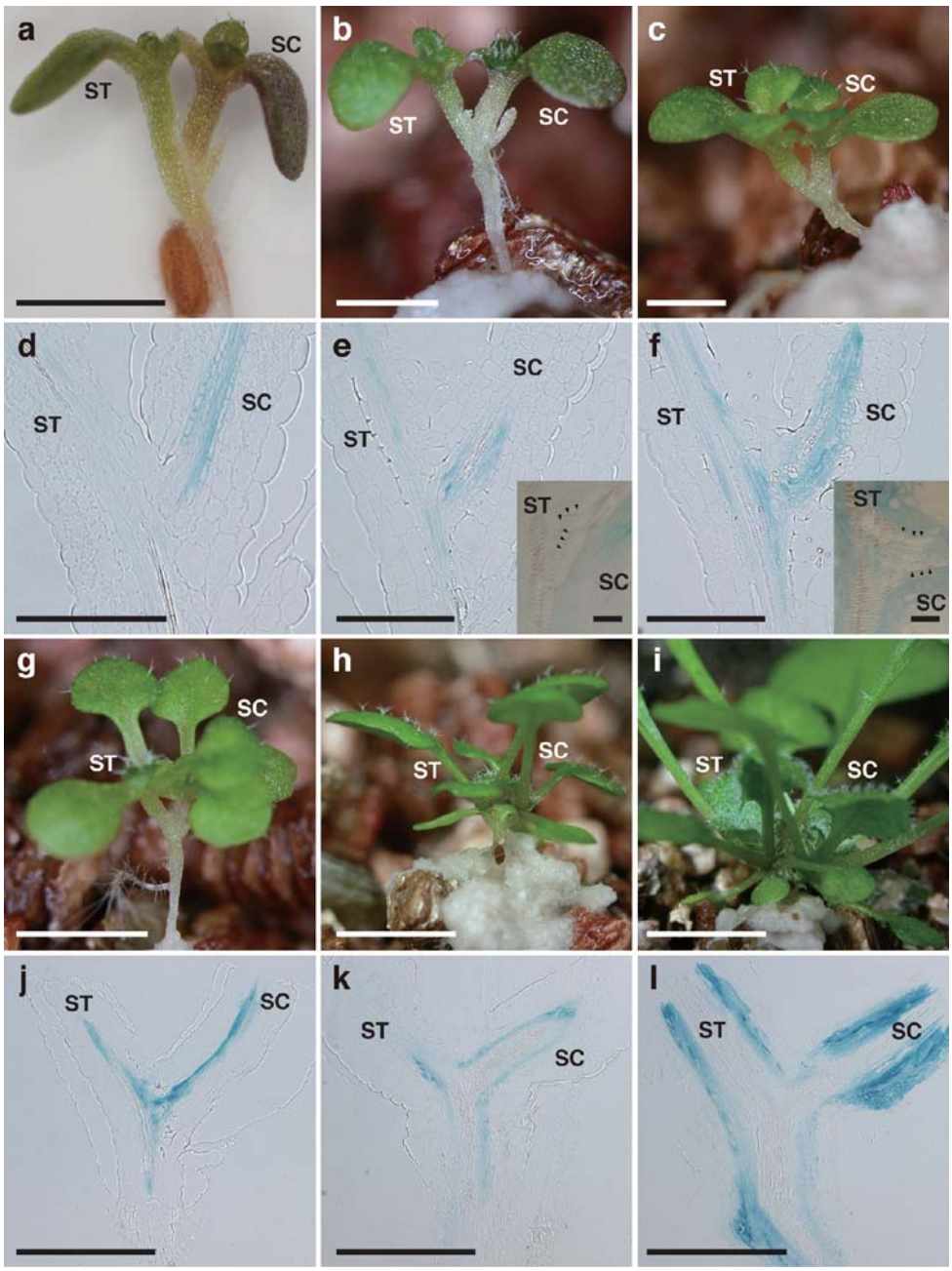

Fig. 2 Establishment of phloem continuity in grafted plants. Histological analysis of graft junctions on hypocotyls. **a–c, g–i** Grafted *rolC::GUS* plants. **d–f, j–l** Transverse sections of hypocotyls of *rolC::GUS* grafts stained for GUS activity. Grafted plants were grown for 4 (**a, d**), 7 (**b, e**), 10 (**c, f**), 14 (**g, j**), 21 (**h, k**), and 28 (**i, l**) days after grafting. Insets in **e** and **f** show another section through the xylem tissues of the same plant. *Arrowheads* Regenerated xylem tissues, *ST* stock, *SC* scion. *Bars* **a–c** 1 mm; **d–f** 200 μm; *insets* of **e**, **f** 20 μm; **g–i** 2 mm; **j–l** 500 μm

