Regulation of Flowering in the Long-Day Grass *Lolium temulentum* by Gibberellins and the FLOWERING LOCUS T Gene

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Seasonal control of flowering often involves leaf sensing of daylength coupled to time measurement and generation and transport of florigenic signals to the shoot apex. We show that transmitted signals in the grass *Lolium temulentum* may include gibberellins (GAs) and the FLOWERING LOCUS T (FT) gene. Within 2 h of starting a flarially inductive long day (LD), expression of a 20-oxidase GA biosynthetic gene increases in the leaf; its product, GA20, then increases 5.7-fold versus short day; its substrate, GA19, decreases equivalently; and a bioactive product, GA5, increases 4-fold. A link between flowering, LD, GAs, and GA biosynthesis is shown in three ways: (1) applied GA19 became florigenic on exposure to LD; (2) expression of *LtGA20ox1*, an important GA biosynthetic gene, increased in a florally effective LD involving incandescent lamps, but not with noninductive fluorescent lamps; and (3) paclobutrazol, an inhibitor of an early step of GA biosynthesis, blocked flowering, but only if applied before the LD. Expression studies of a 2-oxidase catabolic gene showed no changes favoring a GA increase. Thus, the early LD increase in leaf GA5 biosynthesis, coupled with subsequent doubling in GA5 content at the shoot apex, provides a substantial trail of evidence for GA5 as a LD florigen. LD signaling may also involve transport of FT mRNA or protein because expression of *Lift* and *Liconstans* increased rapidly, substantially (>80-fold for FT), and independently of GA. However, because a LD from fluorescent lamps induced *Lift* expression but not flowering, the nature of the light response of FT requires clarification.

It has been more than 90 years since Tournoius, in 1914, and, independently, Garner and Allard, in 1920, proposed that seasonal control of flowering was a response to daylength; long-day (LD) species flower with an increase in the daily hours of light, and short-day (SD) species flower in shorter daylengths. The leaf was the predominant organ of daylength perception, and, based on his grafting studies, Chailakhyan, in 1937, coined the term florigen for the florally inductive signals transported from the leaf to the shoot apex (for review, see Lang, 1965). Based on recent studies with Arabidopsis (*Arabidopsis thaliana*), one such florigen may be the mRNA or protein of the FLOWERING LOCUS T (FT) gene (Huang et al., 2005). For the grass *Lolium temulentum*, another florigen could be the GA class of plant hormones (King and Evans, 2003).

How many transported factors regulate flowering has been a matter of ongoing debate (see Bernier, 1988), but, in seeking an answer, some logical rules can be applied. According to Jacobs (1959) and others (see Weyers and Patterson, 2001), there are four key requirements for identifying florigens that act in the photoperiodic induction of flowering: (1) the compounds should replace the photoperiodic requirement; (2) the compounds should increase in the leaves at the time of floral induction; (3) the compounds should be transported to the shoot apex at or before the time of floral evocation; and (4) concentrations in the shoot apex should be sufficient to cause flowering.

To study floral signaling, there are a number of advantages to using *L. temulentum*. It flowers on exposure to a single LD of about 14 to 16 h of light, but remains strictly vegetative in SD. Its leaf response is rapid and is completed after 17 to 22 h. At this time, floral signals are exported out of the leaf and start arriving at the shoot apex by 24 h (McDaniel et al., 1991). Furthermore, early biochemical and molecular...
changes at the shoot apex begin by 26 to 32 h (for review, see Evans and King, 1985).

As for the role of FT, the use of a heat shock promoter sequence to drive FT expression and flowering in transgenic plants of Arabidopsis elegantly demonstrated its involvement as a floral signal transmitted from the leaf to the shoot apex (Huang et al., 2005). This finding also provided a link between flowering and CONSTANS (CO) action in the leaf (Mouradov et al., 2002). For L. temulentum, such genetic and transgenic approaches are difficult, but the timing of the earliest events of its flowering is so well characterized (see above) that any rapid and early increase in FT would imply a role as a floral signal (requirement 2). At the same time, such a study could help to define the role of GAs as a floral signal in L. temulentum.

Our claim that the GA class of plant hormones plays a role in LD signaling in L. temulentum is based on a number of observations. First, several applied GAs, especially GA5 and GA4, evoke flowering of L. temulentum in SD without causing excessive stem elongation (Evans et al., 1990; King et al., 2003) and so act like exposure to a single LD (requirement 1). Second, applied, tetradeuterated GA5 is transported intact from the leaf to the shoot apex, and, the more applied to the leaf and received at the apex, the greater the flowering response (King et al., 2001; requirements 3 and 4). Third, when summed because they are equally bioactive, the endogenous content of GA5 and GA4 in the shoot apex increases 4-fold on the day after the LD (King et al., 2001, 2003) to reach a combined concentration of approximately 10^{-7} M.

Only a minute fraction (about 10^{-6}) of leaf-applied GA reaches the shoot apex, in part because the apex is such a small sink, but probably there are also restrictions on leaf uptake. However, the use of excised shoot apices of L. temulentum has avoided these problems and allowed us to show that a GA dose exceeding 10^{-7} M was sufficient for flowering and that a 2- to 3-fold increase saturated the response (King et al., 1993). These findings match very closely those for concentrations and increases in shoot apex bioactive GAs that occur during flowering induced by a LD (King et al., 2001, 2003; requirement 4).

Overall, such studies make a strong case for GA5 and GA4 as LD florigenic stimuli in L. temulentum; however, an essential piece of information is missing, namely, that a LD increases leaf GA5 content (requirement 2). It was also important to link any GA increase in the leaf to the activity of GA biosynthetic enzymes.

