Spatial control of flowering by DELLA proteins in Arabidopsis thaliana

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SUMMARY
The transition from vegetative to reproductive development is a central event in the plant life cycle. To time the induction of flowering correctly, plants integrate environmental and endogenous signals such as photoperiod, temperature and hormonal status. The hormone gibberellic acid (GA) has long been known to regulate flowering. However, the spatial contribution of GA signaling in flowering time control is poorly understood. Here we have analyzed the effect of tissue-specific misexpression of wild-type and GA-insensitive (della17) DELLA proteins on the floral transition in Arabidopsis thaliana. We demonstrate that under long days, GA affects the floral transition by promoting the expression of flowering time integrator genes such as FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF) in leaves independently of CONSTANS (CO) and GIGANTEA (GI). In addition, GA signaling promotes flowering independently of photoperiod through the regulation of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes in both the leaves and at the shoot meristem. Our data suggest that GA regulates flowering by controlling the spatial expression of floral regulatory genes throughout the plant in a day-length-specific manner.

KEY WORDS: Gibberellic acid, Flowering, DELLA, Arabidopsis thaliana

INTRODUCTION
Since its discovery in the 1930s, gibberellic acid (GA) has been shown to affect such diverse biological processes as seed germination, root development, cell elongation, flower development and flowering time (Davies, 2004). However, only recently have we begun to understand the molecular mechanisms that underlie GA signaling. GA is perceived by its receptor, GID1, which undergoes conformational changes after binding to bioactive GA. These changes facilitate the interaction between GID1 and DELLA proteins, which ultimately results in their degradation (Fu et al., 2004; Griffiths et al., 2006; Willige et al., 2007; Murase et al., 2008). The DELLA proteins have been named after a conserved motif of five amino acids in their N-terminal region (Peng et al., 1997; Silverstone et al., 1998; Dill et al., 2001), which were later shown to be required for interaction with GID1 (Griffiths et al., 2006; Willige et al., 2007; Murase et al., 2008). Deletion of the DELLA motif confers dwarfism and dark green color, similar to mutants with impaired GA biosynthesis, such as ga1-3. However, in contrast to ga1-3, deletion of the DELLA domain cannot be fully rescued by exogenous GA (Koornneef and van der Veen, 1980; Koornneef et al., 1985; Peng et al., 1997).

The Arabidopsis thaliana genome contains five DELLA genes, GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF ga1-3 (RGA), RGA-LIKE1 (RGL1), RGL2 and RGL3, that exhibit partial functional redundancy (Dill and Sun, 2001; Lee et al., 2002; Bolle, 2004; Gallego-Bartolome et al., 2010). Gene expression analysis has demonstrated that hundreds of genes are differentially expressed in response to GA and that this response is DELLA-dependent (Ogawa et al., 2003; Willige et al., 2007). However, DELLA proteins exert their function mainly by regulating transcription factor activity through protein-protein interactions (Daviere et al., 2008; de Lucas et al., 2008; Feng et al., 2008).

The role of GA in regulating flowering was first studied by the application of GA to plants (Lang, 1957; Langridge, 1957). Only later, after the isolation of GA biosynthesis and signaling mutants, such as ga1-3, could the GA-mediated control of flowering be investigated in detail (Koornneef and van der Veen, 1980; Sun et al., 1992; Wilson et al., 1992). ga1-3 mutants completely failed to flower when grown under short-day (SD) conditions, whereas flowering was only moderately delayed under long-day (LD) conditions (Wilson et al., 1992), suggesting that GA was not required to induce flowering under inductive photoperiod. However, more recent analyses strongly indicate that GA contributes to the regulation of flowering time in A. thaliana in response to LD conditions after all (Griffiths et al., 2006; Willige et al., 2007; Hisamatsu and King, 2008; Osnato et al., 2012; Porri et al., 2012).

The role of FLOWERING LOCUS T (FT) in mediating flowering in response to inductive photoperiod has well been documented. It is now widely accepted that the FT protein acts as a florigen and conveys the information to induce flowering from the leaves to the shoot meristem (Wigge et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007; Liu et al., 2012). At the shoot meristem, FT interacts with 14-3-3 proteins and the bZIP transcription factor FD to form a heterotrimeric complex that is thought to bind to the regulatory regions of target genes to trigger the transition to the reproductive phase (Abe et al., 2005; Wigge et al., 2005; Taoka et al., 2011).