Grafted plants and age-matched control plants grown under the same conditions as the grafted plants were subjected to tracer applications (Fig. 3a). When the dyes were applied to the cut edge of a single cotyledon of control plants, fluorescence spread rapidly throughout the whole plant. In the first leaf, CF fluorescence was detected in the veins of the leaf, and HPTS fluorescence was broadly observed in the marginal region of the leaf because of the unloading properties of the dyes (Fig. 3a). Next, grafted plants that were classified as "good" grafts (with a vigorous scion) or "poor" grafts (with a poor scion) were tested. The tracers were applied to an incision on a single cotyledon of the scions and the fluorescence of the dyes was monitored in the first true leaf of the stock plants. Fluorescence was detected in the first leaf of the stocks with a healthy scion, but not in the leaves of stocks with a poor scion (11 "poor" grafts were examined; Fig. 3a). These results indicate that a functional phloem connection was established between hypocotyls of the stock and scion of a fraction of the grafts that may eventually turn out to be successful by 14 days after grafting.

To examine the capacity of the established phloem connection in trafficking macromolecules, we investigated whether GFP, with its well known feature of long-distance

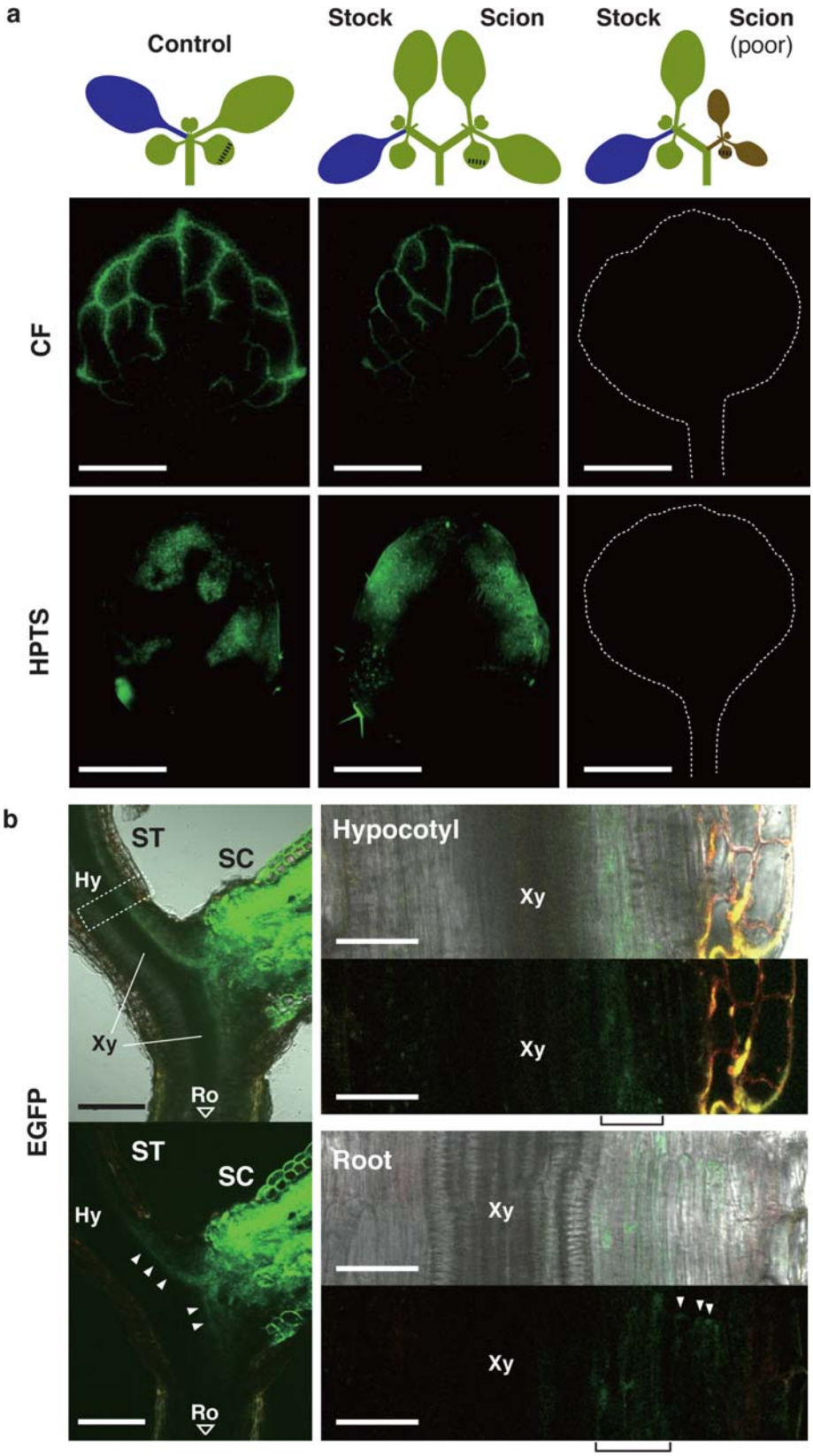

Fig. 3 Functional connection of phloem system in grafted plants. **a** Transport of phloem-specific tracer dyes, carboxyfluorescein (CF) and 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS). *Left column* Control plants, *middle column* successful grafts with a vigorous scion, *right column* unsuccessful grafts with a poor scion. *Top row* Schematic diagrams of the plants, site of tracer application and the first leaf observed for dye fluorescence. Tracers were applied to a cut edge (*black dashed line*) of a single cotyledon of the scion plants grown for 14 days after grafting or control plants of the same age; 30 min after application, the fluorescence of the tracers was observed at the first leaf (*blue*) of the stocks or the control plants. *Middle and bottom rows* Fluorescence of CF and HPTS, respectively, in the first leaf. The *white dashed lines* show the leaf contour (*right*). **b** Transport of enhanced green fluorescent protein (EGFP) from a scion over-expressing