Here, we show that a LD rapidly increases endogenous GA5 in the leaf blade. In addition, there are rapid increases in expression of a GA 20-oxidase, a likely candidate for up-regulation of GA biosynthesis by LD. By contrast, there was little evidence of a decrease in expression of a catabolic GA 2-oxidase whose down-regulation would have allowed a GA increase. Last, we show rapid and large early increases in expression of LICO and LIFT in the leaf, which fit best with an independent role for FT in floral signaling.

RESULTS

Characteristics of LD-Induced Flowering of L. temulentum

Specificity, precision, and rapidity of response to a LD are essential for any identification of important molecular and biochemical changes associated with flowering. Exposure of the leaf of L. temulentum to one LD from incandescent lamps that exceeds 14 h in duration induces flowering, but in SD the plants remain vegetative (Fig. 1; Evans, 1960; Evans and King, 1985). Thus, L. temulentum shows an obligate LD flowering response. The precision in the LD response of L. temulentum is shown in Figure 1 based on the average values of percentage of plants flowering from many studies and in daylengths of various durations (see Evans and King, 1985). The inset shows the same timing of response in an experiment performed as part of these studies and where we recorded floral scores. Flower development occurs rapidly, and after 3 weeks (Fig. 1) the inflorescence of plants exposed to a LD has formed another initials.

Whereas the threshold LD for flowering of Lolium requires a 14- to 15-h LD light exposure, flowering increased with a LD up to 24 h in duration (Fig. 1; see Evans and King, 1985). Over this period, signal synthesis and export proceeds to completion because leaf removal at hour 24 no longer prevents flowering (see Evans and Wardlaw, 1966; Evans and King, 1985). Thus, in making comparisons between LD and SD at any one time, we focus on the 12- to 18-h period when leaf photoinduction, signal synthesis, and export are occurring. In circadian terminology, the zero time

![Figure 1. Flowering of L. temulentum grown vegetatively in 8-h SD and then exposed to a single incandescent extension of the light period ranging in duration from 2 to 16 h. In the main figure, the data were averaged from experiments carried out at different times over many years and, hence, combined as percentage of plants flowering. The inset reports the effect of photoperiod length on flowering based on the floral score determined 3 weeks after the single LD; these plants were part of the study reported in Figure 4. The scale used to determine the floral score is presented by McDaniel et al. (1991). There were 14 plants per treatment, and error bars were smaller than the symbols.](https://www.plantphysiol.org/doi/abs/10.1104/pp.141.2.499)
point is often the daily light-on signal; thus, in comments on daylength duration, this time is used as the zero (CT 0) reference point.

LD Flowering and Leaf GA Content

Applied GAs can induce flowering in SD but show large differences in effectiveness on stem elongation and flowering. For example, GA₅ and GA₆ are good endogenous candidates as LD florigens because they induce flowering but have little effect on stem elongation, as does LD exposure (Evans et al., 1990; King et al., 2003). By contrast, some of the more generally used bioactive GAs (e.g. GA₅, GA₆, and GA₇ [see Hedden and Phillips, 2000]) cause extensive stem elongation but less flowering in *L. temulentum* and so are unlikely to be endogenous regulators of flowering (Evans et al., 1990). For this reason, we have focused our analysis on endogenous GA₁, GA₅, GA₁⁰, GA₁⁹, and GA₂₀ because they cover various key biosynthetic steps, as shown below in an abbreviated GA metabolic pathway, and include three relevant enzymes (adapted from Hedden and Phillips, 2000). Structures of these GAs can be found at www.plant-hormones.info/.

\[
\begin{align*}
\text{GA}_5 & \rightarrow \text{GA}_4 \sum \rightarrow \text{GA}_5 \rightarrow \text{GA}_6 \sum \rightarrow \text{GA}_5 \rightarrow \text{GA}_3 \\
\rightarrow & \text{2-oxidase} \\
\rightarrow & \text{3-oxidase} \\
\rightarrow & \text{2-oxidase}
\end{align*}
\]

Preliminary studies of metabolism of GA₅ in *L. temulentum* showed its conversion to GA₆ but not to GA₃ (King et al., 2004). Although applied GA₅ is as flорally effective as GA₅ and it increases in LD apex along with GA₅ (King et al., 2001, 2003), its detection was unreliable in the leaf so no results could be presented. As for GA₅, it does not meet the criteria of being florigenic because its floral activity is associated with substantial stem elongation (see Evans et al., 1990; King and Evans, 2003).

The LD-induced changes include rapid (approximately 4 h) and significant shifts in the content of GAs in the leaf (Fig. 2). By 12 to 16 h, the content of GA₅ was up to 4-fold greater in LD than in plants harvested at the same time in SD (Fig. 2C). In parallel, there was 5.7-fold more GA₂₀ in LD (Fig. 2B); this GA is a known precursor of GA₅. Conversely, there was a matching decrease in LD in the content of GA₁⁹, the immediate precursor of GA₂₀ (Fig. 2A). At the peak at 16 h after the light-on signal (CT 16), GA₂₀ content had increased by 18 ng g⁻¹ over 8 h and GA₁⁹ content had decreased by a similar amount (23 ng g⁻¹).