Besides FT, the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors have been shown to regulate flowering (Cardon et al., 1997; Wang et al., 2009; Yamaguchi et al., 2009). The A. thaliana genome contains 17 SPL-like genes, 11 of which are targets of microRNA156 (miR156) (Rhoades et al., 2002; Guo et al., 2008). The levels of mature...
miR156 decrease as a plant ages. As a consequence, SPL transcripts become more abundant, which ultimately induces flowering (Wang et al., 2009). The regulation of flowering by SPLs is in part due to the induction of miR172 (Wu et al., 2009). miR172 targets mRNAs of APETALA2-like (AP2-like) genes, which regulate flowering by directly binding to and repressing genes such as FT and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) (Aukerman and Sakai, 2003; Schmid et al., 2003; Chen, 2004; Schwab et al., 2005; Mathieu et al., 2009; Yant et al., 2010).

In contrast to this detailed picture of the regulation of flowering by photoperiod and age, little is known about how the floral transition is regulated by GA. To address this question we carried out a comprehensive analysis of the regulation of flowering by DELLA proteins under both SD and LD conditions. Our results indicate that under LD conditions the DELLA proteins regulate the expression of flowering time genes in leaves and at the shoot meristem. By contrast, the effects of DELLA proteins on flowering under SD conditions seem to be limited to the shoot meristem.

**MATERIALS AND METHODS**

**Plant material**

Wild-type plants used in this work are of the Columbia (Col-0) and Landsberg erecta (Ler) accesses. The mutants gai-1, rga-24, gai-t6, rga-t2, rgl1-1, rgl2-1, gai-1 and syl1-10 are in Ler background and have been described (Koornneef et al., 1985; Sun et al., 1992; Peng and Harberd, 1993; Peng et al., 1997; Silverstone et al., 1998; Lee et al., 2002; McGinnis et al., 2003; Achar and Aukerman, 2007). The triple gid1a-c mutant, fl-10, tsf-1, pFT:GUS and p35S:MM172 are in Col-0 background (Takada and Goto, 2003; Michael et al., 2005; Yoo et al., 2005; Willige et al., 2007; Todesco et al., 2010). Genotypes were confirmed by PCR using published oligonucleotides (supplementary material Table S1).

**Growth conditions and plant transformation**

All plants were grown in chambers in controlled photoperiod at 16°C or 23°C, 65% humidity and a mixture of Cool White and Gro-Lux Wide Spectrum fluorescent lights, with a fluence rate of 125 to 175 μmol m⁻² s⁻¹. LD conditions are defined as 16 hours light/8 hours dark and SD conditions as 8 hours light/16 hours dark.

Plant transformation was carried out as previously described (Clough and Bent, 1998). Transgenic T1 plants were raised on soil or MS medium supplemented with 0.1% glucose (BASTA) or 50 μg/ml kanamycin, respectively, after stratification for 4 days at 4°C in darkness. For germination of gid1a-1 gid1b-1 gid1c-2 triple mutant, the seed coat was manually removed. gai-3 plants were germinated by treatment with 50 μM GA3 in 0.1% agarose. GA3 stock solutions were prepared in pure ethanol and working solutions containing 0.01% (v/v) Tween-20 (Sigma-Aldrich) were prepared in distilled water. After 3 days of incubation in darkness at 4°C, the seeds were washed at least ten times with distilled water to remove excess GA3. Treatment of plants was performed by spraying with 50 μM GA3.

**Molecular cloning**

All nucleotides and constructs used in this work are listed in supplementary material Tables S1 and S2. All constructs were confirmed by Sanger sequencing. For misexpression of GA2ox8, the open reading frame (ORF) was amplified from cDNA using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and oligonucleotides G-31688 and G-31689. The fragment was purified and ligated into the Gateway-compatible vector pJLSmart to create pVG-412, and subsequently used for recombination into pGREEN-IIS destination vector (Mathieu et al., 2007) containing the pJLSmart to create pVG-417.

The complete ORFs of the five DELLA genes (RGA, GAI, RGL1, RGL2, RGL3) were amplified directly from A. thaliana genomic DNA with specific oligonucleotides. The amplified PCR products were cloned into Gateway-compatible vector pJLSmart using T4 DNA ligase (Fermentas) to create the entry vectors pVG-104, pVG-105, pVG-118, pVG-119 and pVG-120. Expression vectors suitable for plant transformation were created by recombination into pGREEN-IIS plant binary destination vectors (Mathieu et al., 2007) containing the SUC2, FD and CLV3 promoters, respectively (supplementary material Table S2).

