CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of *Arabidopsis*

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Summary

Flower development at the shoot apex is initiated in response to environmental cues. Day length is one of the most important of these and is perceived in the leaves. A systemic signal, called the floral stimulus or florigen, is then transmitted from the leaves through the phloem and induces floral development at the shoot apex. Genetic analysis in *Arabidopsis* identified a pathway of genes required for the initiation of flowering in response to day length. The nuclear zinc-finger protein CONSTANS (CO) plays a central role in this pathway, and in response to long days activates the transcription of *FT*, which encodes a RAF-kinase-inhibitor-like protein. We show using grafting approaches that *CO* acts non-cell autonomously to trigger flowering. Although *CO* is expressed widely, its misexpression from phloem-specific promoters, but not from meristem-specific promoters, is sufficient to induce early flowering and complement the *co* mutation. The mechanism by which *CO* triggers flowering from the phloem involves the cell-autonomous activation of *FT* expression. Genetic approaches indicate that CO activates flowering through both FT-dependent and FT-independent processes, whereas *FT* acts both in the phloem and the meristem to trigger flowering. We propose that, partly through the activation of *FT*, *CO* regulates the synthesis or transport of a systemic flowering signal, thereby positioning this signal within the established hierarchy of regulatory proteins that controls flowering.

Key words: Flowering, *Arabidopsis*, Phloem, CONSTANS, FT, Grafting, Photoperiod

Introduction

The aerial organs of adult plants are derived from the shoot apical meristem (SAM), a collection of stem cells at the apex of the shoot that are formed during embryonic development. In *Arabidopsis*, organ primordia are continuously formed on the flanks of the SAM during post-embryonic development. Initially, these primordia give rise to leaves. However, after the transition to reproductive development, flowers develop from the primordia formed on the flanks of the SAM and inflorescences develop from meristems present in the axils of leaves. In many plants, the transition from vegetative to reproductive development is controlled by environmental signals such as day length or temperature. Although each of these environmental signals results in floral development at the SAM, the signals are detected in different organs of the plant. For example, day length is perceived in the leaves, whereas temperature is detected at the SAM (Michaels and Amasino, 2000). Perception of day length in the leaf suggested that a systemic signal, often called the floral stimulus or florigen, is synthesised in the leaf and transmitted to the SAM where it triggers flower development (Knott, 1934; Zeevaart, 1976). In *Perilla crispa*, which is induced to flower by exposure to short days (SDs), grafting a leaf exposed to SDs onto a plant grown in long days (LDs) was sufficient to trigger flowering (Zeevaart, 1985). The floral stimulus is transmitted through the phloem sieve elements, which connect the photosynthetic leaves to the growing parts of the plant (King et al., 1968; King and Zeevaart, 1973). However, the inducing substance has proven elusive despite extensive attempts to purify it from phloem extracts (Corbesier et al., 1998), and is usually believed to represent a complex mixture of substances (Bernier et al., 1993).

Mutants impaired in the flowering response to day length may provide a route to identifying the transmissible substance, to explaining how its synthesis and transport are regulated, and to defining the mechanism by which it induces flower development. In pea, mutations that alter flowering and impair the formation of graft transmissible substances were identified (Beveridge and Murfet, 1996; Weller et al., 1997), but the corresponding genes have not been isolated. In addition, the *INDETERMINATE* gene of maize is required for flowering and encodes a transcription factor expressed only in leaves (Colasanti et al., 1998), suggesting that it may affect the synthesis or transport of the floral stimulus. However, the

molecular genetics of the photoperiodic control of flowering is best understood in *Arabidopsis*, where a pathway of genes required to activate flowering specifically in response to LDs has been identified (Hayama and Coupland, 2003; Yanovsky and Kay, 2003), and their global effects on gene expression at the shoot apex have been described (Schmid et al., 2003).

The *Arabidopsis* photoperiod pathway was initially defined by late-flowering mutants, including *gigantea* (*gi*), *constans* (*co*) and *ft* (Koornneef et al., 1998). Analysis of the genes impaired by these mutations demonstrated that *GI* encodes a large nuclear protein of unknown function, which is required for the activation of *CO* transcription (Fowler et al., 1999; Park et al., 1999; Huq et al., 2000; Suarez-Lopez et al., 2001). In turn, *CO* encodes a nuclear zinc-finger containing protein that activates the expression of *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*; also known as *AGL20* – The Arabidopsis Information Resource) (Putterill et al., 1995; Samach et al., 2000). *FT* encodes a RAF-kinase inhibitor-like protein and *SOC1* encodes a MADS-box transcription factor (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000). All of these genes are regulated by the circadian clock, and overexpression of *CO*, *FT* or *SOC1* causes extreme early flowering (Borner et al., 2000; Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000; Onouchi et al., 2000). This pathway is highly conserved in rice, and presumably in other Angiosperms. Orthologues of each of the genes were identified in rice, and *OsGI*, *Heading date 1* (*Hd1*; an orthologue of *CO*) and *Heading date 3a* (an orthologue of *FT*) were shown to regulate photoperiodic flowering by acting in a genetic pathway in the same order as their orthologues do in *Arabidopsis* (Hayama et al., 2003; Izawa et al., 2002; Kojima et al., 2002; Yano et al., 2000).

Although the detection of day length occurs in the leaves of *Arabidopsis* (Corbesier et al., 1996), the tissues in which the components of the genetically defined pathway act to regulate flowering have been difficult to assess because of their low expression levels or general patterns of expression. Here we show that CO, a nuclear zinc-finger protein that plays a central role in the photoperiod-response pathway (Hayama and Coupland, 2003; Yanovsky and Kay, 2003), acts in the phloem companion cells to trigger floral development at the apex, and controls a systemic signal that crosses graft junctions. The mechanism by which *CO* acts in the phloem involves cell-autonomous activation of its target gene *FT* and, based on analysis of a GFP:CO fusion protein, does not require movement of the CO protein. These data identify CO as a regulator of the floral stimulus, and place the floral stimulus within the network of regulatory proteins that control flowering in response to day length.

Materials and methods

Plant material and growth conditions

Plants were grown on soil in controlled environment rooms under LDs (10-hours light/6-hour day extension/8-hours dark) or SDs (10-hours light/14-hours dark) as described (Putterill et al., 1995), or on MS agar under true LDs (16-hours light/8-hours dark). Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem of at least 20 individuals. Data are expressed as mean±s.e.

Both the *ANT::LhG4* and the *CLV1::LhG4* activator lines were obtained from Dr T. Laux (University of Freiburg, Germany) in L*er* (Schoof et al., 2000). The structure of the activator constructs was described by Schoof et al. (Schoof et al., 2000). They were introgressed into the *co-2* mutant to generate homozygous activator lines.

Plasmid constructions

To allow the site-specific CRE recombinase to excise *35S:GUS*, direct repeats of the *loxP* sites were inserted flanking the gene. Two pairs of oligonucleotides containing the repeats with accompanying restriction sites were synthesised (sequences available on request). The complementary primers were annealed and cloned in a pUC derivative carrying *35S::GUS*. *loxR* was cloned in the *Eco*RI site 5′ of *35S::GUS* and *loxH* in the *Hin*dIII site 3′ of the marker gene, generating pGUSLOX. *35S::GUS* flanked by the *loxP* sites was inserted in the *Eco*RI site in the *CO* intron using a 4.3 kb sub-clone of *CO* (Onouchi et al., 2000). A fusion of the *CRE* gene to the heat-shock promoter was kindly provided by Dr E. Meyerowitz (Sieburth et al., 1998).

To construct the *Op::GUS-Op::CO* tandem reporter plasmid, the GUS-coding sequence was inserted into pUBOP (gift of I. Moore, University of Oxford). The *Op::GUS* fragment was then inserted into pGreen0029 to yield plasmid pGreen0029-*Op::GUS*. Similarly, the full-length *CO* cDNA was cloned into pUBOP. The *Op::CO* fragment was then purified and cloned into pGreen0029-*Op::GUS* generating pGreen0029-*Op::GUS-Op::CO*. Details of the cloning are available upon request. This reporter construct was introduced into *co-2* and lines that segregated 3:1 for the T-DNA identified in the T2 generation. Homozygous lines were identified in the T3 generation. These plants flowered at the same time as *co-2* mutants, so *Op::CO* did not promote flowering prior to activation. Expression of *GUS* and *CO* was transactivated in crosses with activator lines.