Growth-active GAs, such as GA₁ and GA₄, accumulate in the leaf of *L. temulentum* and *Lolium perenne* after exposure to 2 LD (Gocal et al., 1999; MacMillan et al., 2005). Here, particularly because we sampled frequently and overnight, we detected increases in leaf GA₁ during the single LD (Fig. 2D). However, the increase in GA₁ was 8 to 12 h later than for GA₅ or GA₂₀ (Fig. 2). A single-step hydroxylation by 2-oxidase enzymes converts GA₁ to GA₈, a relatively inactive GA. The minor and often nonsignificant changes in GA₈ levels in LD (Fig. 2E) suggest there is little effect of daylength on GA catabolism; however, the possibility of change in 2-oxidasen cannot be excluded. The increase in LD in GA₁ content favors this suggestion, and this fits

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**Figure 2.** Diurnal changes in the content of five GAs in the leaf blade of *L. temulentum*. Plants were grown in SD (○, solid line) or exposed to a single flорally inductive LD (□, broken line). The white boxes on the time scale indicate the daily 8-h natural light period, the hatched box indicates the 16-h LD extension with incandescent lamps, and the black boxes indicate the SD dark periods. GA content is expressed as ng g⁻¹ dry weight (dw). The LSD (0.05) is for the three replicate extractions for all 11 times of sampling for any one GA. The classes of enzyme responsible for these three biosynthetic conversions are indicated near the arrows.
with our evidence (see data presented later) of a small increase in LD in expression of one 2-oxidase. On the other hand, because the pool of GA\textsubscript{19} appears to be small relative to that of GA\textsubscript{8}, its conversion to GA\textsubscript{9} might be hard to detect.

**LD Flowering and Inhibitors of GA Biosynthesis**

To establish causality in the relationship between LD, GA biosynthesis, and flowering, we used paclobutrazol (PAC), an inhibitor of early steps of GA biosynthesis (Rademacher, 2000). PAC inhibited flowering only when applied 2 d or more prior to the single LD (Fig. 3; Table I). This shows that GAs are required for flowering and, more specifically, that they are required at the time of LD exposure rather than at later times when the flower is developing. In support of an early requirement for GAs, transfer studies with excised apices cultured on agar with or without GA showed that GAs were essential only over the first 2 to 3 d of culture (King et al., 1993).

PAC studies do not show which steps of GA biosynthesis are LD up-regulated, but the decrease in leaf GA\textsubscript{19} content and the matching increase in GA\textsubscript{20} (Fig. 2) are indicative of LD regulation of a 20-oxidase biosynthetic gene. Therefore, we examined the activity of GA\textsubscript{19}, a 20-oxidase substrate, to reverse the inhibition of flowering by PAC. Applied GA\textsubscript{19} reversed the inhibition by PAC in LD (Table I), but in SD GA\textsubscript{19} was essentially ineffective, a floral score of 2 being required for designation as a floral response. As an aside, when applied in SD to plants not treated with PAC, GA\textsubscript{19} is ineffective (Evans et al., 1990). Taken together, these findings point both to increased 20-oxidase enzymatic activity in LD and to LD specificity; this fits with our evidence (see below) that LD does stimulate expression of a 20-oxidase gene in the leaf.

Application of the inhibitor trinexapac-ethyl (TNE) provided another way to examine LD regulation of GA metabolism because TNE inhibits 2- and 3-oxidases but not 20-oxidases (Rademacher, 2000). In our studies, there was no inhibition of flowering by TNE, so this excludes a role for 3-oxidases. On the other hand, promotion of flowering by TNE (Fig. 3) indicates involvement of 2-oxidases; inhibition of 2-oxidases by TNE would reduce GA inactivation and so account for the small increase in flowering. However, LD and 2-oxidase activity are not linked because TNE promoted flowering for applications both before and well after the LD (Fig. 3). Evidence presented later also indicates that none of the changes in 2-oxidase expression over a day explain LD increases in GA. Previously, we have reported a strong promotion of flowering by TNE applied just before LD exposure (Evans et al., 1994), but, as here, not all of our studies showed a similar time-dependent promotion and we have no explanation for such differences.

**Expression of GA Metabolic Genes**

When the daily SD light period of 8 h was lengthened using far-red (FR)-enriched light from incandescent lamps, transcripts of a GA 20-oxidase (\textit{Liga20ox1}) increased dramatically to reach a maximum after 8 h (CT 16), with expression levels 30-fold greater than at the same time of day for plants in SD (Fig. 4A). The timing of this increase, first evident 2 h after starting the FR-enriched LD exposure (i.e. CT 10), coincides with increases in GA\textsubscript{20} and GA\textsubscript{9} and a decrease in GA\textsubscript{19} (Figs. 2 and 4). It also precedes by some hours the threshold duration of the LD required for floral induction (Fig. 1) and for increase in \textit{Liga20ox1} expression (see below).

\textit{Liga20ox1} transcript levels were low either during daytime hours or when LD exposure was from red (R)-rich fluorescent lamps (Fig. 4). This latter finding is important because it shows that expression of GA metabolism genes parallels the effectiveness of a LD for flowering. A FR-enriched LD from incandescent lamps is florally effective (Fig. 1; Table II, experiment I) and up-regulates 20-oxidase expression (Fig. 4). A comparable 16-h LD exposure but from R-rich fluorescent lamps is florally effective (Fig. 1; Table II, experiment I) and for increase in \textit{Liga20ox1} expression (see below).
values are means to a LD extension from fluorescent lamps is shown in Table II. The flowering response experiment, the flowering response to increasing daylength from incandescent or fluorescent lamps or the SD dark period. For this natural light period, and the hatched box either the 16-h LD extension intensity ( at 10 $\mu$mol m$^{-2}$ s$^{-1}$) or from fluorescent lamps at the same low intensity ( ). The white boxes on the time scale indicate the daily 8-h natural light period, and the hatched box either the 16-h LD extension by incandescent or fluorescent lamps or the SD dark period. For this experiment, the flowering response to increasing daylength from incandescent lamps is shown in Figure 1 (inset); the flowering response to a 16-h LD extension from fluorescent lamps is shown in Table II. The values are means ± s of three replicate assays. Confirmatory results were obtained in five biological repeat experiments.

