Spatially distinct regulatory roles for gibberellins in the promotion of flowering of Arabidopsis under long photoperiods

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SUMMARY
The plant growth regulator gibberellin (GA) contributes to many developmental processes, including the transition to flowering. In Arabidopsis, GA promotes this transition most strongly under environmental conditions such as short days (SDs) when other regulatory pathways that promote flowering are not active. Under SDs, GAs activate transcription of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and LEAFY (LFY) at the shoot meristem, two genes encoding transcription factors involved in flowering. Here, the tissues in which GAs act to promote flowering were tested under different environmental conditions. The enzyme GIBBERELLIN 2 OXIDASE 7 (GA2ox7), which catabolizes active GAs, was overexpressed in most tissues from the viral CaMV 35S promoter, specifically in the vascular tissue from the SUCROSE TRANSPORTER 2 (SUC2) promoter or in the shoot apical meristem from the KNAT1 promoter. We find that under inductive long days (LDs), GAs are required in the vascular tissue to increase the levels of FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF) mRNAs, which encode a systemic signal transported from the leaves to the meristem during floral induction. Similarly, impairing GA signalling in the vascular tissue reduces FT and TSF mRNA levels and delays flowering. In the meristem under inductive LDs, GAs are not required to activate SOC1, as reported under SDs, but for subsequent steps in floral induction, including transcription of genes encoding SQUAMOSA PROMOTER BINDING PROMOTER LIKE (SPL) transcription factors. Thus, GA has important roles in promoting transcription of FT, TSF and SPL genes during floral induction in response to LDs, and these functions are spatially separated between the leaves and shoot meristem.

KEY WORDS: Arabidopsis, Flowering, Gibberellins

INTRODUCTION
Flowering occurs when the shoot apical meristem (SAM), from which all aerial tissues are derived, undergoes a developmental transition that allows the production of flowers instead of leaves. In Arabidopsis thaliana, this transition is controlled by several pathways that are regulated by endogenous developmental signals or by external environmental cues (Fornara et al., 2010). These pathways include the photoperiodic pathway that promotes flowering in response to long days (LDs) characteristic of summer, and the response pathway to the growth regulator gibberellin, which has its strongest effect under short days (SDs).

In the photoperiodic pathway, transcription of the FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF) genes is activated specifically under LDs (Kobayashi and Weigel, 2007; Turck et al., 2008). These genes encode small proteins that are members of the CEN1, TFL1, FT (CETS) family related to phosphatidyl-ethanolamine-binding proteins (Kardailsky et al., 1999; Kobayashi et al., 1999; Pnueli et al., 2001). FT has been demonstrated to move through the phloem system to the SAM (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). FT and TSF interact with the bZIP transcription factor FD, which is expressed at the shoot apical meristem (Abe et al., 2005; Wigge et al., 2005). Genetic analysis demonstrated that FT, TSF and FD all contribute to characteristic changes in gene expression at the SAM during floral transition, including induction of transcription of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS (SOC1) and FRUITFULL (FUL), which encode related MADS box transcription factors and are among the first genes to be activated after exposure of plants to LDs (Abe et al., 2005; Jang et al., 2009; Samach et al., 2000; Searle et al., 2006; Wang et al., 2009; Wigge et al., 2005). After induction of SOC1, expression of many flowering genes is rapidly induced in the meristem. These include members of the family of genes encoding the SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPLs) transcription factors. Three members of this family, SPL3, SPL4 and SPL5, are direct targets of SOC1 and FD (Jung et al., 2011), while transcriptome profiling and in situ hybridization demonstrate that their expression also requires FT TSF and SOC1 FUL function (Schmid et al., 2003; Torti et al., 2012). Ectopic expression of SPL3 accelerates flowering, supporting the idea that they are part of the floral inductive process (Cardon et al., 1997; Wang et al., 2009; Yamaguchi et al., 2009). Similarly, suppression of the function of many SPL genes through overexpression of miR156, which targets SPL mRNAs, delays floral transition (Schwab et al., 2005; Schwarz et al., 2008; Wu and Poethig, 2006). In turn, the floral meristem identity genes APETALA 1 (API) and LEAFY (LFY), as well as the flowering-time gene FRUITFULL (FUL) are directly activated by SPL3 (Wang et al., 2009; Yamaguchi et al., 2009), whereas API and LFY confer floral identity on developing primordia (Bowman et al., 1993). Thus, a series of direct interactions in the shoot meristem linking SOC1, SPLs and floral meristem identity genes reveals one route from floral induction by LDs to floral development.
Genetic analysis suggests that gibberellins have their most important function in flowering under SD. The ga1-3 mutant, which is impaired in GA biosynthesis, fails to flower in SD but shows a relatively weak late-flowering phenotype under LD (Wilson et al., 1992). The stronger effect of GA under SDs, is probably due to the photoperiodic pathway masking the effect of loss of GA signalling under LDs (Reeves and Coupland, 2001). A mechanistic basis for the interaction between the photoperiodic and GA pathways is suggested by the convergence of both pathways on the promotion of SOC1 transcription in the meristem (Achard et al., 2004; Moon et al., 2003; Searle et al., 2006). Furthermore, flowering of soc1 mutants shows reduced sensitivity to GA treatments (Moon et al., 2003). Previous reports demonstrated that GA activates later events in the meristem during flowering, such as the activation of LFY transcription (Blazquez et al., 1998), although it is now unclear whether these are an indirect consequence of increased SOCI expression. In addition, GA has been reported to affect flowering by other mechanisms, but these are not yet clearly integrated into the flowering network. GA increases expression of miR159 and of its target mRNA encoding the MYB transcription factor MYB33 (Achard et al., 2004), which has been proposed to regulate LFY expression (Achard et al., 2004; Gocal et al., 2001; Woodger et al., 2003). In addition, the GATA NITRIDE INDUCIBLE CARBON-METABOLISM INVOLVED (GNC) and GNC-LIKE (GNL) genes encode GATA factors that inhibit flowering, and are repressed by GAs (Richter et al., 2010). Finally, FT transcript is reduced in the strong GA biosynthetic mutant ga1-3 after transfer from SD to far-red enriched LD (Hisamatsu and King, 2008). The relevance of this observation to floral induction under standard white light LD conditions has not yet been demonstrated. Overall, GA may regulate flowering of Arabidopsis by different mechanisms that are not clearly distinguished.