The *AtSUC2*, *AtKNAT1*, *AtSTM*, *AtUFO* and *AtML1* promoters were PCR amplified from Columbia genomic DNA using specific primers with GATEWAY tails. The forward primers contain the AttB1 tail (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT), reverse primers contain the AttB2 tail (5′-GGGGACCACTTTGTACAAG-AAAGCTGGGT). Specific sequences for each primer pair were:

SUC2-F, 5′-AAAATCTGGTTTCATATTAATTTCA-3′;
SUC2-R, 5′-ATTTGACAAACCAAGAAAGTAAGA-3′;
KNAT1-F, 5′-GATCTAGAGCCCTAGGATTTGA-3′;
KNAT1-R, 5′-ACCCAGATGAGTAAAGATTTGAG-3′;
STM-F, 5′-GTGTGTTTGATTCGACTTTTGT-3′;
STM-R, 5′-CTTCTCTTTCTCTCACTAGTA-3′;
UFO-F, 5′-GAATTCTCTGTTTTAATTGCCCCA-3′;
UFO-R, 5′-TTAGCTGAAAAATGAAAAGA-3′;
ML1-F, 5′-AAGCTTATCAAAGAAAAAAACAAGA-3′; and
ML1-R, 5′-AACCGGTGGATTCAGGGAGTTTCT- 3′.

The *35S* promoter was PCR amplified from the pBI121 plasmid using the specific primer sequences 35S-F (5′-AGGTCCCCAGA-TTAGCCTT-3′) and 35S-R (5′-TCCCCCGTGTTCTCTCCAA-3′). The *ROLC* and *TobRB7* promoters were gifts from A. Heyer and I. Moore, respectively, and were also PCR amplified from the corresponding plasmids with the following primer pairs:

rolC-F 5′-GAAAAAGGCAAGTGCCAGGGCC-3′ and rolC-R 5′-TACCCCATAACTCGAAGCATCC-3′; and

TobRB7-F 5′-CCCCTTATTGTACTTCAATTA-3′ and TobRB7-R 5′-TTTCCAAGTTTCACATAACCT-3′.

All PCR products were introduced into the GATEWAY™ pDONR207 (Invitrogen) vector through BP reactions, generating promoter entry clones. The GATEWAY™ vector conversion fragment rfA was fused upstream of *GUS* in pGPTV-BAR, or *CO* and *FT* cDNA in pGreen0229, to generate the binary destination vectors. Different promoter fusions were produced by LR reactions.

A 2451 base pair *CO* promoter fragment purified from plasmid pBCOPL was fused in frame to the *GUS* ORF to yield plasmid pCOGUSL. A 4.61 kb fragment containing the *CO::GUS* fusion was purified from pCOGUSL and cloned into the binary vector pSLJ1714 to yield plasmid pSLCOGUSL.

Heat-shock induction of CRE-mediated recombination

For heat shock, *co-2* transgenic plants containing *CO(35S::GUS)* and *HS::CRE* were exposed to a temperature of 39°C for 1-3 hours. Heat shock of developing embryos was performed by exposing plants with siliques at different days after pollination to cycles of 1 hour at 39°C-1 hour at room temperature, up to a maximum of 3 hours at 39°C. Immediately after heat shock, plants were transferred to standard growth conditions or imbibed seeds were sown on soil. Experiments involving the generation of mosaic plants to determine the activity of *CO* were performed using F_1 embryos/seeds from crosses between plants homozygous for *co-2 HS::CRE CO(35S::GUS)* or *co-2 HS::CRE*. The resulting F_1 carries one copy of the *CO(35S::GUS)* construct, facilitating the detection of GUS-negative sectors after excision.

Plant transformation

All plasmids, except pSLCOGUSL, were introduced into *Agrobacterium* strain GV3101(pMP90) (Koncz and Schell, 1986) and transformed into L*er* or *co-2* plants by floral dip (Clough and Bent, 1998). Plasmid pSLCOGUSL was introduced into *Agrobacterium* strain C58C1(pGV2260) and transformed into L*er*.

Grafting experiments

Y-grafts (two shoots on a single root system) were constructed as described by Turnbull et al. (Turnbull et al., 2002). Seedlings were grown initially on half-strength MS salts, then transferred to compost (Levingtons F2S/vermiculite 4:1). Temperature was 23°C, with a light level of approx 120 μmol m^{-2} s^{-1} for 16 hours (LD) or 8 hours (SD).

Photoperiod induction across Y-grafts was tested using grafted wild-type Col plants grown for 70 days under SDs. One shoot was exposed to 7 LDs (the donor shoot) while keeping the other under continuous SDs by covering with a blackened foil cap for all except 8 hours per day. The shoots under SDs (termed receivers) were partially defoliated to enhance their sink strength. After the LD treatment, plants were returned to SDs. Flowering of both shoots was assessed 17 days after the start of LD treatment. Defoliation controls indicated this manipulation did not retard the flowering of plants induced under LDs.

Sucrose transport across Y-graft unions was measured by applying [U-^{14}C]sucrose (3.7 MBq, 1.5 nmol) to a single mature leaf on one shoot. After 2 hours, both shoots were dissected, and the radioactivity in leaf, stem and root segments was analysed by scintillation counting of ethanol extracts. Data were expressed relative to the total radioactivity recovered at sites away from the fed leaf.

Graft rescue of flowering time in *co* mutants was tested by Y-grafting *co-2* shoots onto wild-type L*er* plants under LDs. Grafts with weak *co-2* shoots were excluded. Controls included self-grafts of *co-2*, and ungrafted *co-2* plants.

Histochemical analysis of GUS expression

Analyses were carried out on plants grown on soil under LDs. After heptane treatment, samples were processed as described by Sieburth and Meyerowitz (Sieburth and Meyerowitz, 1997). For histological analysis, samples were dehydrated through an ethanol series into Histoclear (National Diagnostics), and embedded in Paramat Extra (Gurr®, BDH). Eight μm sections and whole seedlings were viewed after deparaffinisation under bright field on a Leica microscope.

In situ hybridisation

Methods of digoxigenin labeling of mRNA probes, tissue preparation and in situ hybridisation were as already described (Bradley et al., 1993) with small modifications. Protease treatment was not performed with Pronase but with Proteinase K [1 μg.ml^{-1} in 100 mM Tris (pH 8), 50 mM EDTA] at 37°C for 30 minutes, and the post-hybridisation washes were preformed in 0.1×SSC.

Probes used to detect the *CO* and *FT* transcripts were prepared from p21CO containing the full-length *CO* cDNA and from pD301 containing 450 base pairs of the 5′ *FT* cDNA, respectively.

Analysis of *CO* and *FT* mRNA abundance

At day 15, emerging true leaves of 100 plants per sample were collected from soil-grown plants 16 hours after dawn, cotyledons were discarded. RNA was analysed by RT-PCR. For synthesis of cDNA, 3 μg of total RNA was primed using dT_{15} primer. cDNA was diluted to 150 μl with water, and 3 μl of diluted cDNA was used for PCR. *CO* was amplified using primers CO53 and COoli9 as described (Suarez-Lopez et al., 2001). *FT* was amplified using primers FT-RTPCR-F (5′-AGAAGACTTTAGATGGCTTCTT-3′) and FT-RTPCR-R (5′-TTATCGCATCACACACTATATAAG-3′). UBQ10 was amplified (Blazquez and Weigel, 1999) and used as a control to normalise the amounts of cDNA. For *CO*, *FT* and *UBQ*, 17, 20 and 17 cycles were used, respectively. PCR products were separated on agarose gels, transferred to filters and hybridised with radioactively labelled probes. Images were visualised using a Phosporimager (Molecular Dynamics), band intensities were quantified using ImageQuant software and values were normalised to *UBQ10*.

