GA₄ Is the Active Gibberellin in the Regulation of LEAFY Transcription and Arabidopsis Floral Initiation

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Flower initiation in Arabidopsis thaliana under noninductive short-day conditions is dependent on the biosynthesis of the plant hormone gibberellin (GA). This dependency can be explained, at least partly, by GA regulation of the flower meristem identity gene LEAFY (LFY) and the flowering time gene SUPPRESSOR OF CONSTANS1. Although it is well established that GA₄ is the active GA in the regulation of Arabidopsis shoot elongation, the identity of the GA responsible for the regulation of Arabidopsis flowering has not been established. Through a combination of GA quantifications and sensitivity assays, we show that GA₄ is the active GA in the regulation of LFY transcription and Arabidopsis flowering time under short-day conditions. The levels of GA₄ and sucrose increase dramatically in the shoot apex shortly before floral initiation, and the regulation of genes involved in GA metabolism suggests that this increase is possibly due to transport of GAs and sucrose from outside sources to the shoot apex. Our results demonstrate that in the dicot Arabidopsis, in contrast with the monocot Lolium temulentum, GA₄ is the active GA in the regulation of both shoot elongation and flower initiation.

INTRODUCTION

Arabidopsis thaliana is a facultative long-day plant, meaning that it flowers more rapidly under long-day conditions than under short days. During the last decade, we have gained detailed knowledge about the molecular mechanisms whereby Arabidopsis senses long days and how this perception is translated into floral initiation (Yanovsky and Kay, 2003; Searle and Coupland, 2004). In comparison, we have much poorer knowledge about how flowering time is controlled under noninductive short-day conditions.

According to current models, Arabidopsis flowering is controlled by the interplay between three different pathways: the long-day pathway, which is responsible for the induction of flowering as a response to long days; the autonomous pathway, which controls flowering time under both long- and short-day conditions; and the gibberellin pathway, which is the most important for floral induction under short days (Mouradov et al., 2002; Boss et al., 2004; Putterill et al., 2004; Simpson, 2004). Under long days, the CONSTANS (CO) protein is stabilized by light, and this leads to induction of the floral activators FLOWERING LOCUS T (FT) and SUPPRESSOR OF CONSTANS1 (SOC1) (Putterill et al., 1995; Samach et al., 2000; Suárez-López et al., 2001; Valverde et al., 2004). By contrast, the autonomous pathway genes mediate their activity through the floral repressor FLOWERING LOCUS C, which represses the transcription of both FT and SOC1 (Michaels and Amasino, 1999, 2001; Sheldon et al., 1999; Samach et al., 2000; Searle et al., 2006).

Arabidopsis plants that are unable to synthesize the growth hormone gibberellic acid (GA), such as the mutant ga1-3, fail to flower when grown under short days (Wilson et al., 1992), suggesting that GAs play a central role in the control of flower initiation under short days, a role that is much less important under long days, in which the flowering of ga1-3 is only marginally delayed (Wilson et al., 1992; Reeves and Coupland, 2001). It has been shown that one of the reasons why ga1-3 mutants fail to flower under short days is because they cannot upregulate expression of the flower meristem identity gene LEAFY (LFY) (Blázquez et al., 1998). The GA effect on LFY is mediated through a GA-response site in the LFY promoter with similarities to a GA-myb binding site, and when this site is mutated, a minimal LFY promoter fails to be upregulated in short days but still responds to the long-day signal (Blázquez and Weigel, 2000). Furthermore, LFY expression from a constitutive promoter induces flowering in a ga1-3 mutant background (Blázquez et al., 1998), proving that LFY acts downstream of the GA signal. This is also true for SOC1, which is also downregulated in a ga1-3 background and can induce flowering when constitutively expressed (Moon et al., 2003). However, although soc1 mutants are late flowering in short days, they are not delayed to such a dramatic extent as ga1-3 mutants (Onouchi et al., 2000). In the absence of data showing the extent to which SOC1 acts upstream of LFY, currently available data suggest that GAs regulate flowering in short days by regulating both LFY and SOC1 and that the regulation of these genes is at least partly independent (Lee et al., 2000; Moon et al., 2003).

LFY is expressed in young leaf primordia and is gradually up-regulated during growth under short days until the plant reaches a threshold level when floral initiation is induced (Blázquez et al., 1997). It is not known whether this gradual increase in LFY
(and SOC1) activity is caused by a concomitant increase in the shoot apical levels of active GAs. However, in a ga1-3 mutant background, this gradual upregulation does not occur (Blázquez et al., 1998).