Bioactive GAs, particularly GA1, GA4 and GA3, are synthesized through a complex pathway (Yamaguchi, 2008). Genes encoding the enzyme GA20 oxidase, which is required to synthesize bioactive GA, are widely expressed in the plant, suggesting that GA is synthesized in most tissues (Rieu et al., 2008b). In addition, GA4 content increases 100-fold in the Arabidopsis shoot apex during the transition to flowering, although this could not be correlated with increased expression of biosynthetic enzymes (Eriksson et al., 2006). The levels of active GAs are also reduced by 2-β hydroxylation catalyzed by GA2 oxidases (GA2oxs) (Rieu et al., 2008a; Schomburg et al., 2003). In Arabidopsis, two classes of GA2oxs have been identified. Class I and II GA2oxs act directly on bioactive GA1 and GA4 to generate inactive hydroxylated forms. By contrast, Class III GA2oxs act earlier in the biosynthetic pathway to reduce the abundance of precursors of bioactive GAs. Overexpression of either class of GA2ox from the viral CaMV 35S promoter reduces the levels of bioactive GAs in vivo and causes phenotypes associated with GA depletion (Rieu et al., 2008a; Schomburg et al., 2003).

GAs regulate gene expression through a relatively short signal transduction pathway (Harberd et al., 2009). This pathway influences gene expression by promoting the degradation of DELLA proteins (Dill et al., 2004; Fu et al., 2004; McGinnis et al., 2003; Nakajima et al., 2006; Willige et al., 2007). This removal of DELLA proteins releases transcription factors that are otherwise prevented from binding DNA by DELLAs, including PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PIF5 (de Lucas et al., 2008; Feng et al., 2008).

Here, we assess the effect on flowering of overexpressing GA2ox and thereby depleting GA in specific tissues and demonstrate spatially distinct functions in the promotion of flowering under LDs.

MATERIALS AND METHODS

Growth conditions and plant materials

Plants were grown on soil under controlled conditions of LDs (16 hours light/8 hours dark) and SDs (8 hours light/16 hours dark) at 20°C. The level of photosynthetic active radiation was 60 μmol m⁻² s⁻¹ under both conditions. For quantitative PCR, leaves of 12-day-old seedlings were collected every 3 hours in a 24 hour cycle under LDs, and mRNA was extracted. For in situ hybridization, plants were grown for 3 weeks in SD, then shifted to LD, and apices were collected 8 hours after dawn before transfer, and after 3, 5 and 9 LDs. These analyses were performed in three biological replicates.

GA treatment

GAs (Sigma) was stored in ethanol 100% with final concentration of 1 mM. Two solutions were then prepared: (1) GA1 10 μM, Tween 0.1%; and (2) pure ethanol 1%, Tween 0.1%. GA treatment was carried out by brushing leaves, apices or seedlings of 10 individual plants with solution 1, while solution 2 was applied to the mock plants.

Flowering time determination

Flowering time was determined by counting the number of cauline and rosette leaves of at least 10 individual plants. Data are reported as mean leaf number ± s.d. and were measured from homozygous lines. Four independent transformants were used for each overexpressor plant.

Plasmid construction, plant transformation and transformant selection

The full-length GA2ox7 and gai cDNAs were amplified by PCR and used to generate an entry clone via BP reaction (Invitrogen, http://www invitrogen.com). The entry clones were used to generate an expression clone via the LR reaction. The plasmids were then introduced into Agrobacterium strain GV3101 (pMP90RK) and transformed into WT Columbia by floral dip.

Determination of chlorophyll concentration

Chlorophyll concentration was estimated by using SPAD-502 leaf chlorophyll meter (Markwell et al., 1995).