**Expression analysis**

Total RNA was extracted using either the RNeasy Kit (Qiagen) or TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. At least 600 ng total RNA was treated with DNase I and used for cDNA synthesis using oligo (dT) and the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCR (qPCR) was performed using the Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) and specific oligonucleotides (supplementary material Table S1) on an MJR Option Continuous Fluorescence Detection System. Expression was normalized against A. thaliana ß-TUBULIN or ACTIN 2, and expression differences were calculated using the ΔΔCT method. For each sample, material from a minimum of 15 seedlings was pooled per replicate and at least two biological and two technical replicates were used for the analysis. A minimum of 40 apical meristems was dissected for each biological replicate for RNA extraction.

Small RNA northern blots were performed using 2 μg total RNA resolved on a 17% polyacrylamide gel in denaturing conditions (7 M urea). The RNA was transferred to HyBond-N+ membranes and hybridized with digoxigenin-labeled oligonucleotides (supplementary material Table S1). Probe labeling was carried out using the DIG Oligonucleotide 3‘-End Labeling Kit, 2nd generation (Roche). microRNA quantitative PCR was performed as previously described (Chen et al., 2005).

GUS staining was performed as described (Blazquez et al., 1997) and pictures obtained using the Leica MZ FLIII microscope. Transcriptome analysis was performed using publicly available data downloaded from AGenExpress (Schmid et al., 2005).

**RESULTS**

DELLA proteins repress flowering under LD photoperiod

Genetic analyses have shown that DELLA genes have partially overlapping function in controlling various aspects of plant development (Dill and Sun, 2001; Lee et al., 2002; Cheng et al., 2004; de Lucas et al., 2008; Feng et al., 2008); however, their relative contribution to the regulation of flowering under inductive photoperiod is still unclear. To address this question we first analyzed the effect of della gain- and loss-of-function mutations on flowering time. We observed that under LD conditions, the loss-of-function mutants gai-1 and rga-24 flowered early with 9.9±0.8 and 9.9±0.5 leaves, respectively, compared with wild type, which produced 11.3±0.6 leaves (P<0.00001; Table 1).

However, these single mutants still flowered later than wild-type plants treated with 50 μM GA3, which produced 7.8±0.9 leaves. In agreement with the notion of functional redundancy among the DELLA genes, early flowering was enhanced in a gai-1 rga-24 double mutant and a gai-3 gai-16 rga-24 rga-24 rgl1-1 rgl2-1 rgl3-1 pentuple mutant, which produced 8.6±0.7 and 7.6±0.9 leaves, respectively (P<0.00001; Table 1 and supplementary material Fig. S1.A-B). By contrast, the semi-dominant GA-insensitive gai-1 allele flowered considerably late with about 16.8±1.0 leaves (P<0.00001; Table 1).
and supplementary material Fig. S1A,B). Similarly, and in agreement with a previous report (Willige et al., 2007), the gid1a-c triple mutant did not flower at all under our LD conditions. Presumably due to high functional redundancy among the GID1 receptors, flowering time was almost, but not completely, recovered (P<0.00001) in the gid1b-1 gid1c-2 double mutant, which flowered with 16.3±1.1 leaves compared with 14.1±1.0 in wild-type plants (Table 1 and supplementary material Fig. S1A,C). Together, our results confirm that DELLA proteins act as repressors of flowering and that their GID1-mediated, GA-dependent degradation contributes to induction of flowering under LD conditions.

Table 1. Flowering time of plants used in this study

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RL</th>
<th>CL</th>
<th>Total</th>
<th>Deviation</th>
<th>Range</th>
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<td>16.3</td>
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<td>11.3</td>
<td>0.9</td>
<td>9-13</td>
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<td>p35S:MIM172 (mock; 23°C)</td>
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<td>4.0</td>
<td>27.7</td>
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<td>23-31</td>
<td>32</td>
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<td>p35S:empty (mock; 23°C)</td>
<td>10.8</td>
<td>2.8</td>
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<td>1.4</td>
<td>10-16</td>
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<td>p35S:MIM172 (mock; 16°C)</td>
<td>45.1</td>
<td>9.4</td>
<td>54.6</td>
<td>3.2</td>
<td>50-60</td>
<td>9</td>
</tr>
<tr>
<td>p35S:empty (mock; 16°C)</td>
<td>20.4</td>
<td>4.8</td>
<td>25.2</td>
<td>1.9</td>
<td>22-29</td>
<td>24</td>
</tr>
</tbody>
</table>