The later steps in the production of biologically active GAs are catalyzed by a set of 2-oxoglutarate–dependent dioxygenases (Figure 1). The precursors of biologically active GAs GA13 and GA12 are converted in parallel pathways through three consecutive oxidations on C-20 by GA20-oxidase (GA20OX), leading to the production of GA8 and GA20, which are further oxidized on C-3 by GA3-oxidase (GA3OX) to form the bioactive GAs GA4 and GA3 (Figure 1) (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000; Olszewski et al., 2002). Furthermore, the biologically active compounds GA3, GA5, and GA6 can be derived from the precursor GA20. The bioactive GA3 and GA4 can be deactivated through oxidation by GA2-oxidase (GA2OX) to the inactive GA34 and GA8 (Figure 1) (Hedden and Phillips, 2000). The Arabidopsis genome contains multiple genes of the enzymes involved in these latter stages of GA metabolism. The GA20OX enzymes are encoded by five genes, GA20OX1 to GA20OX5 (Phillips et al., 1995; Hedden et al., 2001); the GA3OX by four genes, GA3OX1 to GA3OX4 (Hedden and Phillips, 2000), where GA3OX1 and GA3OX2 appear to be the most important (Mitchum et al., 2006); and the GA20OX by eight genes, GA20OX1 to GA20OX8 (Thomas et al., 1999; Hedden and Phillips, 2000; Schomburg et al., 2003), although GA20OX5 does not appear to encode any functional protein (Hedden et al., 2001). High levels of bioactive GA can trigger a feedback mechanism that represses the expression of certain GA20OX and GA3OX genes and upregulates GA20OX (Hedden and Phillips, 2000). However, up until recently, it was unclear how plants perceive GA and how the GA signal is transduced to cause GA-regulated responses. GA signaling has been proposed to be repressed by the action of members of the DELLA subfamily of the GRAS regulatory protein family (Peng et al., 1997; Dill and Sun, 2001; King et al., 2001a). In response to GA, DELLA proteins are targeted for ubiquitination by SCF E3 ubiquitin ligase and subsequent degradation by the 26S proteosome (McGinnis et al., 2003; Sasaki et al., 2003). A recent pivotal discovery from rice (Oryza sativa) identified the protein GIBBERELLIN DWARF1 (GID1) to be a soluble receptor of GA that upon GA binding interacts with the rice DELLA protein SLENDER RICE1 (Ueguchi-Tanaka et al., 2005). GID1 exhibits high binding affinities toward biologically active GAs, whereas it has low affinity, or none at all, for biologically inactive GAs. In Arabidopsis, there are three orthologs to the rice protein GID1 (Ueguchi-Tanaka et al., 2005), and all these orthologs display a much higher binding activity to GA4 than to any other bioactive GA (Nakajima et al., 2006). This finding corresponds well to previous data showing that GA4 is the active GA in the regulation of Arabidopsis cell elongation and shoot growth (Talon et al., 1990; Xu et al., 1997; Cowling et al., 1998). However, there is still no proof that GA4 is the active GA in the regulation of flowering. Indeed, in other species, such as the monocot Lolium temulentum, it has been shown that GA3 and GA4 are the active GAs in the induction of flowering, but they have very little effect on the regulation of stem elongation (King et al., 2001b, 2003), where instead, GA4 shows high activity (Evans et al., 1990; King et al., 2001b). Therefore, it could be speculated that in Arabidopsis, GAs other than GA4, such as GA1, GA3, GA5, or GA6, could be responsible for the regulation of flowering.

Here, we show, by a combination of GA quantifications and sensitivity assays, that GA4 is the active GA in the regulation of LFY transcription and, thus, in the regulation of Arabidopsis floral initiation under short-day conditions. We also show that during growth in short days, shoot apical levels of GA4 and sucrose increase dramatically before floral initiation occurs and that the expression patterns of the genes involved in GA metabolism suggest that this increase in GA4 possibly originates from sources outside the shoot apex.