In situ hybridization and GUS staining

In situ hybridization was performed according to a method already described (Torti et al., 2012) to measure SOCI (Searle et al., 2006), SPL3 and SPL9 (Wang et al., 2009; Wu et al., 2009) and SPL5 (Cardon et al., 1999) expression. Primers to generate GA2ox7, SPL4 probe are in supplementary material Table S1. GUS staining was performed as previously described (Blazquez et al., 1997).

RNA extraction and quantitative real-time PCR

Total RNA was isolated from plant tissues by using RNAeasy extraction kit (Qiagen). Transcript levels were quantified by quantitative PCR (Roche) and PEX4 (At5G25760) was used as a control. Reactions were performed using the primers described in supplementary material Table S2. Total RNA, including small RNAs, was extracted by using miRNAeasy Mini Kit (Qiagen). After DNase treatment (Ambion), the mature form of miRNA156 was then amplified as previously described (Yang et al., 2009) (P. Huijser, unpublished). All quantitative real-time PCRs were performed with at least three independent RNA samples.

RESULTS

Misexpression of GA2ox7 in different tissues causes GA deficiency phenotypes

Overexpression of GA2ox7 mRNA from the CaMV 35S promoter reduces levels of bioactive GAs (Schomburg et al., 2003). To test the effect of reducing GA levels in specific tissues, GA2ox7 cDNA was fused to promoters with specific expression patterns that have been used previously to misexpress regulatory proteins (An et al., 2000). Here, we assessed the effect on flowering of overexpressing GA2ox and thereby depleting GA in specific tissues and demonstrate spatially distinct functions in the promotion of flowering under LDs.
2004; Ranjan et al., 2011). The KNAT1 promoter, which is active in the shoot apical meristem, and the SUC2 promoter, which is specific to the companion cells of the phloem, were used. The CaMV 35S promoter acted as a control to overexpress GA2ox7 in most tissues. The three gene fusions were introduced into wild-type Columbia plants, and independent transformants were selected.

Four independent transformants expressing GA2ox7 transcript at differing levels were identified for each construct. The abundance of GA2ox7 mRNA was measured by qRT-PCR in seedlings of 35S:GA2ox7 (Fig. 1A), in leaves of SUC2:GA2ox7 (Fig. 1B) and in apices of KNAT1:GA2ox7 (Fig. 1C), and was present in each transformant at much higher levels than in wild type. To determine
the spatial expression pattern of GA2ox7 in transformants carrying each transgene, in situ hybridization was performed (Fig. 1D). In wild-type plants, no signal was detected, consistent with the very low level of expression of GA2ox7 mRNA detected by qRT-PCR (Fig. 1A-C). 35S:GA2ox7 plants showed abundant GA2ox7 mRNA in most tissues, including leaves, vasculature and shoot apical meristem (SAM). By contrast, in SUC2:GA2ox7, GA2ox7 mRNA was detected only in the vasculature, whereas, in KNAT1:GA2ox7, it was found only in the shoot meristem (Fig. 1D). Thus, the heterologous promoters CaMV 35S, KNAT1 and SUC2 misexpress GA2ox7 mRNA in the expected patterns.

The transgenic lines were analyzed for height, internode length, leaf radius and chlorophyll content, phenotypes that are strongly impaired in GA-deficient plants (Rieu et al., 2008b). Young transgenic seedlings were darker green and smaller than wild-type plants (Fig. 1E). Misexpression of GA2ox7 from all three heterologous promoters greatly reduced plant height, as measured by the length of the main shoot before senescence (Fig. 1F,G) or the length of the internode between the last rosette and first cauline leaf (Fig. 1H). KNAT1:GA2ox7 had the strongest effect on plant height, demonstrating that depleting GA from the SAM impairs stem elongation.

The leaf radius of each of the transgenic plants was significantly shorter than that of wild-type (Table 1; supplementary material Fig. S1A). The leaves of the transgenic lines also appeared darker green (supplementary material Fig. S1A), and therefore their chlorophyll levels were measured (Table 1). In the leaves of 35S:GA2ox7 and SUC2:GA2ox7, these were ~50% higher than wild type, whereas no significant differences were observed in the KNAT1:GA2ox7 lines. Thus, GA is required to promote leaf growth in the vasculature and at the SAM, but in the regulation of chlorophyll levels an effect was detected only in the leaf vasculature.

KNAT1:GA2ox7 acts at the SAM to deplete GA, so the reduction of leaf size observed in these plants was unexpected. To test whether low level expression of KNAT1:GA2ox7 in leaves could contribute to this phenotype, GA2ox7 mRNA level was measured directly by qRT-PCR. However, GA2ox7 transcript levels were not significantly different in leaves of KNAT1:GA2ox7 plants compared with wild type (supplementary material Fig. S1B). In addition, GA2ox1 transcript levels were also measured in these samples to assess whether GA levels were likely to be changed in the leaves of KNAT1:GA2ox7 plants. This gene is under GA-negative feedback regulation and its mRNA level is therefore increased in tissues in which GA content is reduced (Phillips et al., 1995; Xu et al., 1995). In 35S:GA2ox7 plants, GA2ox1 mRNA levels were more abundant compared with wild type, indicating that as expected these plants contained lower GA (supplementary material Fig. S1C). By contrast, in leaves of KNAT1:GA2ox7, GA2ox1 mRNA levels did not differ significantly compared with wild type (supplementary material Fig. S1D). In addition, GA20ox1 expression was tested in apices of KNAT1:GA2ox7 plants where expression of the transgene is expected to reduce GA levels. In contrast to what was observed in leaves, the level of GA20ox1 transcript was much higher in apices of KNAT1:GA2ox7 compared with wild-type plants (supplementary material Fig. S1E). The above experiment indicated that the leaf phenotypes of KNAT1:GA2ox7 plants cannot be explained by increased expression of GA2ox7 nor by reduced levels of GA in mature leaves.