GFP fluorescence images

Leaves, leaf epidermal cells and vascular tissues of the 7- to 10-day-old *SUC2::GFP*, *SUC2::GFP:CO* and *CO::GFP:CO* seedlings grown on MS agar under LD were analysed using a Zeiss LSM 510 Meta confocal laser scanning microscope. Images were collected using a 5× lens (for whole leaf image), a 40× lens (for leaf epidermis) and a 63× oil-immersion lens (for vascular tissues), as described by Valverde et al. (Valverde et al., 2004). GFP signal (cyan) was separated from background (black and blue) using the emission fingerprinting Linear Unmixing function.

Results

Spatial pattern of *CONSTANS* expression

CO mRNA is expressed at low levels in wild-type plants (Putterill et al., 1995). The temporal regulation of *CO* mRNA by the circadian clock was studied by RT-PCR (Suarez-Lopez et al., 2001), and initial characterisation of the spatial pattern by in situ hybridisation detected *CO* mRNA in the SAM and young leaf primordia (Simon et al., 1996). To study the pattern of *CO* expression in more detail, a fusion of a *CO* promoter fragment to the *GUS* marker gene was constructed and introduced into wild-type *Arabidopsis* plants. A similar fusion to the *CO* cDNA is sufficient to complement the *co-2* mutation (H.A., unpublished). Staining of whole seedlings detected *CO::GUS* expression in the vascular tissue of the hypocotyl, the cotyledons and the leaves (Fig. 1). Expression was also detected at the apex of the seedlings (Fig. 1A,B), and in all cells of young leaves (Fig. 1B), although this was restricted to the vascular tissue in older leaves (Fig. 1D,E). Cross sections of young inflorescence stems showed staining in the protoxylem and phloem (Fig. 1C), but only in the phloem of older inflorescences. Longitudinal sections of seedlings demonstrated GUS staining in young leaves and in the meristem (Fig. 1B). GUS staining was also detected in the vascular tissue of the root (data not shown). The vascular pattern of *CO::GUS* expression was similar to that described recently for a related *CO::GUS* fusion (Takada and Goto, 2003), except that we also detected expression more widely, particularly in the protoxylem, throughout young leaves and in the meristem (Fig. 1B,C).

Grafting approaches and the generation of somatic sectors indicate that CO induces flowering non-cell autonomously

To identify in which tissues *CO* acts to induce flowering,

genetic chimaeras and grafting approaches were used. A transgenic CRE/LOX recombination system, similar to that described previously (Sieburth et al., 1998), was used to generate chimaeric *co-2* mutant plants. *CO* activity was restored in somatic sectors after excision of a 35S::*GUS* marker inserted in the *CO* intron (Fig. 2; see Materials and methods). Excision was induced by CRE recombinase expressed from a heat-shock promoter (*HS::CRE*). Upon exposure to heat shock during embryo development, 35S::*GUS* was excised from the *CO* intron and *CO* activity restored, so that *co-2 HS::CRE CO(35S::GUS)* plants exposed to heat shock flower earlier and contain somatic sectors lacking GUS activity (Fig. 2A-C). To restore *CO* activity in smaller somatic sectors, plants were heat shocked either after seed imbibition or 5-10 days after germination. In these experiments, a total of 160 plants were exposed to heat shock and five early-flowering plants were recovered. No GUS-negative sectors could be identified in the rosette or cauline leaves of these plants, either by inspecting the entire stained leaf or by making a cross section through the centre of the leaf. This may be because the sectors were small, and could have been present anywhere within the broad expression pattern of *CO*. Nevertheless, seeds were harvested from individual inflorescences of these plants, and the flowering time of the progeny scored to test whether they had inherited active *CO*. Early-flowering progeny were recovered from some inflorescences, whereas three inflorescences produced only late-flowering progeny (Fig. 2E). The presence of inflorescences that produced only late-flowering progeny suggested that inflorescence development does not require *CO* function in the L2 layer, which gives rise to the gametes. These inflorescences might have expressed *CO* in the L1 or L3 layers, but because the axillary meristem that gives rise to the inflorescence is derived from only a small number of L2 cells (Furner and Pumfrey, 1992; Irish and Sussex, 1992) it seems unlikely that all of the inflorescences lacking *CO* in the L2 layer would contain *CO*-positive sectors in the L1 or L3 layers. Therefore, *CO* function is probably not required in the inflorescence after bolting, as was previously shown for the flowering-time gene *FCA* (Furner et al., 1996). However, the difficulty in detecting informative sectors using the CRE/LOX system caused us to focus subsequent experiments on grafting and expression of *CO* from heterologous promoters.

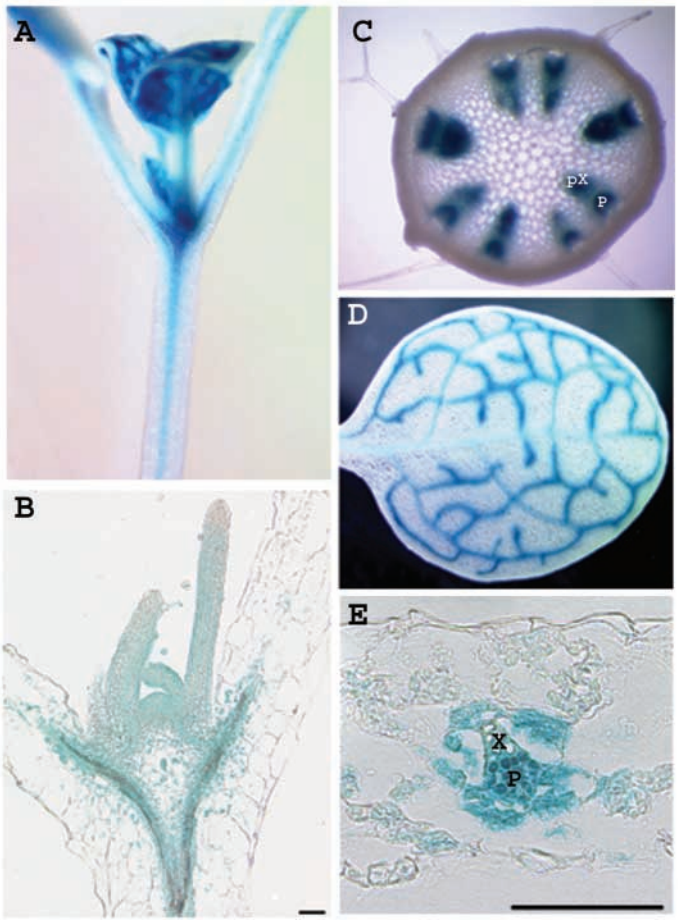

Fig. 1. *CO* expression pattern in *CO::GUS* transgenic plants. Histochemical localisation of GUS activity in *CO::GUS* Landsberg *erecta* (L*er*) plants grown in 16-hour LDs. (A) 12-day-old seedling grown on MS medium. (B) Shoot apex section of an 11-day-old seedling grown on soil. (C) Transverse section of the inflorescence stem of a 38-day-old plant grown on soil. (D) Mature leaf of a 30-day-old seedling grown on soil. (E) Transverse section of an adult leaf. P, phloem; pX, protoxylem; X, xylem. Scale bars: 50 μm.