RESULTS

Levels of GA4 and Sucrose Increase before Floral Initiation

During growth in short days, LFY transcription is gradually upregulated in the young leaf primordium until the time of floral initiation (Blázquez et al., 1997, 1998) (Figure 2A). To investigate whether the levels of certain GAs also gradually increase in the shoot apical regions, we sampled microdissected shoot apices at weekly intervals until flowers could be seen visually. These shoot apical regions were delimited by the oldest leaf primordium expressing LFY to include all LFY-expressing tissues. In addition, to pinpoint the time of floral initiation, we quantified the levels of APETALA1 (AP1) and AP3 transcription in parallel samples. AP1 transcription is one of the earliest markers for floral initiation and can be detected before formation of the floral primordium (Hempel et al., 1997). By contrast, AP3 transcription is first seen in the developing floral primordium at stage 3 (Jack et al., 1992), approximately 2 d after the beginning of stage 1 (Smyth et al., 1990). AP1 transcript could first be detected at very low levels on
day 42 and more strongly on day 49, while AP3 transcription was first seen on day 49 and more clearly on day 56 (Figure 2A). The relative timing of upregulation is consistent with our observations that the first flower primordia could be seen using a stereomicroscope at day 56, and the first flower buds were visible to the naked eye at day 63 (data not shown). From these results, we conclude that floral initiation takes place between days 42 and 49.

The quantification of the GA content in the shoot apices showed that, of all tested GAs, GA4 was present at the highest level at all time points (Figure 2B). The quantification of GA4 in the shoot apices showed that young plants had a relatively high level of GA4, but the levels subsequently dropped to very low levels for 2 to 3 weeks (Figure 2B). The initial high levels of GA4 could presumably be related to the rapid hypocotyl elongation that ends at about this time. Interestingly, just before floral initiation, between days 35 and 42, the shoot apical levels of GA4 increased dramatically and continued to rise until they reached ~100-fold higher levels by day 56. Thereafter, the levels of GA4 stayed at constantly high levels. The levels of the equally bioactive GA3 were nondetectable in this investigation but have in an earlier study of the Landsberg erecta line of Arabidopsis been found to be in the range of 2 to 10 pg/g fresh weight (FW) (T. Moritz, unpublished results). This was at least 1000-fold lower than the levels of GA4 in that investigation and provides a possible explanation to why Talon et al. (1990) did not identify GA3 in shoots of Arabidopsis in their large-scale identification of GAs. The shoot apical levels of sucrose followed a similar pattern to the levels of GA4 (Figure 2C). The concentration of sucrose in the shoot apex stayed constant at ~1 to 2 μg/mg FW during most of the vegetative growth period. However, between days 35 and 42, the levels started to rise strongly and rose almost 10-fold to 12 μg/mg FW at day 56 (Figure 2C). By contrast, the levels of glucose and fructose remained unchanged during the floral transition (Figure 2C). This shows that the levels of both GA4 and sucrose increase dramatically in the shoot apices, in the same tissues that express LFY, just before floral initiation under short-day conditions.

**GA4 Is the Relevant Endogenous GA Regulating LFY Transcription**

Although GA4 is the GA present at the highest level, it does not have to mean that GA4 is the relevant endogenous GA inducing flowering in Arabidopsis. Previously it has been shown that GA4 is more effective than GA1 at shortening the time to visible flower buds of Arabidopsis in short days (Xu et al., 1997). In

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**Figure 2. LFY Expression and GA and Sugar Quantifications during Flower Initiation.**

**A** Real-time RT-PCR analysis of LFY expression in excised shoot apices during growth in short days. Estimated time of flower induction is marked by shaded area. Values are expressed relative to 18S rRNA. Inset: semiquantitative RT-PCR analysis of AP1 and AP3 expression in excised shoot apices during growth in short days. The 18S rRNA was amplified as an internal control. AP1 can be detected from day 42 and AP3 from day 49. Numbers indicate days after sowing.

**B** Gas chromatography–mass spectrometry (GC-MS) quantifications of the levels of GA1, GA4, and GA5 in excised shoot apices during growth in short days. Microdissected shoot apices from 20 to 30 plants were pooled for each time point. Values are expressed as means ± SE of the mean (n = 3).