EGFP (*35S::EGFP*) to an *ft-1* stock plant across a graft junction at 21 days after grafting. A section of the graft junction is shown in the left panel. Magnified images of a part of the hypocotyl (*boxed area* in *left panel*) and the root (another section of the same graft) are shown in the *right panels*. Each section was viewed in light field (*upper images*) and in dark field (*lower images*). EGFP fluorescence (*arrowheads* and *brackets* in the dark field images) was detected in the phloem tissues of the hypocotyl and the root on the side where the scion was fused. *ST* Stock plant, *SC* scion plant, *Hy* hypocotyl *Ro* root (not shown in the photographs), *Xy* xylem bundles. *Bars* **a** 1 mm; **b** 100 μm (*left panels*), 20 μm (*right panels*)

trafficking via phloem (Imlau et al. 1999), can be transported across a graft junction. A scion over-expressing EGFP (*35S::EGFP*) was grafted onto a non-transgenic *ft-1* mutant stock and EGFP fluorescence was observed in the stock plant at 21 days after grafting (Fig. 3b). In both the hypocotyl and the roots of the non-transgenic stock plants, EGFP fluorescence was detected in the phloem tissues on the side where the scion was fused. EGFP fluorescence could be observed in the stock plant at 14 days after grafting in a similar experiment (Fig. S2). These results suggest that the phloem connection between the stock and the scion becomes capable of trafficking of macromolecules by 14–21 days after grafting.

Application of a micro-grafting technique to the study of long-distance signaling in flowering

In successful grafts obtained through standard procedures, the phloem tissues of the stock and the scion of successful grafts becomes functionally connected by 2 weeks after surgery. The frequency of successful grafts (10–30% of the trials) is high enough to obtain sufficient material for quantitative physiological assays and biochemical analysis. These features enable this micro-grafting technique to be applied to the study of long-distance signaling in flowering via *FT* gene products. Before actually applying the technique to the study of flowering, further investigation was conducted on: (1) the relative timing of the establishment of phloem continuity with respect to floral transition, and (2) whether the graft surgery itself affects the timing of the floral transition of stock and scion plants.