Taken together, the phenotypic characterization data suggest that ectopic expression of GA2ox7 from tissue-specific promoters causes phenotypes associated with GA deficiency. To test this further, the transgenic plants were treated with exogenous GA4. The severity of the GA deficiency phenotypes of the transgenic lines was greatly reduced by the GA applications, supporting the conclusion that reduced levels of bioactive GA are the basis of the phenotypes observed (Fig. 1I).
Fig. 2. Flowering time of the transgenic lines under LDs and SDs. (A-C) Flowering time of plants overexpressing GA2ox7 in all tissues from the CaMV 35S promoter (A), in the SAM from the KNAT1 promoter (B) and in the vasculature from the SUC2 promoter (C) grown in SDs. Data are mean±s.d. of at least 10 plants. (D-F) Phenotypes of transgenic lines grown under SDs are shown below flowering time graphs. (G-I) Flowering time of 35S:GA2ox7 (G), KNAT1:GA2ox7 (H) and SUC2:GA2ox7 (I) plants under LDs. Data are mean±s.d. (J-L) Phenotypes of transgenic lines grown under LDs are shown below flowering time graphs. (M-O) GA4 (10 μM) treatment of seedlings of 35S:GA2ox7 (M), of apices of KNAT1:GA2ox7 (N) and of leaves of SUC2:GA2ox7 (O). GA treatment was performed throughout the growth of the plant twice a week. Data are mean±s.d.
The effect of exogenous GA4 treatment on the late-flowering phenotype of the transgenic plants was also tested. GA4 application accelerated flowering of the transgenic lines under LDs, and at the end of the treatment the transgenic lines flowered with a similar number of leaves to the wild-type mock-treated plants (Fig. 2M,N,O).

To test whether the delay in flowering under LDs caused by KNAT1:GA2ox7 was enhanced by SUC2:GA2ox7, the two latest flowering transgenic lines were crossed and flowering time was scored in the F1 generation (supplementary material Fig. S1G,H). The double overexpressor KNAT1:GA2ox7 SUC2:GA2ox7 flowered later than either progenitor and at a similar stage to 35S:GA2ox7. Therefore, the effect of overexpressing GA2ox7 in the leaves and meristem is additive on flowering time under LDs.

Taken together, the flowering-time experiments indicate that under LDs GA acts both in the vasculature and at the SAM to promote flowering. However, the requirement for GA at the meristem is reduced in LDs compared with SDs, whereas in the vascular tissue the effect of GA on flowering appears stronger under LDs than SDs.

**FT and TSF mRNA levels are regulated by GA in the phloem under long days**

Many of the genes comprising the photoperiodic flowering pathway are expressed in the phloem companion cells, where the SUC2 promoter is active. Therefore, whether SUC2:GA2ox7 delays flowering by reducing the transcript levels of the photoperiodic pathway genes FT, TSF, CO and GI was tested (Fig. 3A-D). Several of these genes are regulated by the circadian clock so their RNA levels were measured every 3 hours through a 24-hour cycle under LDs. In wild-type plants, FT mRNA level showed the expected diurnal pattern with a strong increase at 12 hours after dawn and a peak at 16 hours. SUC2:GA2ox7 showed a similar diurnal pattern in FT mRNA, but its rise in expression was slightly delayed and its abundance was significantly reduced between 12 and 16 hours after dawn. The SUC2:GA2ox7 transformants with the highest GA2ox7 transcript levels (Fig. 1B) showed the strongest reduction in FT (supplementary material Fig. S1I). A similar but less pronounced effect was observed for the mRNA of the FT paralogue TSF (Fig. 3B). By contrast, the mRNAs of CO and GI,

![Image](https://example.com/image.png)
which act earlier in the photoperiodic pathway than \( FT \) and \( TSF \), were not significantly reduced in \( SUC2:GA2ox7 \) compared with wild type (Fig. 3C,D).