Figure 4. The diurnal pattern of expression of a 20-oxidase (A) and a 2-oxidase (B) gene in the leaf of L. temulentum. The basal 4 cm of the leaf blade was harvested from plants in 8-h SD ( ) or during exposure to a single florally inductive LD extension of 16 h from incandescent lamps at 10 $\mu$mol m$^{-2}$ s$^{-1}$ ( ) or from fluorescent lamps at the same low intensity ( ). The white boxes on the time scale indicate the daily 8-h natural light period, and the hatched box either the 16-h LD extension by incandescent or fluorescent lamps or the SD dark period. For this experiment, the flowering response to increasing daylength from incandescent lamps is shown in Figure 1 (inset); the flowering response to a 16-h LD extension from fluorescent lamps is shown in Table II. The values are means ± s of three replicate assays. Confirmatory results were obtained in five biological repeat experiments.

lamps was not florally inductive (Table II; Evans et al., 1965) and did not enhance expression of the 20-oxidase (Fig. 4).

In contrast to the LD increases in expression of the 20-oxidase late in the 24-h cycle, the GA 2-oxidase, $LG_{A20ox1}$, showed greatest expression during the day, increasing 9-fold to a peak by CT 6 (Fig. 4B). A FR-enriched LD from incandescent lamps up-regulated $LG_{A20ox1}$ expression 4- to 6-fold relative to its level in leaves harvested at the same time in SD (Fig. 4B). Because 2-oxidases show feed-forward up-regulation of their expression by bioactive GAs (see Hedden and Phillips, 2000), the late increase in $LG_{A20ox1}$ transcripts was probably a response to the late increase in GA$_1$. The dynamics of this homeostatic regulation of GA metabolism are also likely to explain why LD might either enhance or suppress 2-oxidase expression as reported for spinach (Spinacia oleracea; Lee and Zeevaart, 2002, 2005). As for $LG_{A20ox1}$, relative to SD plants in darkness, $LG_{A20ox1}$ expression did not increase in leaves of plants exposed to a R-rich fluorescent LD (Fig. 4B).

High-level expression of $LG_{A20ox1}$ during the day is interesting. It may involve circadian regulation because, after CT 6, there was a decline in SD as if in anticipation of the following night (Fig. 4B). A similar diurnal pattern is also evident in Arabidopsis (Hisamatsu et al., 2005), as is diurnal variation in 20-oxidase expression in SD (Hisamatsu et al., 2005; see Fig. 4).

CO, FT, and Photoperiodic Responses

We examined expression of L. temulentum CO because of its known diurnal regulation in L. perenne (Martin et al., 2004) and because it is believed to positively regulate FT expression in the leaf as part of LD-regulated flowering of Arabidopsis (Huang et al., 2005; for review, see Mouradov et al., 2002). The $LtCO$ gene shows high sequence relatedness to LpCO (97% amino acid identity), which, based on complementation studies, is a homolog of CO in Arabidopsis (Martin et al., 2004).

In SD conditions, $LtCO$ was expressed most highly overnight; this expression was elevated in a FR-enriched LD (Fig. 5). The increase in expression over the first hours (CT 8–16) was mostly less than 2-fold. Later, there were large increases (CT 22–32; Fig. 5). In these assays with L. temulentum, we used mouse RNA for standardization (see “Materials and Methods”), as in the assays described previously for 2- and 20-oxidases. In other studies (data not shown), we used GAPDH as

<table>
<thead>
<tr>
<th>Daylength/GA Treatments</th>
<th>FT Expression</th>
<th>Floral Score</th>
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<tbody>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LD (incandescent)</td>
<td>273 ± 5.4</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>LD (fluorescent)</td>
<td>115 ± 30</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>SD</td>
<td>1.7 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LD (incandescent)</td>
<td>377 ± 51</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>LD (incandescent + GA$_4$)</td>
<td>194 ± 7</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>LD (incandescent + GA$_6$)</td>
<td>262 ± 22</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>SD</td>
<td>2.2 ± 0.1</td>
<td>0.7 ± 0.2</td>
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<tr>
<td>SD + GA$_4$</td>
<td>2.8 ± 0.4</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>SD + GA$_6$</td>
<td>2.3 ± 0.1</td>
<td>3.0 ± 0.3</td>
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<tr>
<td>Experiment III</td>
<td></td>
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<tr>
<td>LD (incandescent)</td>
<td>35.6 ± 8.0</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>LD (incandescent + PAC)</td>
<td>25.1 ± 5.3</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>SD</td>
<td>1.0 ± 0.2</td>
<td>0</td>
</tr>
</tbody>
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Table II. Expression of $LTFT$ in the leaf and flowering of L. temulentum grown in 8-h SD and then exposed to one LD at a low intensity from fluorescent or incandescent lamps (10 $\mu$mol m$^{-2}$ s$^{-1}$).
ultimately, Arabidopsis has an orthologous relationship to rice (Oryza sativa). Characterization of LpCO and late (Figs. 1 and 5). In this repeat experiment, we found a large increase to that seen for LpCO. The results of diurnal oscillations in LpCO with the mouse standard, which allowed us to confirm an internal standard and it gave results similar to those with the mouse standard, which allowed us to confirm the earlier findings of diurnal oscillations in LpCO (Martin et al., 2004). Also, confirming the results in Figure 5, in this repeat experiment, we found a large increase in LpCO expression (data not shown). In their earlier study, Martin et al. (2004) exposed their plants to many cycles of LD, and CO expression was evident at other times and in leaf tissue proximal to the point of GA application (data not shown). There were no effects of GA on LpCO expression in this study (data not shown).