**C** GC-MS quantifications of the levels of soluble sugars in excised shoot apices during growth in short days. Microdissected shoot apices from 20 to 30 plants were pooled for each time point. Values are expressed as means ± SE of the mean (n = 3). Shaded area indicates estimated time for flower initiation.
L. temulentum, GA8, and GA9 have been proposed to be the GAs responsible for the induction of flowering (King et al., 2001b, 2003). To check if GA8 and GA9 or other GAs with biological activity could be involved in the regulation of flowering in Arabidopsis, we treated wild-type and GA-deficient ga1-13 plants with GA during growth in short days. Only GA3 and GA4 significantly decreased the total number of leaves formed before flowering of the wild type (Figure 3) and induced flowering in the ga1-13 mutant (Table 1). The effect of GA4 on the wild type was the same as previously shown (Xu et al., 1997). To further investigate the effect of other GAs on flowering in short days, we chose to analyze the effect of application of GA on LFY transcription. Using this system, we determined dose–response curves for all the potentially bioactive GAs to evaluate their ability to induce a LFY:β-glucuronidase (GUS) transgene (Figure 4A). While increasing levels of the inactive GA8 did not lead to increased GUS activities, all the potentially bioactive GAs were able to increase LFY transcription. GA9 and GA7 were the most active, followed by GA1, GA9, and GA9 with the lowest activity. To be able to get better resolution on the activity of the different GAs we chose to repeat the dose–response experiment with a previously described in vitro assay (Blázquez et al., 1998). This in vitro assay is based on plant seedlings submerged in a medium and therefore allows a better control over the uptake and concentration dependence of the various added GAs. In spite of the different developmental stages of the plants between these two experiments, the result from the in vitro experiment (Figure 4B) was similar to the result from application of GA to shoot apices (Figure 4A), suggesting that GA regulation of LFY expression uses a similar mechanism in seedlings as in 4- to 8-week-old plants grown in short days. There were dramatic differences in the sensitivity to the various GAs. The most active GA was GA1, which induced a half-maximum response at ~4 mM, while GA1, GA9, and GA8 induced the same response at ~4 μM—a 1000-fold difference. GA3 displayed an intermediate dose response with a half-maximum response at ~0.3 μM (Figure 4B). These data suggest that GA9 and GA7 are the most active endogenous GAs in terms of being able to induce LFY transcription. Taken together with the fact that GA4 is present at significantly higher levels in the shoot apices during floral initiation than GA3 (Figure 2B), we conclude that GA4 is the relevant endogenous GA in the regulation of LFY transcription and the control of flowering time of short-day-grown Arabidopsis plants.

Transcriptional Activity of Genes Involved in GA Metabolism

An interesting issue raised by the strong increases observed in the levels of GA8 in the shoot apex is whether they are due to local changes in GA metabolism or if GAs are transported into the shoot apex from outside sources. Since transcription of the genes controlling GA metabolism is also subject to regulation by active GAs (Hedden and Phillips, 2000), the expression patterns of these genes can give valuable insights into the cause of changes in GA concentrations. While several of the GA20OX and GA3OX genes are negatively feedback regulated by active GAs, several of the GA20OX genes are positively regulated. When the transcriptional activity of the GA20OX genes was determined in parallel samples to those used to quantify GAs and sugars, it was found that the expression of these genes remained unchanged from day 35 to day 42 when the highest relative increase in GA levels was detected (Figure 5A). After day 42, the GA20OX expression started to gradually increase (Figure 5A). The same pattern was seen for the GA3OX1 gene, the activity of which decreased at day 42 and then gradually increased until day 56 (Figure 5B). By contrast, the GA3OX2 gene, which has been shown not to be feedback regulated by high levels of GAs (Yamaguchi et al., 1998), showed increased expression only at day 49 (Figure 5B). The activity of the GA20OX genes gradually increased from day 35 to day 56, with no significant down-regulation at day 42 (Figure 5C). Taken together, these data show that the increase in the shoot apical levels of GA8 at day 42 can be explained neither by a local induction of GA20OX nor by a decrease in the activity of the GA20OX genes, suggesting that the dramatically increased amounts of GA8 may be derived from sources outside the plant apex. It has already been demonstrated that tetradeuterated GA8 can be transported from leaf to

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**Table 1. Flowering Time of ga1-13 Plants after GA Treatment**

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<tr>
<td>GA3</td>
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<tr>
<td>GA6</td>
<td>&gt;130a</td>
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<td>GA8</td>
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A single leaf was treated every third or fourth day with 5 μL of a 10 μM GA solution. Flowering time was determined as the time when 50% of the plants had visible flower buds.

a No plant had started to flower before the experiment was terminated.
shoot apex in the grass *L. temulentum* (King et al., 2001b). To test the possibility of transport of GA from *Arabidopsis* leaves, we applied the bioactive GA$_4$ on a single leaf of wild-type plants and analyzed the effect on the total number of leaves formed by the plant. This reduced the number of leaves formed before flowering from 73.8 ± 4.9 to 63.6 ± 3.7 (mean ± sd, n = 10). Furthermore, after application of deuterium-labeled GA$_4$ to a single leaf, labeled GA$_4$ could be detected at the shoot apex (Table 2). Although not conclusive, given our poor knowledge about the location of GA biosynthesis and the nature of GA transport, these observations suggest that GAs can be transported from leaf to shoot apex to induce flowering both in *Lolium* and *Arabidopsis*.