RL, rosette leaves; CL, cauline leaves; n.a., plants did not flower in the course of the experiment.
DELLA proteins regulate flowering under LD conditions in the leaf vasculature

The control of flowering can be spatially divided into processes that occur in leaves, such as perception of photoperiod, and those that occur at the shoot meristem (Kobayashi and Weigel, 2007). The analysis of publicly available microarrays (Schmid et al., 2005) revealed a dynamic regulation of the five DELLA genes in different plant tissues, including the leaves and the shoot meristem (Fig. 1A), indicating that the DELLA proteins could affect flowering in either of those two tissues. To investigate their spatial contribution to the regulation of flowering we employed tissue-specific expression of wild-type (GAI, RGA, RGL1, RGL2 and RGL3) and GA-insensitive versions (gaiΔ17, rgaΔ17, rgl1Δ17, rgl2Δ17, rgl3Δ17) of the DELLA cDNAs. The latter were created by introducing a 17-amino-acid deletion into the DELLA cDNAs, analogous to the one originally identified in the gai-1 mutant (Fig. 1B) (Peng et al., 1997).

Transgenic T1 plants expressing del117 from the phloem companion cell (PCC)-specific SUC2 promoter (Stadler and Sauer, 1996) exhibited the dark green color typically observed in GA-deficient mutants. We found that pSUC2:rgaΔ17, pSUC2:rgl1Δ17, pSUC2:rgl2Δ17 and pSUC2:rgl3Δ17 delayed flowering more strongly than pSUC2:GAIΔ17, although late-flowering individuals were occasionally observed among the latter (P<0.00001; Fig. 1C,D; supplementary material Figs S2, S3). Furthermore, transgenic plants expressing full-length DELLA ORFs also displayed an intermediate dark green color and late-flowering phenotype (P<0.00001; Fig. 1C,D). In particular, pSUC2:RGA and pSUC2:RGL1 flowered almost at the same time as pSUC2:rgaΔ17 and pSUC2:rgl1Δ17 (Fig. 1C,D; supplementary material Figs S2, S3).

To ensure that also the endogenous DELLA proteins regulate flowering in the leaf PCCs, we expressed the GA catabolic enzyme GA2ox8 under control of the SUC2 promoter (Stadler and Sauer, 1996; Olszewski et al., 2002; Rieu et al., 2008). The reasoning for this is that it would reduce the pool of bioactive GA, resulting in higher DELLA protein levels specifically in the PCCs. Indeed, transgenic T1 plants expressing pSUC2:GA2ox8 displayed a dark green color and flowered later (14.1±1.5 rosette leaves) than control plants (10.4±0.8; P<0.00001; supplementary material Fig. S4). Taken together, these observations suggest that the DELLA proteins regulate flowering in response to GA under LD conditions in the leaf PCCs.

CO- and GI-independent regulation of FT by DELLA proteins in the vasculature

The FT gene has been shown to be specifically expressed in leaf vasculature in response to inductive photoperiod (Kobayashi and Weigel, 2007; Turck et al., 2008). To test if the late flowering observed in the pSUC2:del117 lines (Fig. 1C,D; supplementary material Figs S2, S3) was due to a reduction in FT expression, we introduced pSUC2:rgl3Δ17 into a pFT:GUS reporter line (Takada and Goto, 2003). T2 plants derived from seven independent T1 lines that varied in their flowering time from wild-type-like to late flowering were analyzed and a clear anti-correlation between flowering time and expression of the endogenous FT gene was observed (Fig. 2A). FT expression was strongly reduced in late-flowering pSUC2:rgl3Δ17 T2 lines, whereas lines flowering at the same time as the control plants had almost wild-type-like FT expression (Fig. 2A). Similarly, the
pFT:GUS reporter showed a much decreased expression and staining in the vasculature of late-flowering plants (Fig. 2B,C).

As FT, as well as its closest paralog TWIN SISTER OF FT (TSF), are under the control of the circadian clock, we analyzed the diurnal expression of these two genes in the late-flowering pSUC2:rgl3Δ17 line. Quantitative analysis showed that both FT and TSF maintained their diurnal expression but at a reduced level (Fig. 2D,E). By contrast, expression of GIGANTEA (GI) and CONSTANS (CO), which act upstream of FT, was unchanged in pSUC2:rgl3Δ17 and in the strong gid1a-c mutant compared with wild type (Fig. 2F,G,H). Together these results suggest that the DELLA proteins participate in the regulation of FT and TSF expression in PCCs and contribute to their regulation under LD conditions independently of CO and GI.

