Long-Day Induction of Flowering in *Lolium temulentum* Involves Sequential Increases in Specific Gibberellins at the Shoot Apex

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One challenge for plant biology has been to identify floral stimuli at the shoot apex. Using sensitive and specific gas chromatography-mass spectrometry techniques, we have followed changes in gibberellins (GAs) at the shoot apex during long day (LD)-regulated induction of flowering in the grass *Lolium temulentum*. Two separate roles of GAs in flowering are indicated. First, within 8 h of an inductive LD, i.e. at the time of floral evocation, the GA$_4$ content of the shoot apex doubled to about 120 ng g$^{-1}$ dry weight. The concentration of applied GA$_5$ required for floral induction of excised apices (R.W. King, C. Blundell, L.T. Evans [1993] Aust J Plant Physiol 20: 337–348) was similar to that in the shoot apex. Leaf-applied [${}^3$H]$_5$GA$_5$ was transported intact from the leaf to the shoot apex, flowering being proportional to the amount of GA$_5$ imported. Thus, GA$_5$ could be part of the LD stimulus for floral evocation of *L. temulentum* or, alternatively, its increase at the shoot apex could follow import of a primary floral stimulus. Later, during inflorescence differentiation and especially after exposure to additional LD, a second GA action was apparent. The content of GA$_1$ and GA$_4$ in the apex increased greatly, whereas GA$_5$ decreased by up to 75%. GA$_4$ applied during inflorescence differentiation strongly promoted flowering and stem elongation, whereas it was ineffective for earlier floral evocation although it caused stem growth at all times of application. Thus, we conclude that GA$_1$ and GA$_4$ are secondary, late-acting LD stimuli for inflorescence differentiation in *L. temulentum*.

Plants of *Lolium temulentum* remain vegetative when grown in short days (SD), but flower after exposure of their leaves to a single long day (LD). The leaf gibberelin (GA) content increases in LD (Gocal et al., 1999) and applied GAs can cause flowering in noninductive SD (Evans, 1964; Pharis et al., 1987; Evans et al., 1990). Thus, GAs mimic LD responses and they could be a transmissible endogenous floral stimulus in this LD plant. A number of other LD plants but not all (for summary, see Metzger, 1995) flower in response to GA, and recent genetic and molecular studies with Arabidopsis support such a role for endogenous GAs in flowering in LD (Wilson et al., 1992; Weigel and Nilsson, 1995; Bláñquez et al., 1997). Furthermore, where there are effects of LD exposure on stem elongation there are clear increases in the GA content of leaves, petioles, and shoot tips (Talon and Zeevaart, 1990; Talon et al., 1991; Zeevaart et al., 1993).

Inflorescence initiation in *L. temulentum* after one LD precedes any acceleration of stem elongation. Therefore, if GAs were to play a role endogenously in floral evocation, they should have little effect on stem elongation. From application studies, we previously identified a number of GAs, including GA$_5$, that meet this criterion of inducing flowering but with little or no effect on stem elongation (Evans et al., 1990, 1994a, 1994b). However, in a broad context, three lines of evidence should be obtained to confirm a role for GAs in floral evocation. First, exposure to LD should increase the levels of florally active endogenous GAs in the shoot apex. Second, there should be evidence that inhibitors of GA biosynthesis block flowering. Third, there should be molecular/biochemical linkages between GA and floral initiation at the apex. Elsewhere, we and others have addressed the latter two issues (Evans, 1969; Evans et al., 1994a; Weigel and Nilsson, 1995; Bláñquez et al., 1997; Gocal et al., 1999).

Considering the requirement for change in GA content, not only did the endogenous content of several GAs increase in leaves of *L. temulentum* soon after exposure to two or more florally inductive LD (Gocal et al., 1999), but bioassayable GA-like activity at the shoot apex increased within 8 h of the end of the LD (Pharis et al., 1987), i.e. at the time when floral evocation occurs (McDaniel et al., 1991). Furthermore,
GAs do act at the shoot apex because plants of *L. temulentum* flower in SD when GA$_3$ is applied either to the leaf, near to the shoot apex of intact plants (Evans, 1964), or to cultured apices excised from plants in SD (King et al., 1993). The latter study was particularly persuasive because, without added GA, the excised shoot apex continued to grow vegetatively and only formed leaves.