Several repressors of \( FT \) transcription have been described, including \( SVP \) (Lee et al., 2007; Li et al., 2008), \( FLC \) (Searle et al., 2006), \( TEM1 \) and \( TEM2 \) (Castillejo and Pelaz, 2008). Increased expression of the mRNAs of these repressors in \( SUC2:GA2ox7 \) plants could explain the reduced level of \( FT \) and \( TSF \) transcripts, and therefore these mRNAs were quantified in the transgenic plants (Fig. 3E-H). No significant difference between \( SUC2:GA2ox7 \) and Col wild type was observed for \( SVP \), \( TEM1 \) and \( TEM2 \) transcript levels, indicating that increased levels of these mRNAs cannot explain the reduced expression of \( FT \) and \( TSF \). \( FLC \) mRNA levels were slightly increased at the beginning of the light period in the \( SUC2:GA2ox7 \) plants, suggesting that the increase in abundance of this mRNA may be the cause of the reduced levels of \( FT \) and \( TSF \) mRNAs (Fig. 3F). To test this further, \( flc \) mutant and wild-type plants were treated with paclobutrazol (PAC), an inhibitor of GA biosynthesis, and \( FT \) transcript levels were quantified. Interestingly, \( FT \) transcript was reduced to similar levels in wild-type and \( flc \) PAC-treated plants (supplementary material Fig. S1J). This result supports the idea that lowering GA content reduces \( FT \) expression and suggests that the effect of GA levels in regulating \( FT \) is likely to be independent of \( FLC \).

Finally, \( GNC \) and \( GNL \) were recently described to act as repressors of flowering downstream of GA (Richter et al., 2010). \( GNL \) mRNA levels did not differ in \( SUC2:GA2ox7 \) compared with Col (Fig. 3I), showing the same diurnal pattern of abundance in both genotypes. \( GNC \) transcript levels slightly increased 18 hours after dawn in \( SUC2:GA2ox7 \) plants compared with Col (Fig. 3J), but this difference is probably not sufficient to explain the reduced levels of \( FT \) transcript, which are observed earlier in the diurnal cycle (12 hours after dawn) (Fig. 3A).

An 8.1 kb fragment has previously been described to contain the \( FT \) promoter and recreates the spatial pattern of expression of \( FT \) (Adrian et al., 2010; Takada and Goto, 2003). The \( SUC2:GA2ox7 \) transgenic line and Col were crossed to an 8.1 kb \( FT \) transgenic plant and GUS expression was analyzed in the F1 plants (Fig. 3K). As expected, 8.1 kb \( FT \) transgenic seedlings showed GUS signal in the vasculature of the cotyledons and leaves. By contrast, in 8.1 kb \( FT \) transgenic seedlings, which were similarly stained, no GUS signal was detected. Thus, in wild-type plants GA acts to increase \( FT \) mRNA through the defined 8.1 kb \( FT \) promoter.

**Ectopic expression of \( FT \) suppresses the late flowering caused by \( SUC2:GA2ox7 \)**

To assess whether the reduced level of \( FT \) and \( TSF \) mRNA was the cause of delayed flowering of \( SUC2:GA2ox7 \) plants, a transgene expressing \( FT \) from a heterologous promoter was introduced into \( SUC2:GA2ox7 \) plants. Ectopic expression of \( FT \) can overcome the effect of loss-of-function of \( FT \) and \( TSF \) (Jang et al., 2009; Michaels et al., 2005; Yamaguchi et al., 2005). The \( GAS1:FT \) construct overexpresses \( FT \) mRNA only in minor veins and to a lesser extent than other phloem-specific promoters (Corbesier et al., 2007; Haritatos et al., 2000). The \( SUC2:GA2ox7 \) × \( GAS1:FT \) plants flowered much earlier than those carrying only \( SUC2:GA2ox7 \) and after producing a similar number of leaves to \( GAS1:FT \) plants (Fig. 4A,B), supporting the idea that the late flowering of \( SUC2:GA2ox7 \) is caused by reduced \( FT \) mRNA levels.

In addition, the effects of impairing GA signalling in the companion cells on \( FT \) expression and flowering time were tested by expressing from the \( SUC2 \) promoter the dominant mutant form of \( GAI \) that represses GA signalling (Peng et al., 1997). \( SUC2:gai-D \) plants were late flowering and showed reduced \( FT \) mRNA levels, similar to the effects observed in the \( SUC2:GA2ox7 \) plants (supplementary material Fig. S2A,B).

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**Fig. 4.** The \( ft \) \( tsf \) double mutant shows less sensitivity to leaf applications of GA in the acceleration of flowering. (A,B) Effect of ectopic expression of \( FT \) in \( SUC2:GA2ox7 \) plants grown in LDs. Col wild-type, \( SUC2:GA2ox7 \) and \( GAS1:FT \) plants were used as controls. (C,D) Effect of \( GA4 \) on flowering time of \( ft \) \( tsf \) and Col wild-type plants under SDs. \( GA4 \) (10 \( \mu \)M) was applied to leaves twice weekly. (E) Effect of \( GA4 \) on \( FT \) expression in \( SUC2:GA2ox7 \) and Col wild-type plants in LDs. GA treatment was carried out in leaves of 10-day-old plants and tissues were collected 24 hours after. Data are mean±s.e.
The above experiments suggested that GA and GA signalling act in the vascular tissue to increase FT and TSF mRNA levels and thereby promote flowering. Therefore, whether FT and TSF are required for the leaf meristem of leaves to promote flowering was tested. Leaves of ft-10 tsf-1 double mutants and wild-type plants grown under SD were treated with GA4 (Fig. 4C,D). Wild-type plants showed significant acceleration of flowering upon GA treatment, producing 20 leaves fewer than the mock-treated plants. By contrast, GA application to leaves of ft-10 tsf-1 mutants caused flowering to occur after production of only 10 leaves fewer than the mock-treated plants. Therefore, ft-10 tsf-1 double mutants still respond to GA leaf treatments, but their response is strongly attenuated compared with wild-type plants. This result is consistent with GA leaf treatments acting partly through FT and TSF to promote flowering. In addition, leaves of SUC2:GA2ox7 and Col wild type were also treated with GA and after 24 hours the FT transcript level was quantified (Fig. 4E). Wild-type plants did not show any significant change in FT expression after GA application, which is probably due to the saturating level of GA at this stage. By contrast, SUC2:GA2ox7 showed an approximately threefold increase of FT transcript in the GA-treated compared with the mock-treated plants. Therefore, depletion of GA in the leaves of SUC2:GA2ox7 caused FT downregulation, which could be restored by applying active GA.