Recently developed grafting techniques for *Arabidopsis* (Turnbull et al., 2002) were used to assess whether *CO* could act non-cell autonomously across a graft junction. In other species the floral stimulus is transferred through the phloem (King et al., 1968; King and Zeevaart, 1973). The transfer of radiolabelled sugars was therefore examined to determine whether Y-grafted *Arabidopsis* plants form phloem connections. Radiolabelled sucrose was applied to a leaf on the graft donor, and after two hours approximately 7% of the transported radiolabel had crossed the Y-graft junction and was detected in the receiver shoot (Fig. 3A). A phloem connection had therefore formed between the grafted shoots. To test whether flowering in response to LDs involves a systemic signal, Y-grafts were made between two wild-type plants (Turnbull et al., 2002) and these were grown under non-inductive SDs for 70 days. One of the grafted shoots was subsequently exposed to 7 LDs, whereas the other, termed the SD receptor, was covered so that it received only SDs. After this treatment, both shoots were transferred back to SDs. Seventeen days later, all of the shoots exposed to LDs were flowering (Fig. 3B). In addition, 73% of the shoots exposed only to SDs but grafted to shoots exposed to long days were flowering (Fig. 3B). As a control for this experiment, Y-grafted shoots were treated in the same way, but the graft unions were severed, before exposure of one shoot to LDs. After this treatment, none of the shoots exposed only to SDs flowered. This suggests that a floral stimulus can cross the Y-graft junction from a shoot exposed to LDs to induce flowering of the SD receptor. Finally, Y-grafts assembled between *co-2* mutant and wild-type plants were grown under LDs. Control *co-2* mutants produced on average six more leaves and flowered later than those grafted onto wild-type plants (Fig. 3C,D). This experiment suggests that a transmissible substance formed in the wild-type donor shoot crosses the graft junction, accelerating flowering of the *co-2* mutant. The grafting experiments suggest that *CO* acts in response to LDs to regulate the synthesis or transport of a signal that induces flowering of *Arabidopsis*.

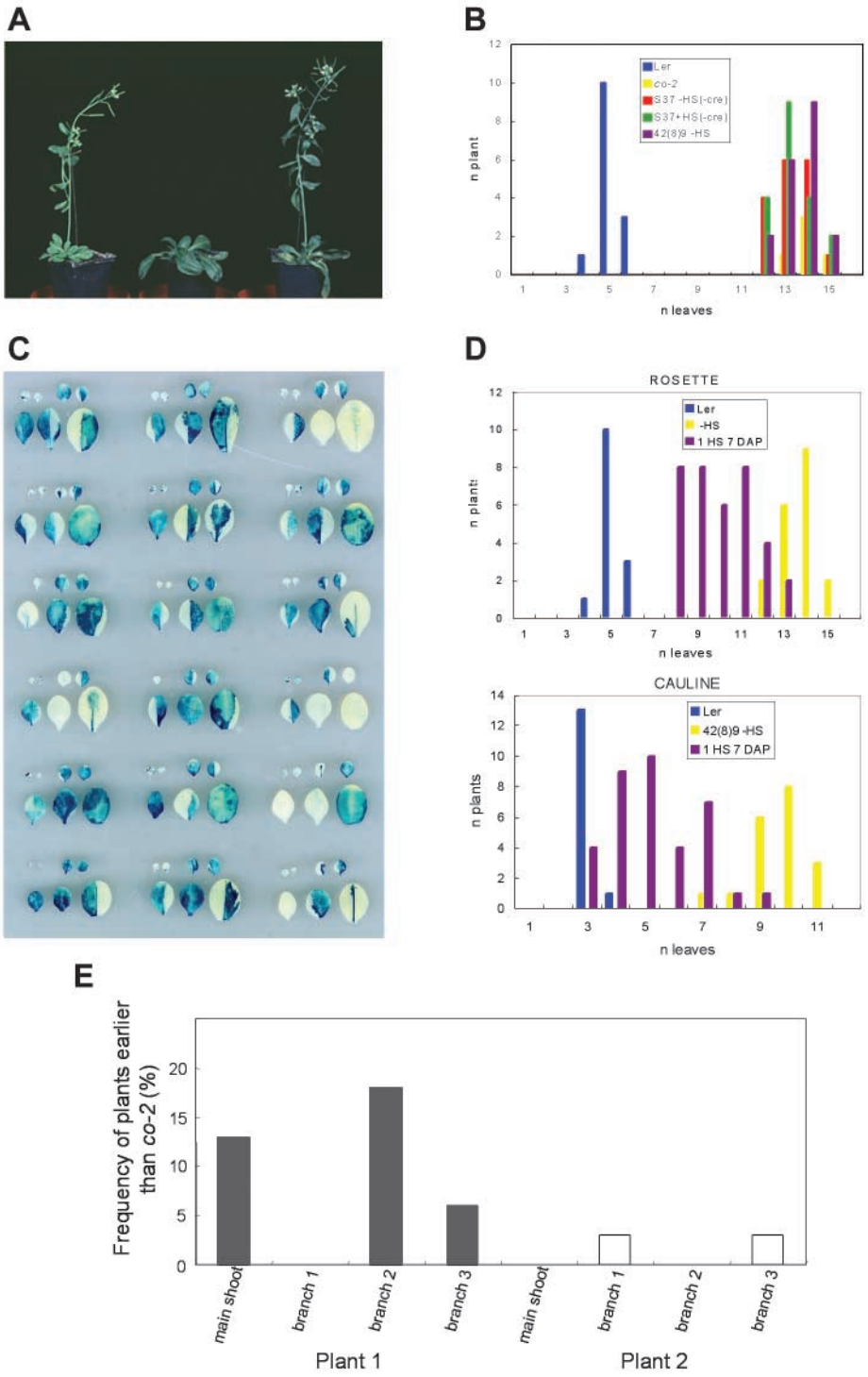

Fig. 2. *CO* somatic sectors created by *Cre*/*lox*-mediated excision of *35S::GUS* from the *CO* intron. (A) Excision of *35S::GUS* from *CO* restores early flowering on *co-2* mutants. (Left) Wild-type plant. (Middle) Plant homozygous for *co-2* and *CO(35S::GUS)* exposed to heat shock flowers late. (Right) Plant homozygous for *co-2*, *HS::CRE* and *CO(35S::GUS)* exposed to heat shock flowers early. This correlates with excision of the *35S::GUS* gene as detected by PCR (data not shown). (B) Comparison of flowering times of progenitor *co-2 CO*(*35S::GUS*) lines with those of wild-type and *co-2* mutant plants. The transgenic progenitor lines do not carry HS::CRE and flower at the same time as co-2 mutants. Heat shock does not affect their flowering time. In the absence of exposure to heat shock, plants homozygous for *co-2 CO (35S::GUS) HS::CRE*, such as line 42(8)9, also flower at the same time as *co-2* mutants. (C) GUS staining illustrates patterns of GUS-negative sectors obtained in plants homozygous for *co-2 HS::CRE* and heterozygous for *CO(35S::GUS)* heat shocked 7 days after pollination. Two cotyledons and five first leaves are stained from 18 different plants exposed to heat shock. Different sector patterns indicate excision of the *35S::GUS* marker at different times during shoot development. (D) Effects on flowering time of heat shocking *co-2 CO*(*35S::GUS*) *HS:CRE* plants 7 days after pollination. The flowering time of heat-shocked plants is intermediate between that of wild type and *co-2* mutants. Flowering time is measured as leaf number. Rosette and cauline leaf number is shown separately. (E) Flowering times of the progeny of two *co-2 CO*(*35S::GUS*) *HS:CRE* plants that were heat shocked either as imbibed seeds (Plant 1) or 10 days after germination (Plant 2). The progeny were harvested from individual inflorescences, and scored as early (similar to wild type) or late (similar to the co-2 mutant). Plants (n=25-40) were scored from each inflorescence, and the proportion of plants scored as early flowering from each inflorescence is shown. Branch 1 of Plant 1, and the main shoot and branch 2 of Plant 2, showed no early flowering plants.

Misexpression from heterologous promoters demonstrates that *CO* activates flowering from the phloem

CO shows a wide spatial pattern of expression that includes the meristem, young leaves, phloem and protoxylem (Fig. 1), and based on the grafting experiments acts non-cell autonomously to induce flowering (Fig. 2). To identify more precisely the tissues in which *CO* controls flowering, misexpression approaches were employed. Promoters driving specific patterns of expression were fused directly to the *CO* open reading frame, or a two component system was used in which the synthetic transcription factor LhG4 (Moore et al., 1998) was expressed in specific patterns and used to drive the expression of both *CO* and the *GUS* marker genes from an effector construct (*Op::GUS-Op::CO*). Using these methods the effect on flowering time of expressing *CO* in specific patterns in the *co-2* mutant could be assessed.