The second approach involved applying GAs to leaves of plants either exposed to LD or kept in SD, with the treated leaf being harvested 12 h later at CT 18. GA4 did not stimulate LpFT expression but did induce flowering in SD (Table II; experiment II). GA20 did not stimulate LpFT expression in SD, but by contrast, was nonflorigenic, as we reported before (Evans et al., 1990). In LD, neither GA enhanced LpFT expression, which was actually reduced. Comparable patterns in LpFT expression were evident at other times and in leaf tissue proximal to the point of GA application (data not shown). There were no effects of GA on LpCO expression in this study (data not shown).

The third approach was the same as in Figure 3 and involved inhibiting GA-induced flowering by applying PAC to the plants 5 d before the LD. The incandescent LD exposure led to the expected increase in LpFT expression, but there was no significant effect of PAC application, although flowering was reduced substantially (Table II, experiment III). The LD increase was lower in this experiment probably because samples were taken at CT 24, when LpFT expression is sometimes lower than at CT 18 (data not shown).

Taken together, these findings show that, as well as increasing GA production, a single LD dramatically enhances LpFT expression. However, LpFT expression is not regulated by GA content, so GAs and LpFT respond independently to a LD.

**DISCUSSION**

Critical components of the photoperiodic control of flowering include a specific spectral response, action of a circadian clock to gate the photoreceptor input, and the production of florigenic signals that are then translocated from the leaf to the shoot apex, where they evoke flowering (for review, see Lang, 1965; Mouradov et al., 2002). Our evidence here for the LD-responsive grass L. temulentum relates to GAs and LpFT, which, apparently, are distinct, leaf-produced floral signals.
1990; King and Evans 2003; King et al., 2003) and, from our studies here and previously, four lines of evidence show their endogenous action in LD regulation of flowering: (1) expression of an important GA biosynthetic gene, \(LtGA20ox1\), increased rapidly in the leaf in LD (Fig. 4); (2) almost as rapidly (<4 h), content of the highly florigenic \(GA_5\) increased (Fig. 2); (3) \(PAC\), an inhibitor of GA biosynthesis, blocked flowering, but only if applied before the LD (Tables I and II; Fig. 3); and (4) subsequently, the \(GA_5\) content of the shoot apex increased to a florally effective concentration (King et al., 2001, 2003).

The increase in \(LtGA20ox1\) expression in the leaf after 2 to 4 h in LD (CT 10–12), followed soon after by a \(GA_5\) buildup, must be close to the earliest event of LD after 2 to 4 h in LD (CT 10–12), followed soon after by a apex at this time (CT 24 and later; Evans and Rijven, expression differences are also evident at the shoot apices excised at the end of the LD (CT 24) or later (Evans and Wardlaw, 1966). Such a delay has also been confirmed by excising shoot apices at different times during the LD and allowing them to form floral primordia in vitro, which only happened with apicesexcised at the end of the LD (CT 24) or later (McDaniel et al., 1991). The first biochemical and gene expression differences are also evident at the shoot apex at this time (CT 24 and later; Evans and Rijven, 1967; Gocal et al., 1999). Thus, \(GA_5\) could well be one transported florigen of \(L. temulentum\), a conclusion that also applies to \(L. perenne\), which requires both vernalization and LD exposure and where leaf \(GA\) content is considerably elevated after exposure to two LDs (MacMillan et al., 2005).

Evidence of spectral specificity in the LD response supports our claim that \(GA\) s are florigens in \(L. temulentum\). It was essential both for flowering and for activating 20-oxidase expression that plants were exposed to a LD from FR-enriched light from incandescent lamps following the high-intensity light period of the SD. Matching low-intensity R-rich light from fluorescent lamps was relatively ineffective for both responses (Table II; Fig. 4; see also Evans et al., 1965; Evans and King, 1985). Furthermore, there is a similar distinction between R and FR light in Arabidopsis, where expression of one 20-oxidase increased within 3 h in a FR-rich LD or after a FR end-of-day exposure. Again, R exposure had no effect; the FR response was unequivocally linked to removal of an inhibition by active phytochrome B Pfr (Hisamatsu et al., 2005).

As detailed in the introduction, some of the critical pieces of evidence in the trail linking LD to \(GA_5\) and its florigenic action relate to \(GA_5\) transport to the shoot apex, to its concentration there, and to the extent of its increase. \(GA_5\) induces flowering when applied once to the leaf (Evans et al., 1990), and, based on studies using tetraduterated \(GA_5\), it is transported intact from the leaf to the shoot apex to give a dose-dependent flowering response (King et al., 2001). Furthermore, when supplied via agar to shoot apices excised from vegetative plants, \(GA_5\) causes flowering above a threshold concentration of about \(10^{-7}\) M and with saturation at a 2- to 5-fold greater dose (King et al., 1993). The endogenous concentration of bioactive \(GA_5\) plus \(GA_6\) in the shoot apex is already close to this threshold (\(10^{-7}\) M; King et al., 2003) and it increases 3- to 4-fold following a LD (King et al., 2001, 2003), which fits with the dose response for flowering of excised apices.