![Figure 4](image)

**Figure 4.** Dose–Response Curves for the Activation of LFY:GUS Expression by Different GAs.

(A) Dose–response curves for the activation of LFY by different GAs in short-day-grown plants. Twenty-four-day-old plants were treated three times, at 2-d intervals, by application of 20 μL of different concentrations of various GAs to the shoot apical part of the plant. Plants were then assayed for GUS activity. Values are shown as the means ± 2 × se of the mean (n = 24).

(B) Three-day-old seedlings grown in half-strength Murashige and Skoog medium supplemented with 0.5% sucrose were treated with different concentrations of GA$_1$, GA$_3$, GA$_4$, GA$_5$, GA$_6$, and GA$_8$ for 3 d and then assayed for GUS activity. Values are shown as means ± 2 × se of the mean (n = 24). MUG, 4-methylumbelliferyl β-D-glucuronide.

![Figure 5](image)

**Figure 5.** Expression of Genes Involved in GA Metabolism in Shoot Apices.

RT-PCR (A) and real-time RT-PCR (B) and (C) analyses of gene expression in excised shoot apices during growth in short days. Values are expressed relative to 18S rRNA.

(A) Expression of GA20OX1, GA20OX2, and GA20OX3. n.d., not determined.

(B) Expression of GA3OX1 and GA3OX2.

(C) Expression of the GA2OX genes. Values are expressed as means ± se of the mean (n = 3).
DISCUSSION

A hormonal response is always triggered by a combination of the concentration of a particular hormone and the plant’s sensitivity to it. The concentration of a given hormone is determined by the balance between its biosynthesis, inactivation through catabolism or conjugation, and its transport in and out of the tissue concerned (Davies, 2004). The sensitivity to a hormone is determined by the concentration and activity of proteins involved in hormone reception and signal transduction. In order to identify the endogenous GA that is relevant for the regulation of LFY transcription (and thus flowering) in Arabidopsis plants examined here, we have determined their sensitivity to, and endogenous concentrations of, various GAs that have been suggested to be biologically active.

Bioactive GAs Regulating Flowering

The result of the in vitro dose–response experiment (Figure 4B) clearly shows that in terms of the regulation of LFY expression, the plant is >100 times more sensitive to GA4, with a half-maximal response at a concentration of ~4 nM, than to any other putatively active GA. GA3 and GA20 are clearly also the most active GAs in inducing flowering (Figure 3) and activating LFY transcription (Figure 4A) when applied to apices of short-day-grown plants. The reason why GA3 displays the same relative activity as GA4 in the experiment with the older plants could be that in this experiment the uptake and endogenous concentration of the applied GA is likely to be much less controlled. GA3 is also much more stable than GA4 since it is not the subject of degradation by GA2 oxidases, which could also result in a difference in endogenous cellular concentrations between the experiments. Nevertheless, the two experiments, although using very different experimental setups and with plants at different developmental stages, still show that GA3 and GA20 are more effective than any of the other GAs. It should be noted that the GA levels used in the dose–response experiment (0.1 nM to 10 μM) fall within the physiological range of GA4 concentrations. We found that the shoot apical content of GA4 varied between 2 and 110 pg/mg FW during growth in short days (Figure 2B). If one assumes that 90% of the fresh weight consists of water, this corresponds to an aqueous concentration of GA4 in the range of 3 to 400 nM. Therefore, it is likely that the effects caused by the exogenous supply of GAs closely reflect those caused by changes in the endogenous levels.

At the time of flowering, the levels of GA4 were found to be higher than those of any of the other bioactive GAs (Figure 2B). Taken together, the findings that GA4 is the most potent activator of LFY transcription and that GA4 is present at higher levels than any other bioactive GA at the time of flowering strongly suggest that GA4 is the relevant active endogenous GA in the regulation of LFY transcription and, thus, Arabidopsis flowering during growth in short days. It is interesting to compare the situation in Arabidopsis with that described for grasses. In the grass L. temulentum, it has been shown that while GA4 appears to be the active GA in the regulation of elongation growth, GA3 and GA6 are active in the regulation of flowering, and the concentrations of these GAs in the shoot apices double within 8 h of an inductive long-day treatment (King et al., 2001b, 2003). By contrast, our results show that GA4 is the active GA in the regulation of both flower initiation and elongation growth in Arabidopsis. Therefore, it will be interesting to see if this represents a general distinction between monocot and dicot plants.