Direct expression of *CO* from the *AtSUC2* and *rolC* promoters complemented the *co-2* mutation. The *AtSUC2* promoter is expressed specifically in the companion cells of the

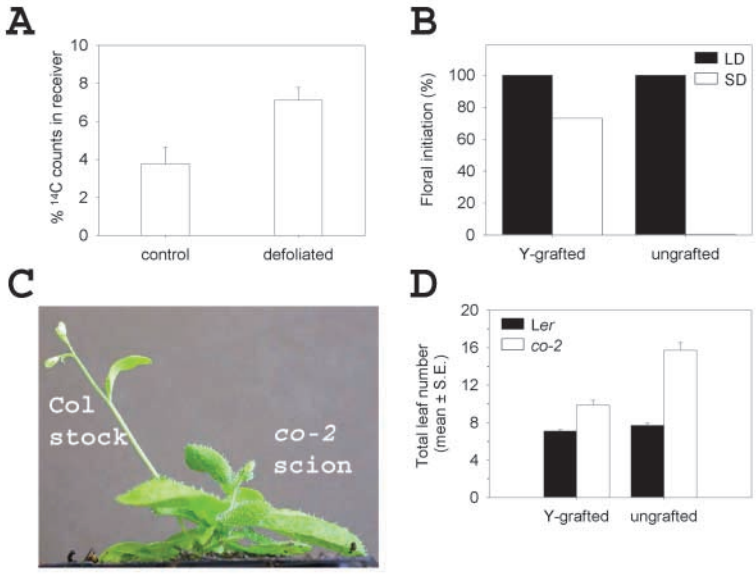

Fig. 3. Analysis of photoperiod response by grafting. (A) Transport of phloem-mobile ^{14}C-sucrose across graft union. Tissues were harvested 2 hours after feeding ^{14}C-sucrose (1 μCi, 1.5 nmol) to leaf on graft partner (donor). Graph shows the proportion of the mobile fraction recovered on the other side of the graft union. Defoliation of the receiver shoot was expected to increase the transfer of photosynthate to the receiver shoot, and a significant effect of defoliation is observed (P=0.05 by t-test). (B) Transmission of a photoperiod stimulus across a graft union. Y-grafted Col wild-type plants grown in 8-hour SDs for 70 days were transferred to 16-hour LDs for 7 days. During this time one of the shoots, the SD receptor, was partially defoliated and covered for part of the day so that it was only exposed to SDs. After the 7 days in LDs, the grafted plants were returned to SDs. Flowering was scored 17 days after the start of LD treatment. Disconnected Y-graft plant pairs were treated exactly as grafted except the graft union was severed. Under these conditions, none of the plants exposed only to SDs flowered. (C) Photograph of Y-grafted *co-2* mutant and wild-type plants. Developing flower buds on *co-2* shoot (right) grafted to Columbia-5 (left) under LD (27 days). (D) Flowering-time of grafted plants. Y-grafts were assembled on 4- to 5-day-old seedlings. The *co-2* mutant grafted to the wild-type plants flowered earlier after producing fewer leaves than the *co* mutant control (P<0.001 for acceleration of flowering in *co* grafts versus *co* controls). Plants were held under 16-hour LD (n=9-16). Bars are mean±s.e.

phloem and not in the meristem or in young leaf primordia (Fig. 4A) (Imlau et al., 1999; Stadler and Sauer, 1996), and the *rolC* promoter is expressed specifically in the phloem (Booker et al., 2003). Transgenic *co-2* mutant plants carrying *AtSUC2::CO* or *rolC::CO* exhibited extremely early flowering, indicating that *CO* expression in the phloem was sufficient to trigger flowering and that expression in the meristem or leaf primordia was not required (Fig. 4A-C). These transgenes caused early flowering under both LDs and SDs, as was previously shown for *35S::CO* transgenic plants.

To determine whether expression of *CO* in the meristem was also able to promote early flowering, *CO* was expressed from the promoters of the *UFO*, *KNAT1*, *STM* and *CLV1* genes, all of which are expressed in the meristem (Clark et al., 1997; Ingram et al., 1995; Lee et al., 1997; Lincoln et al., 1994; Long et al., 1996). Direct fusions of *CO* to the *KNAT1* promoter did not complement the late-flowering phenotype of *co-2* mutants (Fig. 4B,C), although a higher abundance of *CO* mRNA was detected in the meristem of these plants by in situ hybridisation (Fig. 4D). Similar results were obtained with the *STM* and *UFO* promoters (not shown), suggesting that expression of *CO* in the meristem is not sufficient to induce early flowering. By contrast, *co-2* mutant plants containing *CLV1::LhG4* and *Op::GUS-Op::CO* flowered early (Fig. 4C). *CLV1* is expressed in the meristem (Clark et al., 1997), but analysis of the transgenic plants also indicated expression of GUS in the vascular tissue (Fig. 4A), which taken together with the results obtained using other promoters suggests that expression of *CO* in the vascular tissue of these plants is responsible for the early-flowering phenotype.

In *CO::GUS* plants staining was also detected throughout the young leaf primordia (Fig. 1B), and therefore *CO* was expressed in these organs using a fusion of the *AINTEGUMENTA* (*ANT*) promoter (Elliott et al., 1996; Klucher et al., 1996) to *LhG4* to drive *CO* expression in *ANT::LhG4 Op::GUS-Op::CO co-2* plants. These plants flowered late, at a similar time to *co-2* mutants, suggesting that *CO* was not able to drive early flowering when expressed in leaf primordia (Fig. 4A,C). Finally, fusion of the *ML1* promoter, which is expressed specifically in the epidermis (Abe et al., 2001), or the *TobRB7* promoter, which is expressed in the root (Yamamoto et al., 1991), to *CO* did not complement the *co-2* mutation (Fig. 1B and data not shown). The misexpression data therefore indicate that *CO* acts specifically in the phloem to promote flowering of *Arabidopsis*.

CO activates *FT* cell-autonomously in *SUC2::CO* plants

CO promotes the expression of downstream genes (Samach et al., 2000), particularly *FT*, which encodes a RAF-kinase-inhibitor-like protein (Kardailsky et al., 1999; Kobayashi et al., 1999). Expression of *FT* from the viral CaMV35S promoter corrects the late-flowering phenotype of *co-2* mutants (Kardailsky et al., 1999; Kobayashi et al., 1999). Whether the mechanism by which *CO* activates flowering from the phloem companion cells involves *FT* was therefore examined. The abundance of *FT* and *CO* mRNA 16 hours after dawn was examined in LD-grown wild-type, *SUC2::CO* and *35S::CO* plants by RT-PCR (Fig. 5A). *CO* mRNA abundance was much higher in *35S::CO* and *SUC2::CO* than in wild-type, with *35S::CO* showing the highest levels of *CO* mRNA. *FT* mRNA levels were also elevated in *35S::CO* and *SUC2::CO* plants, with *SUC2::CO* showing the higher levels. This supports the idea that expression of *CO* in the phloem from the *SUC2* promoter causes increased *FT* expression, as was previously shown for *35S::CO* (Samach et al., 2000). Furthermore, the higher level of *FT* expression in *SUC2::CO* than *35S::CO* plants suggests that specific expression of *CO* in the phloem may be more effective in activation of *FT* than general expression from the *35S* promoter.