Here, we examine changes in the spectrum of GAs and in their content in the shoot apex of *L. temulentum* following exposure of the leaf to florigerative LD. High-resolution (HR)-mass spectrometry (MS) and selected reaction monitoring (SRM)-MS provided the specificity and high sensitivity required for measurements of GAs (Moritz and Olsen, 1995). These MS methods allowed various precursors, active GAs, and their catabolites to be measured simultaneously. The high sensitivity of MS meant that femtogram amounts of individual GAs could be detected in batches of 40 shoot apices (about 200 µg total dry weight). The selectivity of HR-MS and SRM-MS coupled with the use of deuterated GAs as internal standards allowed analyses with minimal purification and, thereby, minimal losses.

RESULTS

Shoot Apex GAs

Due to the low shoot apex tissue amounts, the identification of GAs was based on HR-selected ion monitoring (SIM). Identity was certain when both the ratio of the ions (at a resolution of 10,000) and the retention time matched the standard. The GAs identified included GAs 1, 3, 4, 5, 6, 8, 9, 19, 20, 24, and 34, many of these having also been identified by full-scan MS in leaves of *L. temulentum* (Gocal et al., 1999). In general, on a dry weight basis, the shoot apex contained up to 50-fold more GA than the leaf.

It was not possible, routinely, to analyze all the GAs we had identified both because of restrictions on data collection (e.g. GA$_3$ and GA$_6$) and, sometimes, because of the presence of contaminating ions (e.g. GA$_{20}$). As a consequence, we quantified only the C-13 hydroxylated GAs 1, 5, and 19 and the non-C-13 hydroxylated GAs 4, 9, 24, and 34. Aside from GA$_5$, these GAs are metabolites of two parallel biosynthetic pathways, with several possible cross-links between them, GA$_5$ being a potential intermediate in the conversion of GA$_{20}$ to GA$_3$ (Hedden and Kamiya, 1997).

In leaves of *L. temulentum*, we previously identified GA$_3$ and GA$_{20}$ (Gocal et al., 1999), but GA$_5$ was not detected with certainty because we only obtained a small peak and a match to three fragment ions (A. Poole, unpublished data). GA$_1$ and GA$_4$ were detected in leaves of *L. temulentum* and especially after exposure to repeated LD (Gocal et al., 1999), but they were hardly detectable in vegetative or pre-inflorescence-stage apices, their content increasing later.

Of the seven GAs analyzed in these vegetative apices, GA$_{10}$ was the most abundant and GA$_1$ and GA$_4$ were the least abundant. As an indication of reproducibility between experiments, for SD shoot apices collected over four matched experiments, the average content of GA$_{10}$ was 96.3 ± 2.0 ng g$^{-1}$ dry weight ($n = 15$), and by visual inspection (Fig. 1) there appears to be no diurnal trend.

Early LD-Induced Changes in GAs at the Shoot Apex

There was an early increase in the GA$_5$ content of the shoot apex following exposure to florigerative LDs (Fig. 1). By the end of the 8-h high-light period after the 1st LD, there was up to a 2-fold increase in apex GA$_5$ content (91 vs 38 ng g$^{-1}$ dry weight, experiment Lt447; 126 vs 74 ng g$^{-1}$ dry weight, experiment Lt454), but with a smaller increase in a further experiment (Fig. 1, Lt446).