A functional connection of the phloem between stock and scion capable of macromolecular trafficking, as judged by trafficking of phloem-specific tracers, was established by 14 days after grafting. These observations suggest that the long-distance flowering signal may begin to be transported from the donor to the recipient plant through the phloem around 14 days after grafting (corresponding to 18 days after germination) in successful grafts. Under the growth conditions used here, expression of a floral meristem identity gene, *APETALA1* (*AP1*), which is a reliable marker for flowering (Hempel et al. 1997), was clearly detected by this timepoint in intact wild-type plants (Fig. S3). Therefore, if wild-type plants are used as recipient plants, they are likely to make a floral transition before establishing the phloem connection with the donor scion. This makes wild-type plants unsuitable as recipients under these experimental conditions. By contrast, *AP1* expression was not clearly detected until 30 days after germination in the *ft-1* mutant, as expected from its late-flowering phenotype (Fig. S3). The combination of establishment of a phloem connection early enough, and a greatly delayed floral transition makes the *ft-1* mutant a suitable recipient in the study of long-distance signaling in flowering.

In a previous report, it seemed that flowering of the *co-2* mutant was accelerated by graft surgery (*co-2* grafted onto *co-2* flowered earlier than intact *co-2*; see Fig. 3d in An et al. 2004). This observation suggests that graft surgery per se may cause acceleration of flowering, at least in some genotypes. To investigate whether flowering of *ft-1* plants is influenced by graft surgery, the flowering-times of *ft-1* stocks and scions were compared with intact *ft-1* plants grown under the same conditions (Fig. 4). Ranges of the leaf number of intact plants and grafted plants overlapped. There were no plants whose flowering was distinctly promoted by graft surgery. There was no significant difference in the flowering time between the scion and the intact plants, but a small delay was observed in the stock plants (Fig. 4). Similar results were obtained in the case of grafts between wild-type stocks and scions (Table S1). These results indicate that grafting itself has no effect on acceleration of flowering of *ft-1* and wild-type plants used as either stocks or scions.

To further confirm that grafting does not accelerate flowering, the effects of graft surgery on the expression of flowering-related genes were investigated. The expression of four floral pathway integrators, *FT*, *TWIN SISTER OF FT* (*TSF*), *SUPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), and *LEAFY* (*LFY*) (Araki 2001; Simpson and Dean 2002; Michaels et al. 2005; Yamaguchi et al. 2005), *FD*, which encodes a bZIP protein acting in the shoot apex with an FT protein (Abe et al. 2005; Wigge et al. 2005), and *AP1*, which is a regulatory target of LFY and FT/FD, was examined at 1 and 24 h after surgery to test for early response, and 10 days after surgery when the histological continuity of the vasculature is established. With the possible exception of *LFY*, whose expression seemed to be increased slightly by the surgery, no consistent differences from intact plants were observed (Fig. 5a). In addition, that the spatial pattern of *FT* expression was unaffected by surgery was confirmed by a *gFT::GUS* reporter (Fig. 5b). These observations suggest that expression of flowering-related genes is not affected by graft surgery.

Based on these results, it was decided to use an *ft-1* mutant stock as a recipient of the long-distance signal from a donor scion with functional *FT* genes (either as transgenes or the endogenous gene) in subsequent experiments. Since analysis of transmission of the physiological effect to promote flowering and the FT protein from the donor scions to the recipient *ft-1* stocks is described in detail elsewhere (Notaguchi et al. 2008), only the exemplary results are presented here. A late-flowering phenotype of the *ft-1* stock plants was rescued by grafting a transgenic scion expressing *FT* either ubiquitously (*35S::FT*) or specifically in the phloem tissues (*SULTR2;1::FT*) (Fig. 6a–c). However, in either case, there