To determine the spatial pattern of expression of *CO* and *FT* in *SUC2::CO* plants, in situ hybridisation was performed. The abundance of *CO* and *FT* mRNA in the vascular tissues of wild-type plants was below the level of detection by in situ

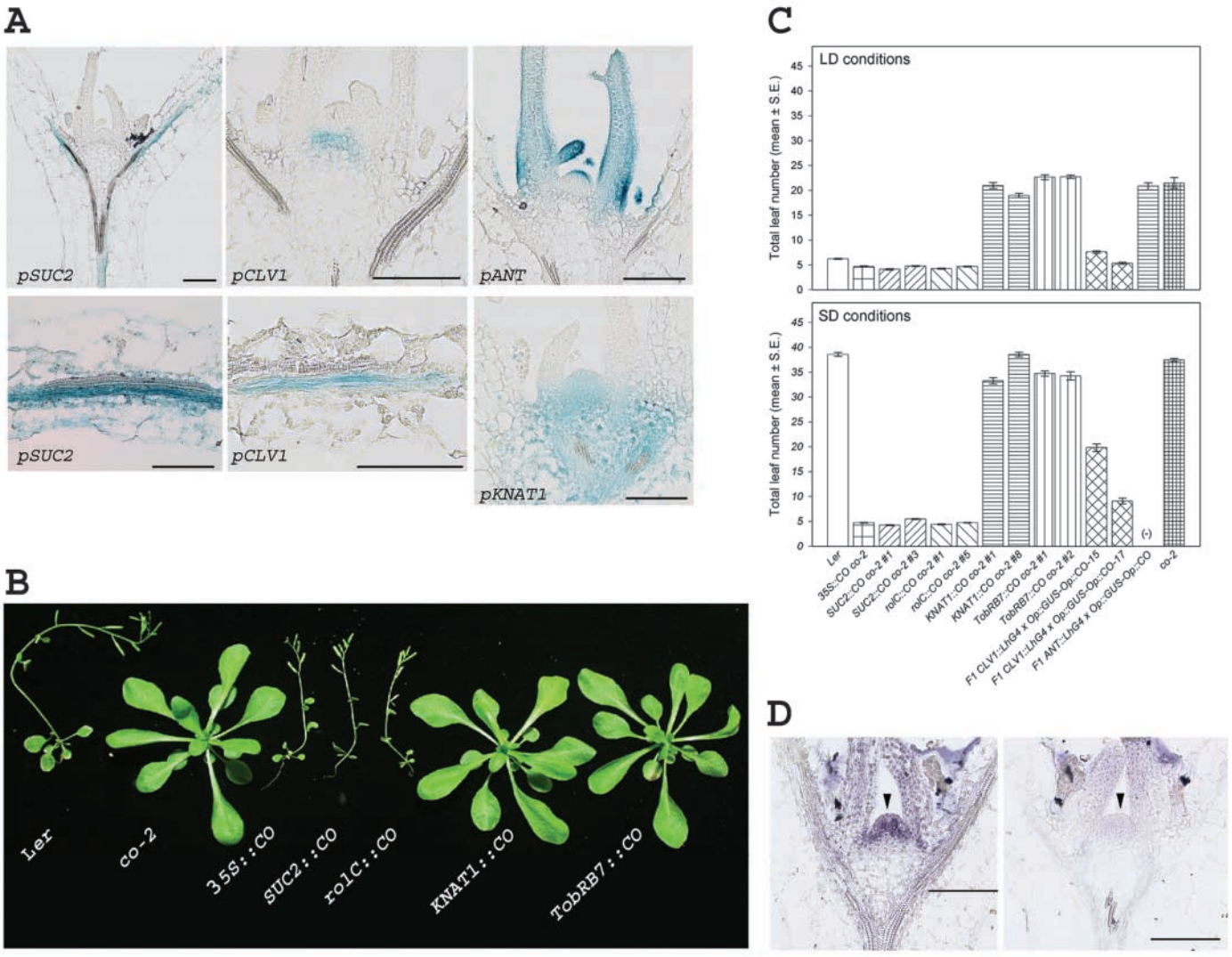

Fig. 4. Misexpression of *CO* from heterologous promoters. (A) Histochemical localisation of GUS activity in longitudinal sections of promoter::*GUS* transgenic plants. Shoot apex and cotyledon section of 9-day-old At*SUC2::GUS* L*er* plant showing phloem-specific expression (*pSUC2*). Shoot apex and cotyledon section of an 11-day-old F1 plant of *CLV1::LhG4×Op::GUS- Op::CO co-2* showing expression in the meristem and phloem (*pCLV1*). Shoot apex section of an 11-day-old F1 plant of *ANT::LhG4×Op::GUS-Op::CO co-2* showing expression in leaf primordia (*pANT*). Shoot apex section of 9-day-old *KNAT1::GUS* L*er* plant showing expression in meristem (*pKNAT*). (B) Phenotype of LD-grown *co-2* plants carrying transgenic constructs driving *CO* expression in specific domains. (C) Flowering time of *co-2* transgenic plants in which *CO* is expressed from tissue-specific promoters. Plants were grown either in LDs or in SDs. The minus sign indicates that an experiment was not conducted under SDs. (D) In situ hybridisation of sections of *KNAT1::CO co-2* (left) and *co-2* mutant (right) plants probed with *CO*. Arrowheads in D indicate SAM. Scale bars: 100 μm.

hybridisation (Fig. 5B) (Takada and Goto, 2003). However, *CO* mRNA was detected in the phloem of *AtSUC2::CO* and *rolC::CO* plants (Fig. 5B). Expression analysis demonstrated that *FT* was upregulated in *AtSUC2::CO* and *rolC::CO* plants specifically in the phloem, and not in adjacent leaf cells (Fig. 5B). This is consistent with the recent observation that mutation in the *TFL2* gene, which encodes a heterochromatin protein 1-like protein of *Arabidopsis*, causes early flowering and increased *FT* expression in the vascular tissue (Takada and Goto, 2003).

Genetic analysis indicates that *CO* activates flowering from the phloem through *FT*

Gain- and loss-of-function genetic experiments were used to determine whether the activation of flowering by *CO* in the phloem involves *FT* (Fig. 6). Introduction of the *ft-7* mutation into *AtSUC2::CO* plants significantly delayed flowering (Fig. 6), indicating that the extreme early flowering induced by expression of *CO* in the phloem requires *FT* activity. However, these plants still flowered earlier than *ft-7* mutants (Fig. 6), thus in the phloem CO does not exclusively function through *FT* activation.

In addition, to determine in which tissues *FT* acts to promote flowering downstream of *CO*, *FT* was expressed from heterologous promoters in the *co-2* mutant. When expressed in the phloem using the *rolC* or *AtSUC2* promoters, FT caused extreme early flowering and complemented the *co-2* mutation (Fig. 6), supporting the idea that the early flowering of

At*SUC2::CO* plants is partially due to activation of *FT* in the phloem. However, whereas *CO* activated flowering only when expressed in the phloem (Fig. 4), *FT* induced early flowering when expressed in several tissues. Fusion of *FT* to the promoters of the meristem genes *UFO*, *KNAT1* and *STM* corrected the late-flowering phenotype of *co-2* mutants (Fig. 6 and data not shown). In addition, expression of *FT* from the epidermis-specific promoter of *ML1* induced early flowering (Fig. 6). Thus activation of *FT* in the phloem is part of the mechanism by which CO promotes flowering, but the effectiveness of *FT* in promoting flowering is not restricted to these cells.