Apex dry weight was essentially unchanged over this 1st d (see later, Fig. 2) and, based on estimates of water content of the *L. temulentum* apex (Rijven and Evans, 1967), the endogenous GA$_5$ concentration in the shoot apex after one LD reached a maximum of

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Diurnal changes in three separate experiments in the GA$_5$ and GA$_{10}$ content (ng g$^{-1}$ dry weight [dwt]) of the shoot apex of *L. temulentum* of vegetative plants in SD (white symbols) and for plants exposed to 1 or 2 florigerative LD (black symbols). The LD low-irradiance day length extension is shown as a stippled bar, darkness as a black bar, and daylight as a white bar.
about 6 \times 10^{-8} \text{ M}. This concentration approaches the threshold of 10^{-7} \text{ M} required in the medium for GA_{5} to induce inflorescence initiation in shoot apices of L. temulentum excised from plants in noninductive SD (King et al., 1993).

For vegetative SD apices, there was some diurnal increase in GA_{5} content in one experiment (Lt454) but not in the other two (Fig. 1). In contrast to GA_{5}, the content of GA_{19} in the same apices showed relatively little variation, with no obvious distinction between SD and LD treatments (Fig. 1). GA_{24} content was relatively unchanged or increased by between 30% and 90%, little or no GA_{1} or GA_{4} could be detected, and GA_{9} content dropped 3-fold (data not shown).

Longer term trends in GA content are shown in Figure 2 for daily samples taken at the start of each day (8:30 AM) up to the stage of floret formation at 18 d. As the inflorescence differentiated, GA content per gram dry weight declined (Fig. 2a), but for any one GA the total content per apex remained high or increased up to 10 d (Fig. 2b). Due to increased apex weight associated with inflorescence differentiation (40-fold dry weight increase by the last harvest in Fig. 2b), the late decline in GA content per gram dry weight shown in Figure 2a therefore represents, in part, a late “dilution” of the GAs. It is noteworthy that apex dry weight was constant over the first 3 d after LD exposure (Fig. 2b) and did not change diurnally (data not shown). Thus, the early changes in Figure 1 in GA_{5} content (“concentration” in Fig. 2a or “amount” in Fig. 2b) relate solely to its metabolism.

To confirm the evidence in Figure 2a of a fall in GA_{5} content by 4 or 5 d after the start of the LD, three experiments were carried out with daily harvests at the diurnal maxima at 4:30 PM. All confirmed the findings in Figure 2 in that there was a clear doubling in the content of GA_{5} by 56 h from the start of the LD followed by a fall (data not shown).

Changes at Later Times in GAs at the Shoot Apex

Three days after the start of two LD, the content of GA_{1} and GA_{4} increased, whereas that of GA_{3} and GA_{19} began to decrease 1 d later (Fig. 2). These findings were confirmed in a further experiment (not shown).

Although inflorescence initiation and flowering of L. temulentum results from exposure to only 1 LD, repeated cycles accelerate floral development, as shown in Figure 3. This enhancement was evident soon after the LD exposure and was matched by the earlier appearance of floral organs (Fig. 3) and by a faster increase in apex dry weight (right-side column in Fig. 3). Plants kept in SD remained vegetative with a final apex length of less than 1.0 mm. Such results confirm the increased flowering response with extra LD (Evans, 1960) and the close relation between shoot apex length and mass previously reported (Rijven and Evans, 1967), which we have used to establish the weight of our batches of apices harvested for GA analysis (see “Materials and Methods”).

In parallel with enhanced flowering response from extra LD (Fig. 3), increasing the number of LD cycles resulted in large changes in the GA content of the shoot apex as shown in Figure 4. Although a single LD exposure led to a small increase in GA_{1} from an initially non-detectable level in the vegetative shoot...
apex, this occurred only after 10 d. Increasing the number of LD cycles greatly increased the content of GA_{1}, GA_{4}, and GA_{9} in the apex and these changes occurred much earlier (Figs. 2 and 4). Such increases in GA content with multiple LD cycles match those we previously detected in the leaf for GA_{1}, GA_{4}, and GA_{9} (Gocal et al., 1999). In contrast, multiple LD cycles led to dramatic decreases in the GA_{19}, GA_{24}, and GA_{5} content of the apex (Fig. 4). The changes in GAs potentially reflect precursor/product conversion of GA_{19} to GA_{2}, and of GA_{24} to GA_{9} and then to GA_{4}. Where complete data were available from three replicate experiments, the content of GA_{24} had changed little after 2 LD (1.6-fold to 39.0 ± 4.3 ng g\(^{-1}\) dry weight), but GA_{9} had increased 7-fold to 24.3 ± 7.0 ng g\(^{-1}\) dry weight, and GA_{4} increased 23-fold to 9.3 ± 5.0 ng g\(^{-1}\) dry weight, whereas GA_{34} showed little change (2.3-fold increase to 25.9 ± 8.1 ng g\(^{-1}\) dry weight).