Up-regulation of a GA 20-oxidase provides a final piece of evidence for the role of \(GA\) in LD-regulated flowering of \(L. temulentum\). Both 20-oxidase expression (Fig. 4) and activity are increased in LD; the activity increase is shown best by the reciprocal changes in the content of \(GA_{19}\) and \(GA_{20}\). The 20-oxidase substrate, \(GA_{19}\), built up overnight in SD but dropped rapidly in LD. In an inverse manner, the content of its product, \(GA_{20}\), dropped in SD and increased in LD (Fig. 2). As a further link between LD, flowering, and 20-oxidase activity, \(GA_{19}\), when applied to the leaf, was highly florigenic, but only on exposure to LD (Table I).

Taken together, our findings meet the four key requirements for establishing the identity of florigens as outlined in the introduction. Clearly, increased 20-oxidase activity in LD leads to \(GA_5\) as a natural, transported florigenic compound (King and Evans, 2003).

The LD effects on 20-oxidase expression were not unexpected. There are many similar reports of FR-rich LD increasing 20-oxidase expression in leaves or petioles of spinach (Lee and Zeevaaart, 2002), Arabidopsis (Wu et al., 1996; Xu et al., 1997; Hisamatsu et al., 2005), potato (\(Solanum tuberosum\); Carrera et al., 1999), and \(L. perenne\) (MacMillan et al., 2005). Furthermore, in landmark studies, not just gene expression, but 20-oxidase activity itself, increased in spinach petioles exposed to LD (Gilmour et al., 1986).

The 2- and 3-oxidase genes of \(GA\) metabolism do not show LD up-regulation of their expression (Lee and Zeevaaart, 2002; compare with Lee and Zeevaaart, 2005) as we also suggest from our inhibitor studies (Fig. 3). Furthermore, in a comprehensive study with Arabidopsis, only one 20-oxidase was important for LD response based on expression assays with 10 \(GA\) biosynthetic genes (Hisamatsu et al., 2005).

An unexpected but perhaps monocot-specific finding in our many studies is the poor florigenic action of highly growth-active \(GA_1\), \(GA_3\), and \(GA_4\). Applying \(GA_1\) and \(GA_4\) to vegetative plants causes weak or no flowering, but the stems elongate excessively, as with \(GA_1\) (Evans et al., 1990; for review, see King and Evans, 2003). Thus, unlike \(GA_5\), these latter GAs (\(GA_1\), \(GA_3\), and \(GA_4\)) can be excused as florigens in \(L. temulentum\) and this fits with the delayed increase in leaf \(GA_1\) content in LD (Fig. 2), which occurred well after the expected time for completion of leaf synthesis and export of floral stimuli (compare with Fig. 1; Evans and Wardlaw, 1966). Also, although \(GA_1\) and \(GA_4\) content increases in LD leaves (Fig. 2; Gocal et al., 1999), neither \(GA_1\) was detectable in the shoot apex.
during early times of floral evocation, although GA<sub>5</sub> content increases at that time (King et al., 2001, 2003). We proposed previously (King et al., 2001; King and Evans, 2003) that differences in catabolism by 2-oxidases could explain the contrasts between the florigenic GA<sub>5</sub>, which promotes flowering but causes little stem elongation, and the growth-active GAs like GA<sub>3</sub>, GA<sub>1</sub>, and GA<sub>4</sub>. Unlike GA<sub>5</sub>, GA<sub>1</sub> and GA<sub>4</sub> would not be chemically protected against inactivation by 2-oxidases, hence, their poor florigenic activity. Our findings here do not address this question, but our ongoing studies (R. King and L. Evans, unpublished data) do indicate localized 2-oxidase expression just below the shoot apex, as shown for rice by Sakamoto et al. (2001). Given such 2-oxidase localization, it is not difficult to explain the floral effectiveness in <i>L. temulentum</i> of a 2-oxidase protected and weakly growth-active GA<sub>5</sub>. Based on our studies, the fact that GA action on elongation and flowering can be experimentally distinguished in rosette plants (Cleland and Zeevaart, 1970) or that GA<sub>6</sub> induces flowering of Arabidopsis (Xu et al., 1997; Gocal et al., 2001), but not of <i>L. temulentum</i>, cannot be used to argue against a role for GAs in flowering of this grass.

<i>LIFT</i> and Floral Signaling

To better focus our claim that GAs are floral signals in <i>L. temulentum</i>, it was essential to address the question of additional/alternative florigens. Therefore, we examined <i>LtCO</i> and <i>LIFT</i> expression, particularly because of the recent demonstration that, in SD, overexpression of <i>FT</i> in the leaf of Arabidopsis induces flowering and that FT mRNA or protein may be a transported floral signal (Huang et al., 2005).

Our evidence for <i>L. temulentum</i> here and for Arabidopsis (T. Hisamatsu, E. Goldschmidt, C. Blundell, and R. King, unpublished data) indicates separate roles for <i>FT</i> and GA in floral signaling. For example, when GA<sub>5</sub> was applied to plants in SD, it induced flowering without increasing <i>LIFT</i> expression (Table II). Thus, GA action does not involve <i>LIFT</i> and GA must independently regulate these two florigenic pathways. This conclusion also fits with our evidence that <i>LIFT</i> expression could increase independently of GA, as seen on exposure to a LD from low-intensity fluorescent light (Table II). Similarly, by inhibiting GA biosynthesis with PAC, we could restrict flowering in an incandescent LD, but there was still an increase in <i>LIFT</i> expression (Table II). However, despite such evidence of separate LD responses of <i>LIFT</i> and GA, we cannot resolve whether <i>LIFT</i> is a true floral signal or whether it facilitates production and transport of other signals. This latter possibility is especially relevant given the evidence that <i>FT</i> is expressed in vascular tissue of leaves (An et al., 2004) and that FT expression can increase without causing flowering of <i>L. temulentum</i> (Table II).