In *SUC2::GFP:CO* or *CO::GFP:CO* plants, GFP:CO fusion protein is detected in the phloem and not in other leaf cells

The non-cell-autonomous induction of floral development by CO when expressed in the phloem may be explained by movement of the protein into other cells, as has been described for GFP (Truernit et al., 1996) and several plant transcription factors (Lucas et al., 1995; Nakajima et al., 2001; Sessions et al., 2000). To test the possibility that CO moves from the phloem companion cells, the location of a GFP:CO fusion protein was tested when expressed from the *AtSUC2* or *CO* promoters. The At*SUC2::GFP:CO* and *CO::GFP:CO* transgenes fully complemented the *co-2* mutation. GFP fluorescence was then examined by confocal microscopy. In control *AtSUC2::GFP* plants, GFP fluorescence was detected in the vascular tissue, and also in the mesophyll and epidermal layers of the leaf (Fig. 5C), indicating that GFP can move freely from the companion cells, as previously demonstrated (Truernit et al., 1996). By contrast, in *AtSUC2::GFP:CO* plants, fluorescence was detected only in the vascular tissue of the leaf (Fig. 5C). In addition, at the apex of *AtSUC2::GFP:CO* seedlings, GFP fluorescence was detected in the vascular tissue, but not in the meristem (Fig. 5D). Therefore, at the level of detection of this experiment, GFP:CO protein remains in the phloem and does not move to adjacent leaf cells, or to the

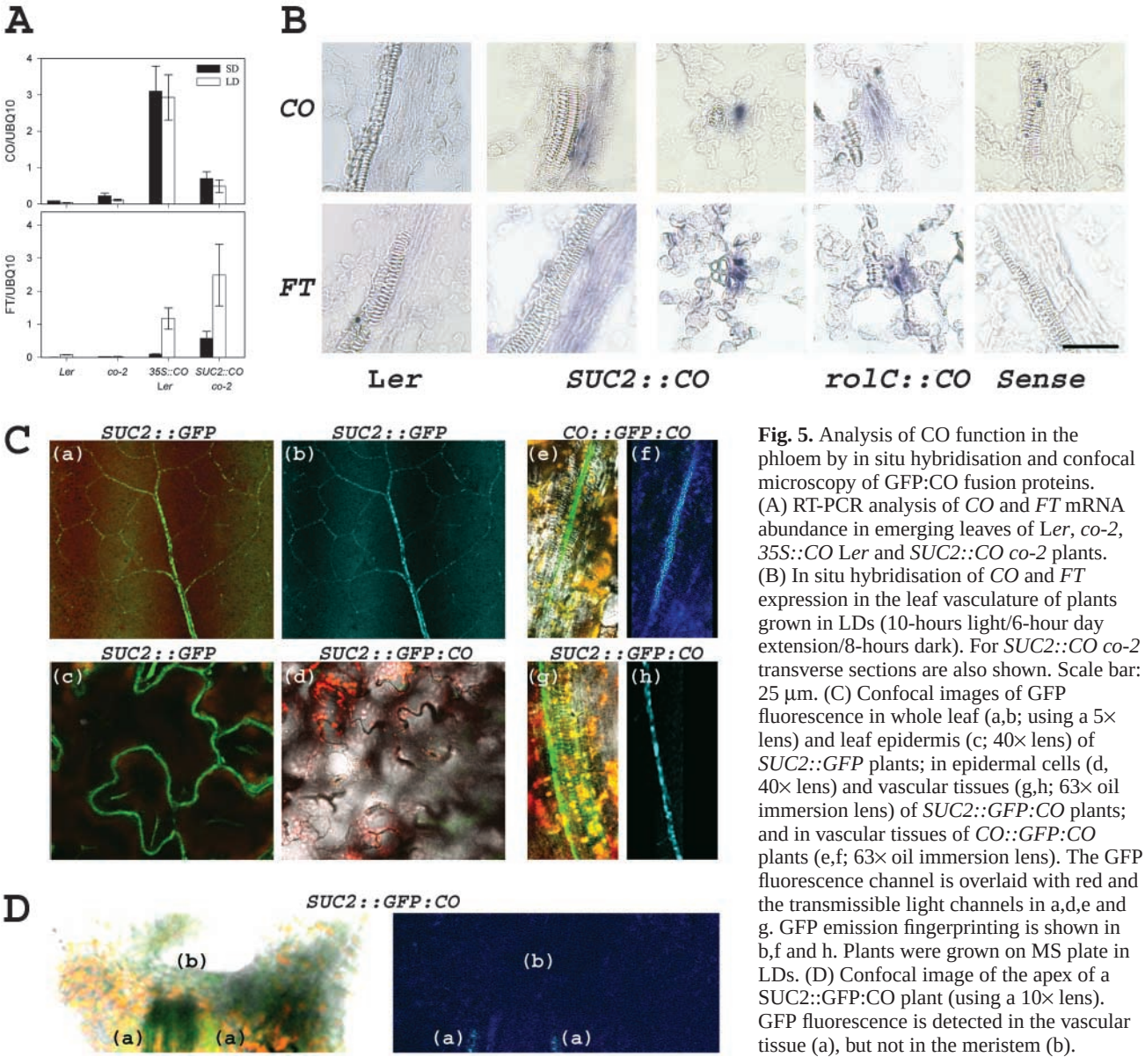

Fig. 5. Analysis of CO function in the phloem by in situ hybridisation and confocal microscopy of GFP:CO fusion proteins. (A) RT-PCR analysis of *CO* and *FT* mRNA abundance in emerging leaves of L*er*, *co-2*, *35S::CO* L*er* and *SUC2::CO co-2* plants. (B) In situ hybridisation of *CO* and *FT* expression in the leaf vasculature of plants grown in LDs (10-hours light/6-hour day extension/8-hours dark). For *SUC2::CO co-2* transverse sections are also shown. Scale bar: 25 μm. (C) Confocal images of GFP fluorescence in whole leaf (a,b; using a 5× lens) and leaf epidermis (c; 40× lens) of *SUC2::GFP* plants; in epidermal cells (d, 40× lens) and vascular tissues (g,h; 63× oil immersion lens) of *SUC2::GFP:CO* plants; and in vascular tissues of *CO::GFP:CO* plants (e,f; 63× oil immersion lens). The GFP fluorescence channel is overlaid with red and the transmissible light channels in a,d,e and g. GFP emission fingerprinting is shown in b,f and h. Plants were grown on MS plate in LDs. (D) Confocal image of the apex of a SUC2::GFP:CO plant (using a 10× lens). GFP fluorescence is detected in the vascular tissue (a), but not in the meristem (b).

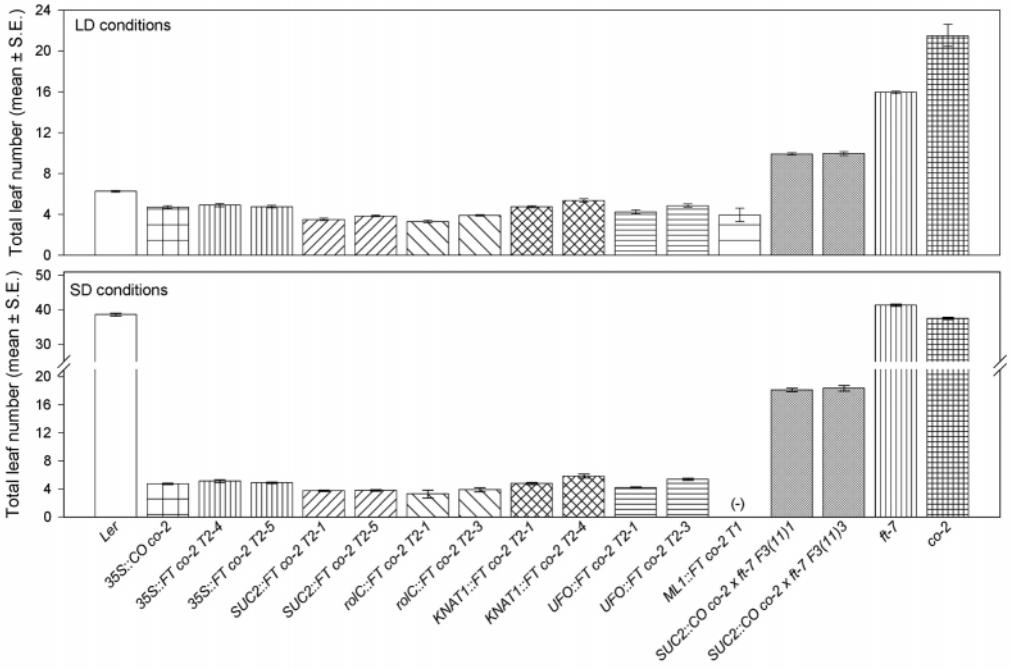

Fig. 6. Flowering time of *co-2* transgenic plants in which *FT* is expressed in specific tissues, and of *SUC2::CO co-2 ft-7* plants. Plants were grown in LDs or in SDs on soil. The minus sign indicates that an experiment was not conducted under SDs.

meristem. Similarly, in *CO::GFP:CO* plants, GFP:CO was detected only in the vascular tissue (Fig. 5C). The localisation of GFP:CO protein to the phloem is consistent with the CO-mediated activation of *FT* expression in the leaves of *AtSUC2::CO* plants only occurring in the phloem (Fig. 5A), and indicates that CO protein acts in the phloem companion cells to induce flowering.