GA4 and Sucrose as Mobile Signals Inducing Flowering

Our data show that the levels of GA4 increase dramatically in the shoot apices of Arabidopsis plants just before flowering is initiated in short days (Figure 2B), just as GA4 levels rise strongly after a short-day to long-day shift in Lolium. Given our findings here that GA4 is the most active GA in the regulation of LFY transcription, it is likely that this increase in the levels of GA4 is necessary for floral initiation to occur since a ga1-3 GA biosynthesis mutant containing very low levels of GA4, and also of other bioactive GAs (King et al., 2001a), fails to flower when grown in short days (Wilson et al., 1992). We also know that this failure to flower is, at least partially, caused by a failure to upregulate the expression of the GA-regulated genes LFY and SOC1 (Blázquez et al., 1998; Moon et al., 2003). This sharp increase in the shoot apical levels of GA4 is surprising since the plants were growing under constant short-day conditions with no environmental trigger for flower initiation. It seems that as the plants grow older they reach a critical age or size at which a rapid increase in the levels of GA in the shoot apex is triggered, regardless of the daylength. A correlation between a sharp rise in the shoot apical content of GAs and floral induction in response to a short-day to long-day shift has also been shown in Silene armeria (Talon and Zeevaart, 1990).

It should be pointed out that our data show that there is no simple correlation between GA levels and the transcriptional activity of LFY. For instance, relatively high levels of GA4 could be detected in the shoot apices of 14-d-old seedlings (Figure 2B), while the expression of LFY at this time is still very low (Figure 2A). This indicates that the young seedling is not as competent to respond to the same level of GA4 as an older plant. This is further corroborated by the finding that even when short-day-grown wild-type or ga1-3 plants are treated with GA throughout development, they still display a gradual increase in the expression of a LFY:GUS construct (Blázquez et al., 1998). However, since a ga1-3 mutant in short days requires this GA treatment in order to display any signs of LFY upregulation, and the LFY upregulation is necessary for flowering (Blázquez et al., 1998), our data still strongly suggest that the dramatic increase in GA4 levels that is

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<th>Sample</th>
<th>Ratio (m/z 420 → 392)/(m/z 418 → 390)</th>
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<tr>
<td>Nonlabeled GA4 standard</td>
<td>0.13</td>
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<tr>
<td>Shoot apices in</td>
<td>0.21</td>
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<tr>
<td>2H2-GA4 leaf-fed plants</td>
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2H2-GA4 transport was detected by measuring endogenous and labeled GA4 in apex samples and GA4 standards with GC-MS in selected reaction monitoring mode and comparing the ratio of endogenous and labeled GA4 in the sample and GA4 standard. m/z, mass-to-charge ratio.

Identification of Leaf-Fed Deuterated GA4 in Arabidopsis Shoot Apices

Table 2.

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correlated with the *LFY* upregulation before floral initiation is relevant for the timing of flowering.

An interesting issue raised by the observed increases in active GA$_4$ is whether the additional amounts are synthesized locally in the shoot apex or are caused by a transport of GAs to the apex from other sources. The fact that transcription of the feedback-regulated GA20OX and GA3OX GA biosynthesis genes appears to be unaffected or reduced at the same time as the GA levels in the shoot apex start to rise (Figures 2B, 5A, and 5B), while expression of the feed-forward-activated GA2OX genes is increased (Figures 2B and 5C), suggests that the increased amounts of GA$_4$ detected in the shoot apex could be derived from the import of GAs from outside sources. This is further supported by the finding that GA$_4$ applications to a single leaf can induce early flowering of short-day-grown plants and that labeled GA$_4$ can move from leaf to shoot apex (Table 2). It is also interesting to note that at the same time that the levels of GA$_4$ started to increase, the shoot apical levels of sucrose also increased markedly (Figures 2B and 2C). Since the shoot apex is a very strong sink for nutrients and has very limited photosynthetic capacity compared with developed source leaves, the increased amounts of sucrose are most likely derived from outside sources. It is possible that the GAs, like sucrose, are transported from source leaves to the shoot apical meristem sink via the phloem (Bernier and Périlleux, 2005).

Recent data suggest that the classic florigen signal moving from leaf to shoot apex to induce flowering can be explained by a movement of the *FT* mRNA through the phloem (Abe et al., 2005; Huang et al., 2005; Wigge et al., 2005). This movement is responsible for the long-day-induced flowering in *Arabidopsis*.