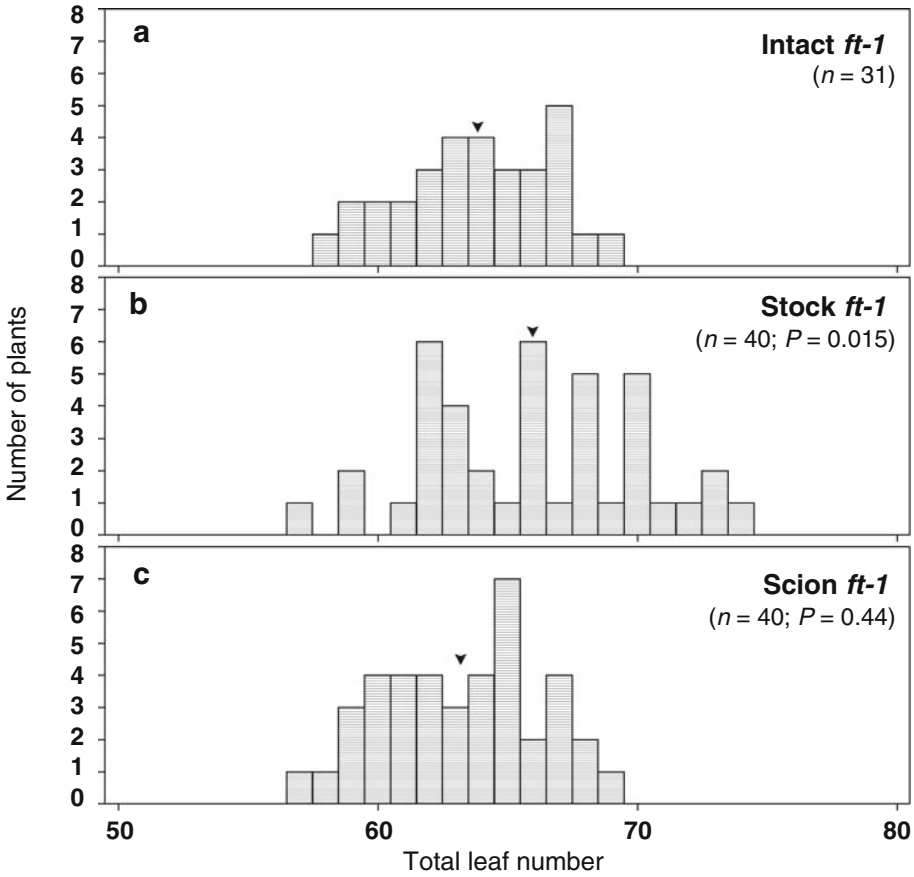

Fig. 4 Effect of graft surgery on the flowering of *ft-1* stock and scion plants. Distribution of flowering time (measured as total leaf number at the time of flowering) of **a** intact *ft-1*, **b** stock *ft-1*, and **c** scion *ft-1* plants. The *arrowhead* in each graph indicates the average of the population. The number of plants examined (*n*), and the *P* value of Student's *t* test with intact *ft-1* (**a**) are shown in each graph

was only a partial rescue and the *ft-1* stock plants flowered much later than intact wild-type plants grown under the same conditions (see Table S1). This is likely due to the fact that floral transition and *AP1* expression have already occurred in wild-type plants by the time the functional phloem connection is firmly established in the grafts under our growth conditions (see above). From these results, we conclude that the action of *FT* to promote flowering is transmissible from a donor scion to a recipient stock through a graft junction and that the phloem serves as an important pathway for the graft-transmissible action of *FT*. Interestingly, grafting of a transgenic scion expressing an FT:EGFP fusion protein specifically in phloem tissues (*SULTR2;1::FT:EGFP* in *ft-1* background), which itself has a very early-flowering phenotype, resulted in no significant promotion of flowering in the *ft-1* stock plants (Fig. 6a, d). This result is in agreement with other results of FT:EGFP (Notaguchi et al. 2008), suggesting the limited ability of the FT:EGFP fusion protein for graft-transmissible action.

Discussion

In the present work, procedures for two-shoot micrografting of Arabidopsis seedlings (Turnbull et al. 2002) were improved and standardized to obtain the large number of successful grafts required for quantitative physiological and biochemical analyses of long-distance signaling (Fig. 1). Three small but critically important technical tips are: (1) keeping the assembled graft standing away from the wet membrane surface, (2) quick preparation of a stock and a scion and assembly of a graft through a standardized procedure, and (3) a short period (5 days) of post-surgery growth on the membrane at 27°C. With application of the technique to the study of flowering in mind, the timing of the histological and functional phloem connections under the growth conditions used was determined (Table 1; Figs. 2, 3). It was also confirmed that the grafting procedure itself has little effect on the expression of flowering-related genes and the timing of flowering of grafted plants (Figs. 4, 5). These results assist in the design of efficient experiments to investigate the transmission of the physiological effects (and the signal molecules) to promote flowering from the donor scions with functional *FT* genes, to recipient stocks in the grafts (Fig. 6 and Notaguchi et al. 2008).