Discussion

Classical physiological experiments demonstrated that the initiation of flowering in response to day length involves a systemic signal formed in the leaves that induces floral development at the SAM (Zeevaart, 1976). A conserved pathway of regulatory proteins that induce flowering in response to day length has been described in *Arabidopsis*, and CO and its target gene *FT* play central roles in this pathway. However, these regulatory proteins have not been integrated into a framework that includes the systemic signal defined by physiological experiments. Recent work demonstrated expression of *CO* and *FT* mRNA in the vascular tissue of *tfl2* mutants (Takada and Goto, 2003), but these mutants exhibit a pleiotropic phenotype (Larsson et al., 1998) and the TFL2 protein regulates chromatin structure to repress the expression of many genes (Gaudin et al., 2001; Kotake et al., 2003), complicating the analysis of the mutant. We used a combination of grafting and specific misexpression of CO or GFP:CO to show that CO acts specifically in the phloem companion cells to trigger flowering non-cell autonomously. *CO* is therefore required for the synthesis or transport of systemic signals that are transported through the phloem to induce flowering at the apex.

The spatial regulation of CO function

Analysis of *CO::GUS* plants detected GUS expression throughout young leaf primordia, in the vascular tissue of mature leaves and cotyledons, as well as in the phloem and the protoxylem of stems. Weaker staining was also detected in the shoot apical meristem, as was indicated by in situ hybridisation (Simon et al., 1996). *CO* expression in the phloem of mature tissues is consistent with a recent report (Takada and Goto, 2003), although we also detected GUS staining more widely (Fig. 1).

Recently, CO was proposed to be part of the mechanism by which *Arabidopsis* distinguishes long and short days, through a combination of circadian-clock regulation and direct responsiveness to exposure to light (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Imaizumi et al., 2003; Valverde et al., 2004). Furthermore, classical grafting experiments suggested that the perception of day length occurs in the leaf, which is consistent with CO acting in phloem cells to promote flowering. This conclusion may have significance beyond flowering-time control, as heterologous expression of *CO* in potato delayed tuberisation, and this effect was graft transmissible (Martinez-Garcia et al., 2002).

The mechanism by which CO promotes flowering in *SUC2::CO* plants appears to involve cell-autonomous activation of *FT* in the phloem. In other tissues, such as the meristem and L1 layer, *CO* expression did not induce flowering, but *FT* expression did. This suggests that CO may only activate *FT* in the phloem, which is supported by the higher abundance of *FT* mRNA in *SUC2::CO* than *35S::CO* plants. Alternatively, in tissues other than the phloem, activation of *FT* by CO may occur at a lower level than by direct fusion of *FT* to specific promoters, and below a threshold level required to induce flowering.

Some plant transcription factors move between plant cells (Lucas et al., 1995; Nakajima et al., 2001; Wu et al., 2003). However, the activation of *FT* specifically in the phloem, and the presence of GFP:CO only in these cells within the leaves and stems of *SUC2::GFP:CO* and *CO::GFP:CO* transgenic plants, suggest that CO protein does not move from the

phloem. The zinc fingers of CO most resemble B-boxes that were described in several animal proteins, and which act as protein-protein interaction domains (Robson et al., 2001). Thus the presence of CO within a larger protein complex may prevent movement of the protein from the phloem companion cells, as was previously proposed for MADS box proteins in floral primordia (Wu et al., 2003). Similarly, there is no evidence that CO contains specific sequences that would enable its translocation between cells, as have been identified for transcription factors such as the maize protein KNOTTED (Lucas et al., 1995).

The role of *FT* downstream of CO

The position of *FT* downstream of *CO* in the photoperiod response pathway was demonstrated genetically and by the analysis of *FT* expression in *co* mutant or *35S::CO* backgrounds (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000). Our data demonstrate that in plants in which *CO* is expressed specifically in the phloem, *FT* is required for the extreme early flowering induced by CO, and is specifically activated by CO in phloem cells. *FT* is probably also activated by CO in the phloem of wild-type plants, although its mRNA abundance is below the level of detection.

Misexpression experiments indicated that *FT* activates flowering when expressed specifically in a wide range of tissues. This may be physiologically significant, as *FT* is regulated by several flowering pathways, as well as by the photoperiod pathway (Blazquez et al., 2003; Cerdan and Chory, 2003; Halliday et al., 2003; Mouradov et al., 2002; Simpson and Dean, 2002). The pattern of FT expression in wild-type plants has not been described, and the tissues in which most flowering-time pathways act to promote flowering have not been defined and may therefore activate *FT* expression in tissues other than the phloem.

FT is a member of a small *Arabidopsis* gene family that includes *TERMINAL FLOWER 1* (*TFL1*), and is related to *CENTRORADIALIS* (*CEN*) of Antirrhinum and *SELF PRUNING* (*SP*) of tomato (Bradley et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999; Pnueli et al., 1998). These proteins, named CETS (CEN, TFL1, FT), share homology to the RAF-kinase-inhibitor proteins of mammals (Kardailsky et al., 1999; Pnueli et al., 2001), and the structure of the CEN protein is related to that of RAF-kinase inhibitors (Banfield and Brady, 2000). The mechanism of action of these proteins was explored by identifying proteins that interact with SP in the yeast two-hybrid system (Pnueli et al., 2001). A NIMA-like kinase, bZIP transcription factors and a 14-3-3 protein that interact with SP were identified, and led to the suggestion that CETS proteins act as adapters in a variety of signalling pathways. How these functions relate to the floral promotive activity of FT is unknown.

The non-cell autonomy of the effect of *FT* on flowering may be due to movement of FT protein between cells, or to the activation of intercellular signalling processes downstream of FT. FT is only 23 kDa (Kardailsky et al., 1999; Kobayashi et al., 1999), smaller than GFP and below the size exclusion limit of plasmodesmata (Imlau et al., 1999), suggesting that it may move freely through plant tissues. Therefore the activation of *FT* in the phloem may precede movement of the protein to the meristem or other tissues. This would be consistent with the observation that *FT* will activate flowering when expressed in a wide range of cell types. Although the floral stimulus is usually not considered to be a protein, classical grafting experiments do not exclude this possibility (Perilleux and Bernier, 2002). However, our data are also consistent with other possibilities, including that FT regulates synthesis of a mobile, small molecule capable of inducing flowering. The target of the *FT*-derived signal in the meristem is unknown, but genetic experiments suggested a close correlation between *FT* and activation of the floral meristem identity gene *APETALA1* (Ruiz-Garcia et al., 1997).

Finally, although *FT* plays a major role in the induction of flowering downstream of *CO*, the flowering time of *SUC2::CO ft-7* plants demonstrates that FT is not essential for *SUC2::CO* to promote early flowering. CO must therefore regulate flowering by both FT-dependent and FT-independent processes. These FT-independent processes might involve other genes previously shown to be upregulated by overexpression of *CO* from the 35S promoter (Samach et al., 2000).

Perspectives

Taken together, the grafting and misexpression data indicate that a systemic signal, analogous to the floral stimulus, induces flowering of *Arabidopsis* in response to LDs, and that this is activated by CO in the phloem companion cells and transmitted through the phloem. FT activates flowering when expressed in many tissues, and may move readily to a critical group of cells in which it promotes flowering or act in almost any tissue to promote the formation of a downstream mobile signal. However, the mechanism by which CO activates flowering from the phloem also involves FT-independent processes, suggesting that CO regulates more than one systemic signal. The identification of CO as a regulator of systemic signals that induce flowering will facilitate the definitive identification of these signals, and the elucidation of the signalling mechanisms underlying this process.

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