Overall, we have established that GAs act as one florigen in the grass <i>L. temulentum</i>. Other florigens could include <i>LIFT</i>, whose expression in the leaf increased dramatically after the increases in GA<sub>5</sub>. Dissimilarity between LD spectral effects on flowering and on <i>LIFT</i> expression, but parallels between LD effects on GA 20-oxidase and flowering, implies separate floral signals, but further analysis is required to establish how many signaling pathways regulate flowering of <i>L. temulentum</i>.

**MATERIALS AND METHODS**

**Plant Material and Growing Conditions**

Plants of <i>Lolium temulentum</i> strain Ceres were grown vegetatively in 8-h, SD photoperiods in sunlit controlled-environment cabinets as described previously (Evans et al., 1990). After 6 to 8 weeks, flowering was induced by exposure to a single LD (14 plants/treatment) involving a low photon flux density (10 μmol m<sup>-2</sup> s<sup>-1</sup>) from incandescent lamps for an 8-h or longer extension of the main 8-h daily SD light period (Evans and King, 1985). In one set of experiments, the same low-irradiance LD was also imposed from R-rich fluorescent lamps. Techniques for measuring flowering and for application of GAs to the leaf were as outlined previously (Evans et al., 1990). PAC ([2RS,3RS]-1-[4-chlorophenyl]-4,4-dimethyl-2-[1,2,4-triazol-1-yl]pentan-3-ol), an inhibitor of an early step of GA biosynthesis, was applied to the roots of plants (0.5 mg/plant). TNE (Primo) [4-(1-cyclopropyl-1-hydroxy)methyl-3,3-dioxygenocyclohexane-1-carboxylate] technical grade (Novartis) was applied to the leaf in 95% ethanol-water (v/v) at a dose of 25 μg/plant.

**GA Analysis**

Over the day of the LD exposure, batches of just fully expanded leaves were harvested and frozen in liquid nitrogen, generally at 4-h intervals. After they were pulverized and lyophilized, a 0.5-g aliquot was extracted overnight with stirring in cold 80% methanol-water (v/v). Debris was centrifuged down, and [17,17<sup>3</sup>H<sub>2</sub>]GAs were added to the supernatant to give an internal standard-protio GA ratio close to 1.0. After removal of the methanol, the residual aqueous phase was partitioned three times at pH 2.8 to an equal volume of ethyl acetate, with the extract dried under vacuum and then further purified through QAE Sephadex and C18 Sep-Pak and HPLC using a C18 column as outlined by Gocal et al. (1999).

Four groupings of the HPLC fractions were dried, methylated, and then silylated prior to high-resolution mass spectrometry using a gas chromatograph coupled to a JEOL JMS-SX/SX102A four-sector tandem mass spectrometer. The ions used for identification and quantitation of selected GAs were outlined previously (King et al., 2001), but with the addition of GA<sub>6</sub> ions. There were three replicate extractions from the original leaf material, and important differences in this experiment have been replicated in a second experiment (data not shown).

**Cloning and Expression of <i>L. temulentum</i> GA-Oxidases, CO, and FT**

Partial-length <i>Lt2ox1</i> and full-length <i>LtGA2ox1</i> cDNA clones were isolated on sequence relatedness to full-length <i>Lolium perenne</i> cDNA sequences, which were isolated using barley (<i>Hordeum vulgare</i>) cDNA clones for each oxidease. The <i>Lt2ox2</i> and <i>LtGA2ox1</i> cDNA sequences were almost identical to the <i>L. perenne</i> coding sequences (>98% nucleotide identity), and we used information from both species in designing primers for real-time PCR assays.

The functional characterization of expressed protein of <i>LpGA2ox1</i> was documented by MacMillan et al. (2005); it converts GA<sub>30</sub> to GA<sub>20</sub> via GA<sub>19</sub> and GA<sub>3</sub> (MacMillan et al., 2005). The protein of <i>Lp2ox2</i> was capable of 2-oxidation of GA<sub>20</sub> as a substrate when transiently expressed in a reticulocyte assay as described by Spielmeyer et al. (2004). GA<sub>3</sub> as a substrate was not metabolized, so additional 2- and probably 20-oxidases are likely to be present in Lolium as in other monocots and dicots (Hedden and Phillips, 2000; Spielmeyer et al., 2004); however, to date, we have not succeeded in cloning these genes from a Lolium cDNA library. There can be complete amino acid identity between...
genes from *L. perenne* and *L. temulentum*. This is also true for CO, although their nucleotide sequences diverge slightly (97% identity). For the 2- and 20-oxidases, our sequence comparison over 60 or more amino acids showed no divergence (data not shown). Thus, although based on an *L. perenne* sequence, our functional assays of GA metabolism genes should also be valid for *L. temulentum*. In further support of this claim, for grasses and, more broadly, for cereals, there can be considerable divergence in amino acid sequences of GA metabolism genes, but they still show identical functionality (see Hedden and Phillips, 2000; Spielmeyer et al., 2004, and refs. therein).