The achieved frequency of successful grafts (10–30%, Table 2) was high enough to enable a quantitative physiological assay to be performed (Fig. 6 and Notaguchi et al. 2008). This is important because grafting has been

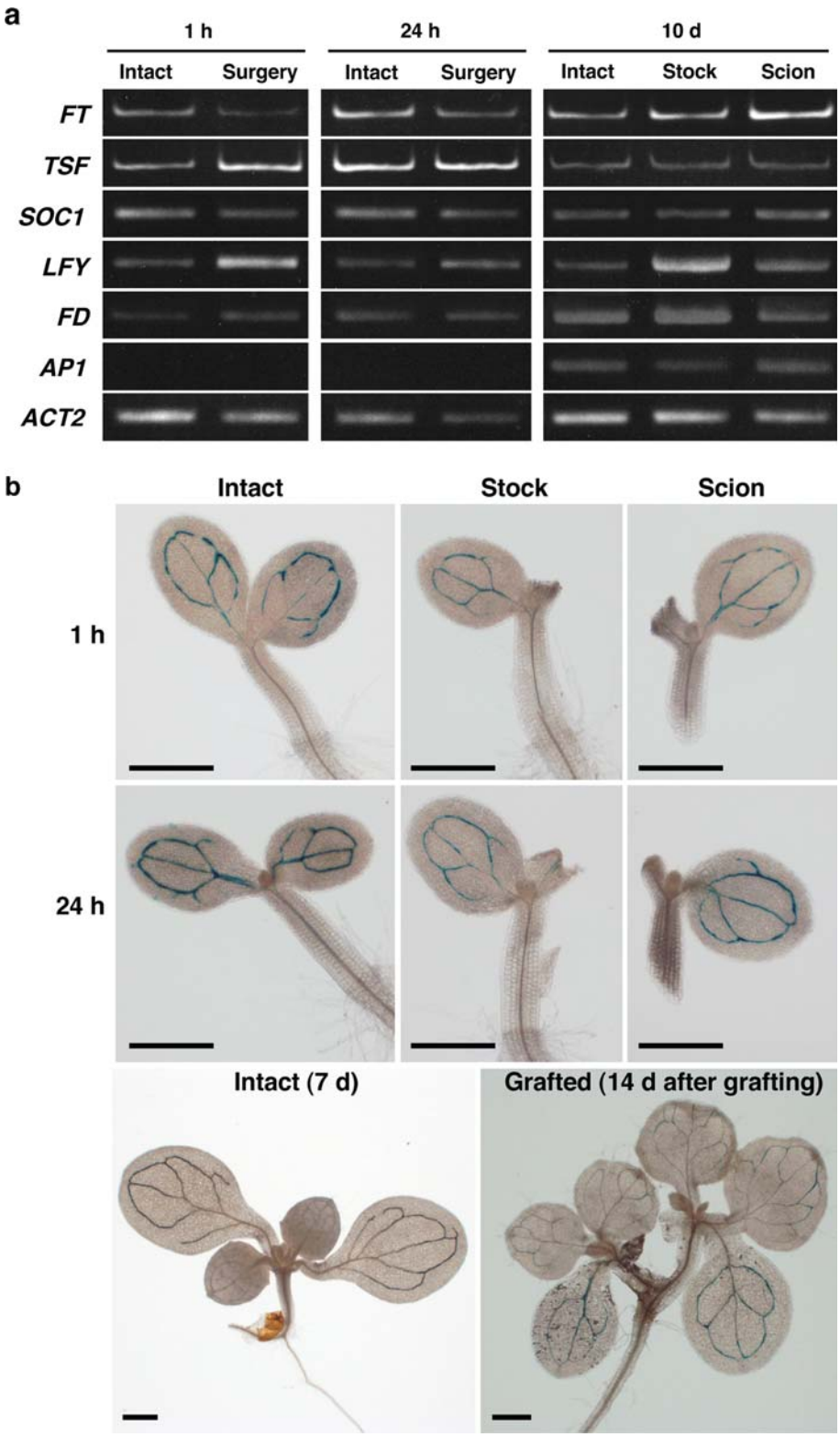

Fig. 5 Effect of graft surgery on expression levels of flowering-related genes. **a** Expression of *FLOWERING LOCUS T* (*FT*); *TWIN SISTER OF FT* (*TSF*); *SUPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*); *LEAFY* (*LFY*); *FD*, which encodes a bZIP protein acting in the shoot apex with an FT protein; and *APETALA1* (*AP1*) genes after graft surgery. *ACTIN 2* (*ACT2*) was amplified as a reference. Plants were harvested at 1 h, 24 h, and 10 days after graft surgery. Intact plants were harvested at the corresponding times. **b** Spatial pattern of *FT* expression after graft surgery. FT-GUS expression pattern in *gFT::GUS* plants (intact, stock, and scion) was analyzed in whole-mount preparations at the indicated times after graft surgery. A 7-day-old intact plant grown on an agar medium was used as a reference for grafted plants at 14 days after graft surgery. *Bars* 1 mm

considered a "demonstrative" tool based on a small number of successful plants (sometimes as few as three) rather than as an analytical tool with a sufficient number of plants. To further improve the efficiency of grafting, addition of indole acetic acid (IAA) and 6-benzylamino-purine (BA) to the growth medium for (1) enhanced callusing at the graft union; (2) retardation of shoot growth, which helps maintain contact at the graft union;

