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 RAP-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 (Bornet 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 (Hdl; 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 means ± s.e.

Both the ANT::LhG4 and the CLV1::LhG4 activator lines were obtained from Dr T. Laux (University of Freiburg, Germany) in Ler (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 homoyzomatic 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 EcoRI site 5′ of 35S::GUS and loxH in the HindIII site 3′ of the marker gene, generating pGUSLOX. 35S::GUS flanked by the loxP sites was inserted in the EcoRI 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 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′-GGGGACAACTTTGACAAAGCGGCGGTTTCT-3′) reverse primers contain the attB2 tail (5′-GGGGACACACTTTGTGACAGAAGGCTGTT-3′); AtSU C2, 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′-GGGGACAACTTTGACAAAGCGGCGGTTTCT-3′) reverse primers contain the attB2 tail (5′-GGGGACACACTTTGTGACAGAAGGCTGTT-3′). Specific sequences for each primer pair were: SUC2-F, 5′-AAAATCTGGTTCATATTTAATTTCA-3′; SUC2-R, 5′-ATTGTGAAACCAAGAGAAATGAA-3′; KNAT1-F, 5′-GACTTGACGCCCTAAAGTT-3′; KNAT1-R, 5′-ACCACAGTGAAGAAATGTT-3′; STM-F, 5′-GGTGTTGTGCAATGTCATTG-3′; STM-R, 5′-CTTCCTTCTCTCTCCTACTAGT-3′; UFO-F, 5′-GGAATTCCTGTTATTTATGCGG-3′; UFO-R, 5′-TTAGCTGAATGACAAAGAAGA-3′; ML1-F, 5′-AACGGTTTACAAGAAAAAACCGAGA-3′; and ML1-R, 5′-AACGGGTTTAGCTGAGGAGGTGCT-3′.

The 35S promoter was PCR amplified from the pBI121 plasmid using the specific primer sequences 35S-F (5′-AGGTTTCCCCAGATTGCTTCCCTTACCTT-3′) and 35S-R (5′-CTCCCGTGTTCTGCTTCCCAA-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′-GAAAAGAGGCAATGATCCGGGCC-3′ and rolC-R, 5′-TCCCCACACCTGCAAGAATGATC-3′; TobRB7-F, 5′-CCCCATTTATGTTTCTCTCAATTA-3′; and TobRB7-R, 5′-TACCCACATCCACATAAATCT-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 pPGTV-BAR, or CO and FT cDNA in pGreen0029, to generate the binary destination vectors. Different promoter fusions were produced by LR reactions.

A 2451 base pair CO promoter fragment purified from plasmid pBOCL 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 ages 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 imibed seeds were sown on soil. Experiments involving the generation of mosaic plants to determine the activity of CO were performed using F1 embryos/seeds from crosses between plants homozygous for co-2 HS::CRE CO(35S::GUS) or co-2 HS::CRE. The resulting F1 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 Ler or co-2 plants by floral dip (Clough and Bent, 1998). Plasmid pSLCOGUSL was introduced into Agrobacterium strain C58C1(pGV2260) and transformed into Ler.

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 (terminated 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-¹⁴C]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 Ler 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 Paraffin 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 0.1xSSC.

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 dT15 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 COol9 as described (Suarez-Lopez et al., 2001). FT was amplified using primers FTRTTPCR-F (5'-AGAAGACTTTAGGCTTC7T3') and FTRTPCR-R (5'- TTATCGCTACACACTATATAAG-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 Phosphorimager (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 40x lens (for whole leaf image), a 40x lens (for leaf epidermis) and a 63x 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.

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.
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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. 2. CO somatic sectors created by Crelox-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.
Fig. 3. Analysis of photoperiod response by grafting. (A) Transport of phloem-mobile 14C-sucrose across graft union. Tissues were harvested 2 hours after feeding 14C-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 Col. (D) Flowering-time of grafted plants. Y-grafted plants 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. Plants were held under 16-hour LD (n=9-16). Bars are mean±s.e.

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; Kluch 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 MLI 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 RAR-kinase-inhibitor-like protein (Kardajlsky et al., 1999; Kobayashi et al., 1999). Expression of FT from the viral CaMV35S promoter corrects the late-flowering phenotype of co-2 mutants (Kardajlsky 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
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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
AtSUC2::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 AtSUC2::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 Ler, co-2, 35S::CO Ler 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 dark extension/8-hours dark). For SUC2::CO co-2 plants, transverse sections are also shown. Scale bar: 25 μm. (C) Confocal images of GFP fluorescence in whole leaf (a,b; using a 5x lens) and leaf epidermis (c; 40x lens) of SUC2::GFP plants; in epidermal cells (d, 40x lens) and vascular tissues (e; 63x oil immersion lens) of SUC2::GFP:CO plants; and in vascular tissues of CO::GFP:CO plants (f; 63x 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 10x lens). GFP fluorescence is detected in the vascular tissue (a), but not in the meristem (b).
CONSTANS induces flowering from the phloem. 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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References


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