Interestingly, it has been suggested that sucrose, like GAs, may also act as a signaling molecule in flowering regulation. There is a synergistic interaction between GAs and sucrose in the activation of *LFY* transcription (Blázquez et al., 1998), and sucrose supplied to the shoot apex has been shown to complement the late-flowering phenotype of the *Arabidopsis* mutants *co*, *gi*, *fca*, *fpa*, and *fve* (Roldán et al., 1999). The only late-flowering mutant that could not be rescued by sucrose in the cited study was *ft* (Roldán et al., 1999). Taken together, these results suggest that *Arabidopsis* flowering time in noninductive short-day conditions is determined by sharp increases in the shoot apical levels of GA$_4$ and sucrose just before flower initiation. These increases could possibly be caused by increased transport of GAs and sucrose from the source leaves coupled to an opening of the plasmodesmatal connections between the end of the phloem and the shoot apical meristem, as demonstrated for long-day-induced flowering (Ormenese et al., 2000). According to this hypothesis, GAs and sucrose can be seen as part of a short-day florigenic signal moving from the leaf to the shoot preceding flower induction.

**Functional Redundancy between GAs and CO/FT**

Another interesting issue to consider is the relevance of these findings to long-day-induced flowering in *Arabidopsis*. In *spinach* (*Spinacia oleracea*) shoots, it has been shown that the levels of GAs increase dramatically after a short-day to long-day shift (Talon et al., 1991). However, this shift, in both *Arabidopsis* and *spinach*, induces very rapid stem elongation (bolting), which is clearly a GA-regulated process. Since this occurs very close in time to flower initiation, it is impossible to tell if the increase in GAs is associated with flower initiation, bolting, or both. In short days, flower initiation and bolting is separated by several weeks (Figure 2A), and we can therefore say that the pronounced increase in GAs seen under short days is much more closely correlated to flower initiation than to bolting. In fact, GAs seem to have a very marginal role in the regulation of flowering time in long days since flowering is only slightly delayed in GA biosynthesis and signal transduction mutants under these conditions (Wilson et al., 1992). Instead, it seems that the role of GAs in short days is taken over by the flowering activators CO and FT in long days (Searle and Coupland, 2004) since in a co mutant, GAs are necessary for flowering and *LFY* regulation in long days (Blázquez and Weigel, 2000). This functional redundancy between GAs and CO/FT is interesting from many perspectives. Both GAs and CO/FT regulate *SOC1*, and both GAs and CO/FT seem to be associated with a mobile flower-inducing signal that moves from the leaf to the shoot apex. Further investigations will help to determine the molecular basis of this functional redundancy and help to establish the relationship between the activities of GAs and CO/FT in the regulation of flower initiation.

**METHODS**

**Plant Growth and Material**

The *Arabidopsis thaliana* *LFY:GUS* line DW150.209 (Blázquez et al., 1997) of the Columbia-0 (Col-0) ecotype was used as wild-type control in this study. The *ga1-13* mutant was isolated by searching the SALK institute Genomic Analysis Laboratory T-DNA express database for *ga1* T-DNA insertion mutants in the Col-0 background (Alonso et al., 2003). T3 seeds corresponding to SALK_109115 were requested from the Nottingham Arabidopsis Stock Centre.

Growth conditions consisted of short days (9 h of light from fluorescent lamps at 130 μmol m$^{-2}$ s$^{-1}$ and 15 h of darkness) and long days (16 h of light and 8 h of darkness) at 23°C.

Seeds were stratified in 0.1% agarose for 2 d before planting. Plants for GA, sugar, and RNA extraction were grown on a 3:1 mixture of soil and vermiculite in short days. For sampling of shoot apices, samples were collected once per week, in the middle of the photoperiod, from 2 weeks after planting until a majority of the plants had started to bolt. Shoot apices carrying leaves smaller than 1 mm were collected from 20 to 30 plants for each sample. Four equal samples, weighing from 4 to 10 mg, were collected per time point. Three samples were randomly selected for GA/sugar quantification and one sample for mRNA extraction. The whole growth experiment was repeated once with similar results.

**GA Activity Measurements**

The in vitro seedling assay for *LFY:GUS* activity was performed essentially as previously described (Blázquez et al., 1998), with the exception that seedlings were grown, 20 to 25 per well, in 12-well cell culture plates, in half-strength Murashige and Skoog medium supplemented with 0.5% sucrose. Three-day-old seedlings grown under long-day conditions were challenged with the addition of various amounts of different GAs (purchased from Lew Mander). *LFY:GUS* activity was analyzed 3 d later on 24 seedlings from each treatment.