The nucleotide sequence of the *CONSTANS* gene of *L. temulentum* was highly homologous to that of *L. perenne* reported by Martin et al. (2004) and was isolated by PCR using the following primers: forward (5′-AGCAC CGATCTACGTAAGCTG-3′) and reverse (5′-TGGGCCCTCTGCTTCCAT GCA-3′); PCR was performed on a *L. temulentum* Uni-ZAP custom cDNA library, titer 1.8 × 10⁹ pfu/ml (Stratagene), made from mRNA isolated directly from inflorescence meristem and leaf tissue using DYNABEADS [oligo(dT25); Dynal], according to the manufacturer’s recommendations. PCR reactions were performed in 50-μL volume with 3.0 μM MgCl₂, 2-μL template, 1× PCR buffer, 0.4 μM of each primer, 10 μM dNTPs, and 2.5 units of *Taq* polymerase (Life Technologies), with an initial denaturing step of 95°C for 5 min followed by 35 cycles of 95°C for 45 s, 65°C for 30 s, and 72°C for 90 s.

*Lt20ox1* gene-specific primers were designed for real-time gene expression analysis based on a relatedness to a *L. perenne* genomic sequence. Amplicon size and sequence were as expected both for the primer pair crossing the second intron and for primers (data not shown) for a more 3′ region of coding sequence, which crossed no intron. Both sets of primers showed the same LD increase in *Lift* expression.

Quantitative Real-Time PCR Analysis of Expression

In repeated sampling every 2 h or longer over a day, a sample was taken of about 70 mg fresh weight of the basal 4 cm of the most recently expanded leaf blade from up to 10 plants. The sample was stored in liquid nitrogen until ground for RNA extraction. Total RNA was isolated using an RNeasy mini kit (Qiagen). For reverse transcription (RT)-PCR of *Lt20ox1* and *LtGA2ox1* in Australia and for some assays of *LCO*, 100 ng of total RNA were reverse transcribed and amplified using a SuperScript one-step RT-PCR kit (Invitrogen) with 1.2 μg of each primer. Routinely the RNA extract was treated on column with DNAase according to the manufacturer’s instructions. In addition, at the end of the extraction, the RNA was precipitated in 2× LiCl, resuspended in water, and freeze thawed three times. This step further reduced the possibility of DNA contamination. For RT-PCR assays performed in Denmark with *LCO* and a *GAPDH* internal standard, 1 μg of total RNA was reverse transcribed into cDNA with oligo(dT) (16-mer) using SuperScript II reverse transcriptase (Invitrogen).

Real-time quantitative PCR expression analysis of *LiCO* and the housekeeping gene *LpGAPDH* was performed with the primers *LICO*-forward (5′-TGGGCCCTCTGCTTCCATGCA-3′) and -reverse (5′-AGCACCGATCTACGTAAGCTG-3′); and *LpGAPDH*-forward (5′-AGCTTCTAGAAGCTGCGA-3′) and -reverse (5′-GACCGCCATATGAGTCG-3′); and *LiFT*-forward (5′-TGGGCCCTCTGCTTCCATGCA-3′) and -reverse (5′-GACGCGATCTACGTAAGCTG-3′). The *LiCO* primers spanned an intron and amplified a product of approximately 200 bp.

Other real-time expression analysis used intron-spanning primers as below: L12ox1-forward (5′-CTCTCTGTCACAATGGCGC-3′) and -reverse (5′-GAAATCCCCTGTAGGCGCA-3′); *LtGA2ox1*-forward (5′-TACATCTGCGCACCACCT-3′) and -reverse (5′-GACGGCGCAATGATGTTCC-3′); and *Lift*-forward (5′-CTCTCAGGCTCCTGATGTAAG-3′) and -reverse (5′-CCGAAACACTCGGACTGAG-3′). The standard for the *Li12ox1*, *LiGA2ox1*, and *LiFT*, and some *LiCO* assays was a commercially available mouse liver RNA (CLONTech) added to each plant RNA sample prior to the step of cDNA synthesis. The mouse primer pair was as follows: MRLt-forward (5′-ACAGCCAACACCAAC-3′) and -RMLt-reverse (5′-GCTACTGCTTGTCACCAACC-3′). The amplicon is 193 bp and, at the concentration of RNA added, its threshold cycle number for expression was in the range of the genes of interest. Equivalence of RNA in each sample was assessed spectrophotometrically by visual checking of rRNA bands on gels and, in some cases, by separate amplifications using *Lolium* 25S RNA primers as detailed by MacMillan et al. (2005).

For quantitative PCR assays, the reaction mixture volumes were 10 or 20 μL containing 3 μM MgCl₂, 1× PCR buffer, 0.5 μM primers, 0.25 μM dNTPs, and 0.5× SYBR Green with *Taq* polymerase (Life Technologies or Sigma-Alrich). Template cDNA was at a 5- to 25-fold dilution. Cycling conditions were as detailed by Klok et al. (2002) and Martin et al. (2004). As noted above, a mouse standard was used in many assays, but in one set of studies of expression of CO a *GAPDH* standard was used, as outlined by Martin et al. (2004). All quantitative PCR was performed on a Rotor-gene 2000 real-time cycler (Corbett Research). Product size after each run was checked on a gel, and any genomic DNA contamination was evident as a shift in band size because the primers spanned an intron. All assays included a no-template sample as a check for contaminants in the reaction. The quantitative PCR analysis was performed using the comparative quantification method with Rotogene 4.5 or 5.0 software (Corbett Research).

The GenBank accession numbers for genes newly described in this article are as follows: *LICO*, AY553297; *LiGA2ox1*, DQ324114; and *LiFT*, DQ309592.

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