**Regulation of FT and TSF by GA**

To confirm that FT and TSF are regulated by GA, and to ensure that the effects we had observed in the pSUC2:rgl3Δ17 line reflected normal DELLA function, we analyzed their expression in GA biosynthesis and signaling mutants. Results obtained in the strong GA biosynthesis mutant ga1-3 had suggested that GA does not substantially contribute to the regulation of flowering time under LD conditions (Wilson et al., 1992). Consistent with this, FT and TSF were expressed normally in ga1-3 under LD conditions (Fig. 3A). By contrast, expression of FT and TSF was reduced approximately twofold in the partially GA-insensitive sly1-10 mutant, which accumulates higher levels of DELLA proteins (McGinnis et al., 2003) compared with wild type (Fig. 3A). Similarly, FT and TSF expression was reduced to ~30% in the non-flowering gid1a-c triple mutant compared with control plants (Fig. 3B).

In agreement with GA regulating FT independently of the photoperiod pathway, we also observed increased levels of FT in a diurnal timecourse in the early-flowering gal1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 mutant compared with wild-type plants (Fig. 3C). Furthermore, FT was precociously expressed in leaves of the gal1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 mutant compared with Ler-1. Expression of FT was comparable between the two genotypes 3

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**Fig. 2. DELLA proteins regulate FT and TSF expression under LD conditions.** (A-C) Repression of FT by RGL3 was confirmed in pSUC2:rgl3Δ17 pFT:GUS (T2) plants by (A, bottom) quantitative RT-PCR of FT, (B) GUS staining, and (C) GUS quantitative RT-PCR. GUS staining represents the third leaf of 10-day-old transgenic plants at zeitgeber (ZT) 16 grown under LD conditions at 23°C. (D-G) Diurnal expression profile of FT, TSF, CO and GI in pSUC2:rgl3Δ17 (T2). Plants were grown under SD conditions for 30 days and shifted to LD conditions for 5 days to induce flowering. Transgenic plants (A-G) are in Col-0 background. The aerial part of the plants was collected every 4 hours for 24 hours. Bars on the top indicate day (white) and night (black) phases. (H) Expression of CO and GI in 3-week-old triple gid1a-c mutant plants growing at 23°C under LD conditions. The error bars indicate the s.d. of rosette leaf number (A, top) and quantitative expression of at least two biological and two technical replicates each (A, bottom; C-H); n indicates the number of plants analyzed. Significance was calculated using the unpaired Student’s t-test: *P<0.05, **P<0.01, ***P<0.001.
days after germination but gradually increased in the _ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1_ mutant (Fig. 3D). To confirm that GA can promote _FT_ expression even under LD conditions, plants containing the _pFT:GUS_ reporter were treated with GA3 or mock-treated every other day for 12 days. In contrast to mock-treated plants, in which the GUS staining was mostly restricted to the peripheral veins, GA3-treated plants displayed a stronger and more dispersed GUS signal (Fig. 3E). This finding was corroborated by quantitative RT-PCR, which revealed a 2.5-fold increase in GUS expression in the _GA_3-treated samples (supplementary material Fig. S5). Taken together, these results suggest that GA substantially promotes the expression of _FT_ and _TSF_ in PCCs and thus the induction of flowering even under LD conditions.

**DELLA proteins repress flowering under LD conditions at the shoot meristem**

Even though plants expressing _dellaA17_ and _DELLA_ cDNAs in the PCCs were clearly late flowering, these plants nevertheless flowered earlier than the triple _gld1a-c_ mutant, suggesting that GA signaling in tissues other than the leaf vasculature contributes to the regulation of flowering. To investigate the contribution of _DELLA_ proteins to flowering-time regulation at the shoot apex, we expressed the _dellaA17_ and _DELLA_ cDNAs under control of the meristem-specific _FD_ (pFD) and the shoot stem cell niche-specific _CLAVATA3_ (pCLV3) promoters (Fig. 4). Expression of _rgaA17, gaiA17, rgl1A17_ and _rgl2A17_ (P<0.00001), but not _rgl3A17_ (P>0.05), at the shoot apex from either pFD or pCLV3 delayed flowering even more strongly than observed in the _pSUC2_ lines (Table 1; supplementary material Figs S2, S3). In general, the delay in flowering was stronger in the _pFD:dellaA17_ lines compared with the _CLV3_ promoter lines, which is probably a consequence of the larger _FD_ expression domain. By contrast, expression of the wild-type _DELLA_ did not significantly affect flowering time (P>0.05; Table 1; Fig. 4B,C; supplementary material Figs S2, S3), suggesting that endogenous GA levels at the meristem are sufficiently high to target misexpressed _DELLA_ proteins for degradation. Taken together, these results highlight the importance of _DELLA_ degradation in promoting flowering at the shoot meristem downstream of the photoperiodic signal produced in leaves.