**Induction of SPLs but not SOC1 transcription is delayed in the meristem of KNAT1:GA2ox7 plants under LDs**

The level of FT mRNA was similar in KNAT1:GA2ox7 and Col plants under LDs (supplementary material Fig. S2C), confirming that the delay in flowering of this plant occurred by a different mechanism than for SUC2:GA2ox7 plants.

During the transition to flowering, expression of many genes is induced at the shoot apex, and this can be synchronized by transferring plants from SDs to LDs. To determine how these gene expression patterns are affected by KNAT1:GA2ox7, the transgenic plants and Col were grown for 3 weeks in SDs and then transferred to LDs. Apices were harvested for in situ hybridization before transfer and then after 3, 5 and 9 days in LDs.

In Col shoot meristems, SOCI mRNA was not detected after 3 weeks in SDs, but increased in the meristem after 3, 5 and 9 LDs (Fig. 5A). Similarly, in the KNAT1:GA2ox7 plants, SOCI mRNA was detected in the meristem after exposure to 3, 5 and 9 LDs. However, unlike Col plants, flower development was not initiated throughout this period. Consistent with this result, an increase in SOCI transcript in apices of Col and KNAT1:GA2ox7 plants was detected after transfer to LDs (Fig. 5B). Thus, the meristem of KNAT1:GA2ox7 plants responds normally to the LD signal in terms of SOCI mRNA induction, demonstrating that GA is required to promote later steps in floral induction.

The SPL genes are expressed in the shoot apical meristem downstream of SOC1 (Jung et al., 2011; Torti et al., 2012) and play important roles in the activation of floral meristem identity genes FUL and AP1 (Wang et al., 2009; Yamaguchi et al., 2009). Therefore, the expression patterns of SPL mRNAs were also studied. In Col plants transferred to LDs, the mRNAs of SPL4 and SPL5 were strongly detected in the rib meristem region after exposure to 3-5 LDs (Fig. 6B,C). Similarly, SPL9 mRNA was detected on the flanks of the meristems of Col plants exposed to 3-5 LDs (Fig. 6D). By contrast, in KNAT1:GA2ox7, expression of SPL4 and SPL9 mRNAs was strongly reduced so that their mRNAs only appeared weakly after exposure to 5 LDs. SPL5 mRNA level was even more strongly affected and was undetectable in the shoot meristem 5 LDs after transfer. SPL3 mRNA was detected throughout the meristem and in leaf primordia in Col plants and increased in abundance during LD induction (Fig. 6A). Conversely, in KNAT1:GA2ox7, SPL3 expression was strongly delayed and transcript was only weakly detectable after 5 LDs in leaf primordia.

These experiments indicate that although KNAT1:GA2ox7 does not prevent the early induction of SOC1 expression in the shoot meristem in response to LDs, it does prevent the subsequent activation of later acting genes such as SPL3, SPL4, SPL5 and SPL9.

The effect of KNAT1:GA2ox7 on SPL gene expression could be exerted at the level of FD, which binds directly to SPL3, SPL4 and SPL5 to promote their expression (Jung et al., 2011). Therefore, fd mutants were treated with active GA and the levels of SPL3 and SPL4 mRNAs were quantified in apices (supplementary material Fig. S2D,E). SPL3 and SPL4 mRNA levels increased in fd mutants treated with GA compared with the mock-treated plants, indicating that GA can activate these SPL genes independently of FD. However, the level of SPL expression is lower than in GA-treated wild-type plants, so a role for FD in this process cannot be excluded (supplementary material Fig. S2D,E).