Activity measurements on plants grown on soil in short day were done by application of 20 μL of various concentrations of different GAs three
times, with 2-d intervals, to the apical region of the plant. GUS activity was analyzed the day after the last application on 24 shoot apices from each treatment.

GA applications for determination of the effect on flowering time were done by applying a solution of GA in 20% ethanol to the shoot apical region, or a single leaf, of individual wild-type Col plants or ga1-13 plants every third or fourth day. For ga1-13 plants, a single leaf was treated with 5 µL of a 10 µM GA solution from day 25, until the first flowers buds could be seen. Flowering time was determined as the time when 50% of the plants had visible flower buds. Wild-type Col plants were treated with 20 µL of a 10 µM GA solution from day 22 until day 49 either on leaves or on the shoot apex. Flowering time was scored as the total number of leaves formed on the primary shoot. Calculation of statistical significance was done with a Student's t test assuming equal means.

GA and Sugar Quantifications

Three replicate samples were analyzed for each time point. Samples (4 to 10 mg) were homogenized and extracted for 2 h in 500 µL 80% methanol, including 2H2-GAs (purchased from Lew Mander), D-Sucrose-13C12 (Larodan Fine Chemicals), D-glucose-1,2,3,6-13C4, and D-fructose-2,3,4,6-13C4 (Cambridge Isotope Laboratories) as internal standards. Fifty microliters of each extract was removed, evaporated to dryness, and trimethylsilylated for quantification of soluble sugars by GC-MS (Ugglä et al., 2001).

Total RNA was isolated from plant shoot apices using RNaqueous-4PCR (Ambion) and treated with DNA-free DNase treatment and removal reagents (Ambion) to remove genomic DNA. Absence of genomic DNA contamination in the DNase I–treated RNA samples was verified by PCR selection reaction monitoring mode using a JEOL JMS MStation as described earlier (Peng et al., 1999).

RNA Isolation and Analysis by PCR

Total RNA was isolated from plant shoot apices using RNaqueous-4PCR (Ambion) and treated with DNA-free DNase treatment and removal reagents (Ambion) to remove genomic DNA. Absence of genomic DNA contamination in the DNase I–treated RNA samples was verified by PCR using LFY primers. Two micrograms of total RNA was subjected to reverse transcription with a first-strand cDNA synthesis kit (Amersham Biosciences) with 200 pg of random hexamer primers. GA20OX1, GA20OX2, GA20OX3, AP1, AP3, and LFY transcription was analyzed with RT-PCR using QuantumRNA 18S internal standard as control (Ambion). The PCR program used was 94°C for 1 min, 94°C for 10 s, 55°C for 30 s, and 72°C for 30 s. Steps 2 to 4 were repeated 33 times. Products were labeled during PCR with DIG (Roche), and fragments were separated on agarose gels and blotted onto Hybond N membranes (Amersham Biosciences). Membranes were probed with Anti-Digoxigenin-AP Fab fragment (Roche) incubated with ECF substrate (Amersham Biosciences), and the resulting signals were detected using a Typhoon 9410 workstation (Amersham Biosciences). All amplifications resulted in a single product of the expected size, except for the GA20ox genes, where two splice variants are amplified. All quantifications were performed on the shorter, completely spliced product.

Expression of the GA3OX and GA2OX genes was analyzed with real-time RT-PCR in a BIO-RAD Mycycler using Syber Green Supermix (Bio-Rad) as previously described (Norberg et al., 2005). The 18S rRNA was amplified as a loading control. All amplifications generated a single product. Relative expression levels were calculated with Q-Gene software (Muller et al., 2002). Primer sequences can be found in Supplemental Table 1 online.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: AP1 (At1g69120); AP3 (At3g45440); GA1 (At4g02780); GA2OX1 (At1g78440); GA2OX2 (At1g30040); GA2OX3 (At2g34555); GA2OX4 (At1g47980); GA2OX6 (At1g02400); GA2OX7 (At1g50860); GA2OX8 (At4g21200); GA2OX1 (At1g15550); GA3OX2 (At1g80340); GA20OX1 (At4g25420); GA20OX2 (At5g51810); GA20OX3 (At5g07200); and LFY (At5g61850).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. Sequence of Forward and Reverse Primers Used for Quantification of mRNA by RT-PCR.

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GA₄ Is the Active Gibberellin in the Regulation of LEAFY Transcription and Arabidopsis Floral Initiation

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