**dellaA17 delay flowering at the shoot meristem under SD conditions**

To better understand the contribution of _DELLA_ proteins in controlling the transition to flowering under non-inductive photoperiod, we scored flowering time in transgenic plants expressing _dellaA17_ and wild-type _DELLA_ in the PCCs (_pSUC2_) and at the shoot meristem (_pFD; pCLV3_) in SD conditions. We observed that expression of _rgaA17, gaiA17, rgl1A17_ and _rgl2A17_ at the shoot meristem caused plants to flower extremely late or not to flower at all even after 6 months of vegetative growth (supplementary material Figs S6, S7). As observed in LD conditions, expression of _rgl3A17_ at the shoot meristem did not affect flowering. However, in contrast to what we had observed in LD conditions, misexpression of _dellaA17_ and _DELLA_ in the phloem companion cells just had a minor effect on flowering time under SD conditions (supplementary material Fig. S6).

**DELLA proteins regulate SPL expression at the shoot meristem**

_SPL_ genes constitute a class of transcription factors that regulates diverse aspects of plant development at the shoot meristem, including the transition to flowering (Cardon et al., 1997; Wang et al., 2009; Jung et al., 2011; Kim et al., 2012). Interestingly, we observed a significant reduction of _SPL3, SPL4_ and _SPL5_ mRNA levels in dissected apices of LD-grown late-flowering _pFD:rgl2A17_ plants compared with Col-0 (Fig. 5A). By contrast, _SPL9_ and _SPL15_ transcripts were downregulated only twofold, and expression of _SPL10_ and _SPL11_ remained nearly unchanged. Supporting the idea that _SPL3, SPL4, SPL5_ and _SPL9_, but not _SPL11_, are targets of GA signaling, we observed reduced expression of these genes in the
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**DEVELOPMENT 139 (21)**

**Fig. 4. Expression of del**

**p35S:MIM172 partially suppress**

The partial suppression of the GA-mediated induction of flowering observed in p35S:MIM172 line suggested that MIR172 itself could be regulated by GA.

**Expression of del**

Expression of del(17) represses miR172. The text discusses the partial suppression of the GA-mediated induction of flowering observed in the p35S:MIM172 line, suggesting that MIR172 itself could be regulated by GA. To test this possibility, the authors analyzed miR172 levels by small RNA northern blot. Under SD conditions, they observed an increase in mature miR172 levels in the pentuple mutant compared with L\(^+\)gai-1er-1 and gai-1 gai-t6 rga-t2 rgl1-1 rgl2-1 mutants. This result supports the hypothesis that miR172 is a direct target of SPL proteins, as previously observed in WT (Yamaguchi et al., 2009).

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**Fig. 5. Phenotypes of**

Fig. 5A shows the phenotypes of del(17) at the shoot meristem delays flowering under LD conditions. In LD-grown del(17) plants, GA also controls flowering in addition to the shoot meristem. GA transcriptionally regulates these three important SPL genes at the shoot meristem. A gene that has been shown to respond strongly to GA under SD conditions is the MADS-domain transcription factor SOC1 (Bonhomme et al., 2000; Moon et al., 2003; Jung et al., 2011). By contrast, expression of these genes remained at low levels in apices of the apical region of early-flowering gid1a-c triple mutant grown under LD conditions (Fig. 5B). In addition, SPL3, SPL4 and SPL5 were precociously expressed in dissected apices of the early-flowering ga1-3 gai-1 rg1-1 rgl1-1 rgl2-1 pentuple mutant compared with wild type (Fig. 5C,D,E). These findings indicate that GA transcriptionally regulates these three important SPL genes at the shoot meristem.