Expression of SPL genes is negatively regulated by miR156 at the post-transcriptional level (Gandikota et al., 2007; Schwab et al., 2005). Therefore, whether downregulation of SPL genes in KNAT1:GA2ox7 was caused by increased levels of miR156 was tested in apices of wild type and KNAT1:GA2ox7 (supplementary material Fig. S2F). Apices were harvested after growing plants in LDs for 6, 9, 11, 13 and 17 days. In Col wild type, the levels of
miR156 progressively decreased along the time course, as previously described (Wang et al., 2009; Wu and Poethig, 2006), reaching the lowest level at 17 LDs (supplementary material Fig. S2F). Similarly, in \textit{KNAT1:GA2ox7}, the expression pattern of miR156 followed the same kinetics as wild type and no significant differences in abundance of miR156 were detected. By contrast, the transcript levels of \textit{SPL3} increased in apices of wild-type plants but not in \textit{KNAT1:GA2ox7} (Fig. 6E). \textit{SPL5} mRNA slightly increased along the time course in \textit{KNAT1:GA2ox7} plants but the transcript levels were significantly reduced compared with wild type (Fig. 6F).

Taken together, the in situ hybridization and the qRT-PCR data suggest that in the shoot apical meristem GA increases \textit{SPL} mRNA levels by acting after \textit{SOC1} mRNA accumulation and not by decreasing miR156 levels.

**DISCUSSION**

The plant growth regulator GA has previously been shown to promote the transition to flowering of \textit{Arabidopsis} mainly under non-inductive SDs. Here, we demonstrated that GA has defined tissue-specific roles during floral induction in response to inductive LDs.

**Effects of tissue specific expression of \textit{GA2ox7} on leaf size and height**

Gibberellins regulate many phases of development, including height, leaf size and chlorophyll content of \textit{Arabidopsis}. The strongest effect on plant height was observed in \textit{KNAT1:GA2ox7} plants, suggesting that the major impact of GA in shoot elongation occurs in the meristem. This effect might be caused by ectopic expression of \textit{GA2ox7} in cells in which it is not normally expressed or due to increased activity of \textit{GA2ox7} in cells in which it is expressed in wild-type plants. The expression patterns of the class III \textit{GA2ox}-encoding genes \textit{GA2ox7} and \textit{GA2ox8} are unknown, but expression of classes I and II \textit{GA2 oxidase} has been detected in the shoot apical meristem of \textit{Arabidopsis}, rice and maize (Bolduc and Hake, 2009; Jasinski et al., 2005; Sakamoto et al., 2001).

The severe short internode phenotype of \textit{KNAT1:GA2ox7} plants is similar to that of loss-of-function GA biosynthetic mutants, consistent with the overexpression of \textit{GA2ox7} depleting GA from the meristem. Bioactive GA is also present within the apex of flowering plants when internodes strongly extend. GA promotes cell division and expansion, suggesting that both contribute to internode elongation in the rib meristem region (Achard et al., 2009; Cowling and Harberd, 1999; Daykin et al., 1997). Although depletion of GA in the meristem showed the greatest effect on stem length and these plants were unable to appreciably extend stem internodes, a significant effect was also observed in the \textit{SUC2:GA2ox7} plants, where GA is depleted in the phloem companion cells. \textit{SUC2:GA2ox7} plants also showed a dark green phenotype associated with increased chlorophyll levels. GA regulates chlorophyll biosynthesis through the transcriptional repressors \textit{DELLA} and the downstream acting proteins GNL and GNC (Richter et al., 2010). Indeed, GA causes downregulation of \textit{GNL}...
and GNC mRNAs leading to reduced levels of protochlorophyllide oxidoreductases (PORs), thus modulating chlorophyll biosynthesis. In agreement with these findings, we showed that overexpression of GA2ox7 causing depletion of GA in the companion cells led to increased chlorophyll levels in the leaves. However, no difference in abundance of GNL and GNC transcripts could be detected in total leaf mRNA. Perhaps if GNC and GNL are expressed throughout the leaf, reduction in expression in companion cells is undetectable in total leaf RNA; alternatively, other genes might be implicated in the regulation of GA-mediated chlorophyll biosynthesis. No effect could be observed on chlorophyll content by lowering GA in the SAM, suggesting that GA levels in the meristem do not affect chlorophyll biosynthesis.

The length of the leaf radius was consistently reduced when GA was depleted in companion cells and in the SAM. This phenotype was similar to that reported for ga20ox1 ga20ox2 double mutants, which show reduced levels of GA4 and GA1 (Rieu et al., 2008a). Our data suggest that GA levels in the companion cells and shoot meristem contribute to this phenotype.