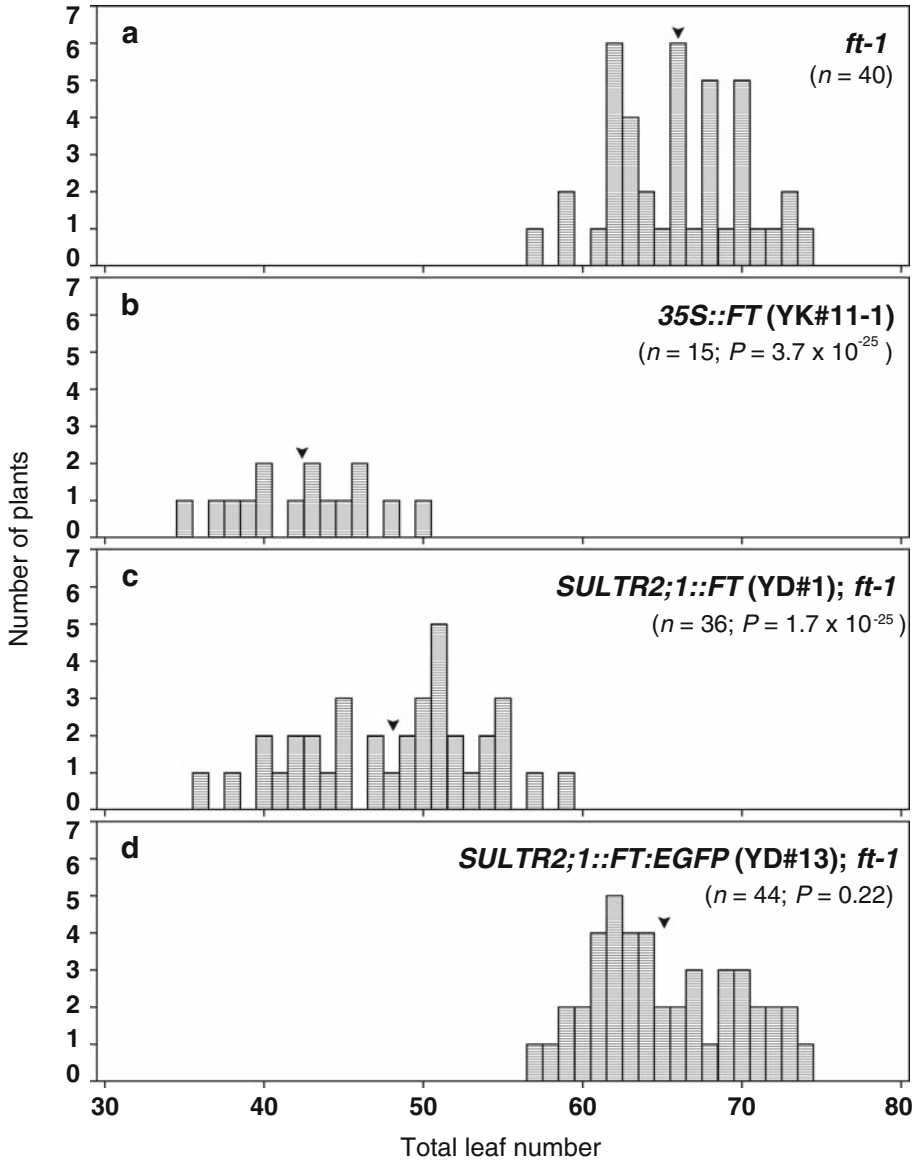

Fig. 6 Graft-transmissible action of the *FT* gene. Distribution of the flowering time of *ft-1* recipient stock plants with a scion of **a** *ft-1*, **b** *35S::FT* (YK#11-1), **c** *SULTR2;1::FT* (YD#1); *ft-1*, and **d** *SULTR2;1::FT:EGFP* (YD#13); *ft-1*. The *arrowhead* in each graph indicates the average of the population. The number of plants examined (*n*), and the *P* value of Student's *t* test with *ft-1* stock plants with *ft-1* scion (**a**) are shown in each graph

and (3) strong reduction in the adventitious root formation from the graft union, as was discussed in a previous report (Rus et al. 2006), may be an option. Although plant growth regulators were not used here to avoid possible physiological effects, if these are expected to be negligible in the phenomenon to be studied, the application of IAA and BA may be helpful in reducing the number of grafts that need to be performed.

The histological continuity of the vasculature was observed at 7–10 days after surgery (Fig. 2, Table 1). A previous report showed that tissue reunion was achieved by 7 days after cutting of hypocotyls in both cucumber and tomato seedlings (Asahina et al. 2002). In grafts of Coleus plants, phloem continuity was established by 6 days after grafting (Stoddard and McCully 1980). The results in this paper are consistent with these observations. In contrast, the establishment of tissue reunion required a longer time (15 days after grafting) in grafts of inflorescence stems of Arabidopsis (Flaishman et al. 2008). This difference in the timing may be a result of different activities of cell division and/or tissue differentiation between seedlings and mature plants. Interestingly, grafting between a stock plant in a Columbia background and a scion plant in a Landsberg *erecta* background resulted in poor adhesion at the graft interface after 5 days of post-surgery growth and eventually a very low frequency of successful grafts (only 13 successful grafts among 1,000 trials, see Table 2, Experiment 7, 2nd combination). This may be due to the graft incompatibility observed in hetero-graft combinations between

Arabidopsis and other plant species (Flaishman et al. 2008). The grafting between these two accessions may provide a good experimental system in which to investigate the genetic basis of graft incompatibility.