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The text concludes by discussing the results of their experiments, which involved analyzing the expression of miR172 and its targets, particularly in the context of GA signaling and flowering time. The results support the hypothesis that miR172 is a direct target of SPL proteins and that GA transcriptionally regulates these genes. The text also highlights the role of DELLA proteins in GA signaling and flowering, and the importance of understanding these regulatory mechanisms for elucidating the molecular basis of flowering time in Arabidopsis.
Similar results were obtained in the late-flowering pSUC2:rgl3Δ17 and pFD:rgl2Δ17 lines. Quantitative analysis showed that the mature miR172 was moderately more abundant throughout the day in Col-0 plants grown under SD conditions for 30 days and shifted to LD conditions for 5 days to induce flowering when compared with pSUC2:rgl3Δ17 plants (Fig. 6E). By contrast, the levels of miR156 were comparable between the two genotypes (Fig. 6F). Similarly, the level of miR172 was reduced in apices of these results indicate that DELLA proteins regulate the PCCs in the leaves, suggesting that at least part of the effect of the DELLA proteins on flowering time in LD conditions is through the regulation of flowering under inductive LD conditions. In agreement with this we observed increased expression of GA-insensitive DELLA proteins (dellaΔ17) in leaves and at the shoot apex consistently demonstrated that these proteins can act as floral repressors in different tissues. For example, we observed that RGL3 reproducibly delayed flowering only when expressed in leaves, but not at the shoot apex. This observation was not completely unexpected, as genetic and molecular analysis of DELLA mutants had previously demonstrated some functional specificity of DELLA proteins, despite their generally high functional redundancy (Dill and Sun, 2001; King et al., 2001; Lee et al., 2002; Piskurewicz et al., 2006; Willige et al., 2007), further strengthens the notion that the accumulation of DELLA proteins contributes substantially to the regulation of flowering under inductive LD conditions.

In addition, expression of GA-insensitive DELLA proteins during flowering in LD conditions but also suggest a certain degree of functional redundancy between the individual proteins. The extreme delay in flowering observed in LD-grown triple gid1a-c mutants, which is due to an increase in DELLA protein (Griffiths et al., 2006; Willige et al., 2007), further strengthens the notion that the accumulation of DELLA proteins contributes substantially to the regulation of flowering under inductive LD conditions.

Interestingly, the delay in flowering observed in pSUC2:rgl3Δ17 plants was clearly correlated with a reduction in FT expression in the PCCs in the leaves, suggesting that at least part of the effect of DELLA proteins on flowering time in LD conditions is through the regulation of FT. In agreement with this we observed increased pFT:GUS expression in response to GA3 application specifically in the leaf vasculature and not in other tissues. In addition, the reduction of FT expression most likely accounts at least in part for

**DISCUSSION**

*Arabidopsis thaliana* controls the transition to reproductive development through a complex regulatory network that integrates environmental and endogenous signals to ensure the correct timing of flowering. The hormone GA has been shown to be essential for flowering under SD photoperiod (Wilson et al., 1992). However, its role in regulating flowering under LD conditions is less well understood. Here we demonstrate that the DELLA proteins, which are key components of GA signaling, contribute substantially to the regulation of flowering under LD conditions. In agreement with previous reports (Silverstone et al., 1997; Dill and Sun, 2001; Dill et al., 2004) we found that the loss of individual DELLA genes resulted in only a minor acceleration in flowering. By contrast, flowering was induced much earlier in higher order mutants. These results not only confirm the importance of the DELLA proteins during flowering in LD conditions but also suggest a certain degree of functional redundancy between the individual proteins. The extreme delay in flowering observed in LD-grown triple gid1a-c mutants, which is due to an increase in DELLA protein (Griffiths et al., 2006; Willige et al., 2007), further strengthens the notion that the accumulation of DELLA proteins contributes substantially to the regulation of flowering under inductive LD conditions.
the late flowering of the gild1a-c mutant, which displays elevated levels of the DELLA proteins. Further evidence that the DELLA proteins repress FT comes from the observation that the early flowering ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 mutant exhibits increased FT expression. By contrast, the targeted reduction of bioactive GAs in the PCCs by the misexpression of the catabolic enzyme GA2ox8 significantly delayed flowering. Taken together, our data strongly indicate that DELLA protein accumulation contributes to the regulation of FT in the PCCs under LD conditions. However, DELLA-mediated GA signaling is only one of several inputs that converge on FT, which probably explains why mutations in the DELLA genes result in only a minor delay in flowering under LD conditions.

Although the delay in flowering we observed in response to misexpression of GA-insensitive DELLA proteins in the PCCs was to be expected based on the phenotypes of dominant DELLA mutants such as gai-1, it was surprising to see that transgenic plants expressing full-length DELLA proteins were also late-flowering. One possible explanation for this finding is that in the misexpression lines, DELLA proteins accumulate to such high levels that they can no longer be efficiently degraded even in the presence of GA, as has been previously demonstrated for GAI (Fleck and Harberd, 2002).