**Effect on floral transition of misexpression of GA2ox7 in phloem companion cells**

The effects of the SUC2:GA2ox7 and KNAT1:GA2ox7 fusions on flowering time were separable at the physiological and molecular levels. Expression in phloem companion cells from the SUC2 promoter caused a relatively stronger delay under LDs than SDs, although the increase in absolute number of leaves was similar under both conditions. By contrast, the KNAT1 fusion caused the strongest effect under SDs, where it prevented flowering. The delay in flowering of SUC2:GA2ox7 under LDs correlated with reduced levels of FT and TSF mRNAs, which were not observed in KNAT1:GA2ox7 plants. A similar correlation between GA levels and FT mRNA abundance was previously observed in the ga1-3 mutant exposed to long days enriched in far-red light (Hisamatsu and King, 2008). However, in those plants, GA levels are strongly reduced in all tissues, and GA depletion in other cell types might affect FT mRNA levels in the companion cells, as was observed for PHYB (Endo et al., 2005). However, our experiments, together with those of Hisamatsu and King (Hisamatsu and King, 2008) strongly suggest that GA is required in the companion cells for normal levels of FT and TSF mRNAs under LDs. We also provide genetic evidence that the reduced levels of FT and TSF mRNAs are causally related to the late flowering of the SUC2:GA2ox7 plants. Introduction of a transgene expressing FT from a heterologous phloem-specific promoter, GAS1, suppressed the late flowering of SUC2:GAox2 plants. Furthermore, GA applications to leaves increased FT and TSF mRNA levels in SUC2:GA2ox7 plants, as previously shown for ga1-3 plants (Hisamatsu and King, 2008), and restored early flowering. The full effect of GA applications to the leaves on flowering time required an increase in FT and TSF mRNA levels, supported by the observation that ft-10 tsf-1 double mutants were less sensitive to GA leaf applications, although they did still respond to the treatment. Previously, Hisamatsu and King (Hisamatsu and King, 2008) discussed an FT independent role of GA applications, and this is probably explained by a spatially separated function for GA in the shoot meristem, as mentioned in the following section. The mechanism by which GA increases FT and TSF mRNA levels is presumably via DELLA protein accumulation. Indeed, we demonstrated that expression of gai-D, a dominant mutant form of the GAI DELLA protein (Peng et al., 1997), in companion cells reduced FT and TSF mRNA levels. Therefore, when DELLA proteins accumulate in the companion cells, they likely inhibit proteins required for transcriptional activation of FT. No effect on mRNAs of previously identified regulators of FT was observed, demonstrating that depletion of GA does not affect the transcription of known repressors or activators of FT, although we cannot exclude that these proteins are regulated at the post-translational level.

**Effect on floral transition of misexpression of GA2ox7 in the shoot meristem**

The role of GA at the apex in the promotion of flowering has mainly been studied under SDs. Under these conditions, GA levels increase at the apex prior to the floral transition, and this correlates with increased expression of the floral meristem identity gene LFY (Eriksson et al., 2006). GA also promotes expression of genes that act earlier in floral induction, particularly increasing transcription of SOC1 (Achard et al., 2004; Moon et al., 2003). Applications of exogenous GA to wild-type plants caused increased abundance of SOC1 mRNA, whereas in ga1-3 and gai mutants, SOC1 mRNA level was reduced. However, all published analyses of SOC1 expression in response to GA were carried out by RT-PCR, and as SOC1 is also expressed in leaves (Michaels et al., 2005), the increase in expression detected in apical samples may not be in the shoot meristem. In addition, the effect of GA on SOC1 mRNA was mainly analyzed at single time points, making it difficult to assess its effect on the dynamics of SOC1 expression during floral induction. By performing in situ hybridization to follow SOC1 mRNA in the meristem over several days after inducing flowering by exposure to LDs, our work identifies a role for GA in the meristem after induction of SOC1.
Transfer of wild-type plants from SDs to LDs causes a rapid induction of SOCI mRNA in the meristem within 1-3 days (Borner et al., 2000; Samach et al., 2000). The SPL genes are induced slightly later, with SPL4, SPL5 and SPL9 mRNAs rising in the meristem 3-5 days after transfer (Torti et al., 2012; Wang et al., 2009). The dynamics of SOCI mRNA induction was not changed in KNAT1-GA2ox7 plants, indicating that reducing GA in the meristem does not affect SOCI induction in the meristem, in contrast to what was observed under SDs (Achard et al., 2004; Moon et al., 2003). However, expression of SPL3, SPL4, SPL5 and SPL9 all occurred markedly later, indicating that GA has a role in floral induction under LDs between activation of SOCI transcription and the activation of SPL gene expression (Fig. 7). By contrast, no effects on SPL9 mRNA or miR156 were detected by RT-PCR in 2-week-old plants treated with GA or in ga1-3 mutants, compared with wild-type (Wang et al., 2009), but this single time point would not have been sufficient to detect the effect of GA on the dynamics of SPL activation. GA-dependent activation of SPL gene expression may contribute to the induction of floral meristem identity genes by GA, because SPLs have been shown to bind directly to floral meristem identity genes such as LFY (Wang et al., 2009; Yamaguchi et al., 2009). As transcription of SPL genes is induced in the SAM both by the photoperiodic (Torti et al., 2012; Wang et al., 2009) and GA pathways, they might both activate LFY transcription via SPL proteins. However, the GA and photoperiod pathways are likely also to have additional independent branches leading to LFY activation, because they were previously shown to activate LFY transcription through independent promoter motifs (Blazquez and Weigel, 2000). The mechanism by which GA regulates SPL expression presumably involves post-translational regulation of transcription factors required to increase SPL expression. These GA regulated factors might act together with SOCI, which has recently been shown to bind directly to SPL genes. Taken together, our data provide a basis for identifying the molecular mechanisms by which under inductive photoperiods GA signalling facilitates the activation of FT transcription in leaves and transcription of the SPL genes in the meristem.

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