Functional continuity of phloem tissue between stock and scion, as assayed by dye trafficking, was established by 14 days after the grafting in presumptive successful grafts (Fig. 3a). An et al. (2004) demonstrated a phloem connection in their grafts by applying ^{14}C-labeled sucrose to a single mature leaf and detecting the radioactivity in the recipient shoot. The results in this study show that a functional connection is established within a sufficiently short period after grafting for use in studying long-distance signaling in various developmental and physiological phenomena. With the aid of EGFP, it was further demonstrated that the phloem connections in the grafts are capable of transporting macromolecules from the donor to the recipient through a graft junction (Fig. 3b, Fig. S2). This knowledge will be useful in designing efficient experiments to investigate the transport of long-distance signaling molecules through a graft junction. Indeed, it was of crucial importance in the demonstration of the transport of the FT protein from a donor scion to a recipient stock (Notaguchi et al. 2008).

The past few years have witnessed rapid progress in the molecular identification of long-distance, systemic signals involved in various physiological and developmental processes, namely, FT protein as the flowering signal (florigen) (Abe et al. 2005; Wigge et al. 2005; Lifschitz et al. 2006; Corbesier et al. 2007; Jaeger and Wigge 2007; Mathieu et al. 2007; Lin et al. 2007; Tamaki et al. 2007; Notaguchi et al. 2008), strigolactones, carotenoid derivatives, as a branching signal (Gomez-Roldan et al. 2008; Umehara et al. 2008), and small RNA molecules as systemic signals involved in such diverse processes as disease response (Brosnan et al. 2007), and phosphate homeostasis (Lin et al. 2008; Pant et al. 2008). However, many other long-distance signals still remain to be discovered and identified. The Y-shaped grafting method can be used to investigate shoot-to-shoot signaling such as disease response and carbon partitioning, as well as root-to-shoot and shoot-to-root signaling. The improved two shoot-to-shoot Y-shaped grafting technique for very young Arabidopsis seedlings reported in this study should be useful for the study of the long-distance action of genes involved in growth and development, and the transport of signal molecules.

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