By contrast, when expressed at the shoot meristem only the GA-insensitive del1a17, and not the full-length DELLA proteins, delayed flowering efficiently. It has been previously shown that bioactive GA accumulates at the shoot meristem before the transition to flowering (Eriksson et al., 2006). Assuming that other factors, such as the GID1 receptors or downstream components, are not limiting at the shoot meristem, this would result in a locally increased capability to degrade DELLA proteins, which might explain why meristem-specific expression of DELLA proteins at the meristem has little effect on flowering. Alternatively, the promotors used in this study (pFD, pCLV3) might be too weak to drive the expression of DELLA proteins beyond the capacity of the endogenous GA-signaling machinery to degrade (Lee et al., 2002).

It has previously been shown that GA signaling controls flowering at the shoot meristem specifically under SD conditions (Blazquez et al., 1998; Blazquez and Weigel, 2000; Moon et al., 2003). By contrast, the finding that pFD:del1a17 and pCLV3:del1a17 lines displayed pronounced late flowering, as well as a recent report describing the effects of GA2ox7 misexpression on flowering (Porri et al., 2012), indicate that the accumulation of DELLA proteins at the shoot meristem contributes to the induction of flowering under LD conditions after all. GA positively regulates SOC1 expression through DELLA proteins under non-inductive SD conditions (Moon et al., 2003). However, we and others (Porri et al., 2012) have observed only a mild effect of GA on SOC1 expression under LD conditions. This is in stark contrast to the strong effect of GA under SD conditions and suggests that under LD conditions GA signaling controls flowering at the shoot meristem predominantly downstream of the photoperiodic pathway and SOC1.

Recently, Wang and colleagues proposed the existence of an endogenous microRNA-regulated pathway that ensures that plants eventually make the transition to flowering even under a non-inductive photoperiod (Wang et al., 2009). This pathway relies on the gradual increase of SPL transcripts in response to the decrease of miR156 level during A. thaliana development. The increase in SPL protein level would ultimately lead to the activation of floral
DELLA-mediated control of flowering

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regulators and transition to flowering (Wang et al., 2009; Yamaguchi et al., 2009). The observation that SPL9 and miR156 level remains unchanged in the ga1-3 mutant when treated with exogenous GA leads to the conclusion that the SPL/miR156 module constitutes a pathway that regulates flowering under SD conditions independently of GA (Wang et al., 2009). Indeed, in our experiments and in agreement with previous work (Jung et al., 2011) miR156 levels remained unchanged in response to GA. However, the expression of the miR156-targets SPL3, SPL4 and SPL5 is significantly altered at the shoot meristem in response to GA, indicating that GA contributes to the regulation of the floral transition by modulating SPL gene expression independently of miR156 under both SD and LD conditions.

In contrast to miR156, there is at least circumstantial evidence for a role of yet another microRNA, miR172, in GA-mediated control of flowering. Plants with artificially reduced miR172 levels were still responsive to treatment with exogenous GA but did not completely recover the early flowering phenotype observed in control plants. One explanation for this behavior could be that the miR172 targets, a clade of AP2-like transcription factors that function as floral repressors (Aukerman and Sakai, 2003; Mathieu et al., 2009; Yant et al., 2010), were expressed too highly in the MIM172 lines for exogenous GA to compensate. In this scenario GA and miR172 would act in parallel signaling pathways that converge on the same targets. However, the observation that miR172 levels were elevated in ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 and reduced in pSUC2:rgl3A17 suggests that DELLA proteins act at least partially through the miR172/AP2-like module.

In contrast to the results observed in LD conditions, regulation of flowering under SD photoperiod seems to be mostly restricted to the shoot meristem. Plants expressing del1a177 proteins from the FD or CLV3 promoters under SD conditions in many cases completely failed to flower, whereas the expression of these proteins in leaves of SD-grown plants seems to have little or no effect. Interestingly, although GAI, RGA, RGL1 and RGL2 seem to be able to repress flowering in SD conditions when ectopically expressed at the shoot meristem, the gai-t6 rga-24 double mutant has been reported to rescue the non-flowering phenotype of ga1-3 in SD (Dill and Sun, 2001), suggesting that these two DELLA proteins are crucial for repressing flowering at the shoot meristem under a non-inductive photoperiod. Taken together, our results demonstrate that under LD conditions GA promotes flowering through the degradation of DELLA proteins in different parts of the plant, whereas its effect under a non-inductive photoperiod seems to be mostly restricted to the shoot meristem.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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