Changes in GA content due to LD exposure may indicate regulation by day length at two steps in GA biosynthesis. The increases in GA_{9} (7-fold) following 2 LD could result from increased activity of a GA 20-oxidase enzyme. The even greater increase in GA_{4} (23-fold) may indicate an added activation of biosynthesis involving a 3β-hydroxylase enzyme capable of converting GA_{9} to GA_{4}. The activity of the 2β-hydroxylase that converts GA_{4} to GA_{34} may not have changed.

### Response to Applied GAs

The physiological relevance for flowering of the early increase in endogenous GA_{5} at the apex and of the later increase in GA_{5} was examined by applying these GAs to L. temulentum plants induced to flower by one LD. Flowering was promoted by a GA_{5} application at the time of the LD (Fig. 5), which supports a physiological role for the early rise at the apex in the content of GA_{5} (Fig. 1). In sharp contrast, applications of GA_{4} at the time of the LD were ineffective for flowering. Four days later, however, at the time when endogenous GA_{4} levels had increased at the apex (Figs. 2 and 4), GA_{4} became florally promotive. There was apparently no restriction on GA uptake and transport to the apex because at all times of application both GA_{4} and GA_{5} caused growth of the stem immediately below the apex (data not shown).

To establish the timing of GA_{5} export from the leaf blade and of import of the intact (unmetabolized) molecule into the shoot apex, we applied either GA_{5} or [\(^{2}\)H\(_{4}\)]GA_{5} to the leaf. Varying the time of removal of the GA_{5}-treated leaf blade showed that for flowering, export from the treated blade had begun by 30 h from the start of the LD and approached completion by 48 h (Fig. 6). A matching export profile was observed when stem length was measured on the same plants (not shown). In parallel treatments but involving [\(^{2}\)H\(_{4}\)]GA_{5} applied to the leaf blade, its arrival at the shoot apex reached a peak at 48 h (Fig. 6). We refer to this peak time because assay sensitivity becomes a problem in defining the threshold for [\(^{2}\)H\(_{4}\)]GA_{5} arrival at the apex. Nevertheless, the match in time between apex arrival of [\(^{2}\)H\(_{4}\)]GA_{5} and leaf export of GA_{5} indicates probable physiological relevance. We detected no [\(^{2}\)H\(_{4}\)]GA_{3} at the apex although it is a logical metabolite of [\(^{2}\)H\(_{4}\)]GA_{5}. Thus, when applied, GA_{5} can be transported from the leaf to the apex within 1 to 2 d and without chemical alteration.

The kinetics of [\(^{2}\)H\(_{4}\)]GA_{5} import by the apex cannot be used to determine the relationship between applied GA_{5} and flowering response. However, we could ascertain if there was such a relationship by varying the amount of [\(^{2}\)H\(_{4}\)]GA_{5} applied to the leaf. As shown in Table I, based on the one study we performed, there was a reasonable correlation between the amount of [\(^{2}\)H\(_{4}\)]GA_{5} at the shoot apex after 48 h and the flowering response after 21 d (\(r = 0.92; n = 4\)). Given the lesser amount of endogenous GA_{5} than [\(^{2}\)H\(_{4}\)]GA_{5} in the apex, there was apparently

### Table I. Effect on apex GA content and on flowering of various doses of [\(^{2}\)H\(_{4}\)]GA_{5} applied once to the leaf blade 6 h after the start of a LD exposure

| Dose (μg) | [\(^{2}\)H\(_{4}\)]GA_{5} Detected at the Apex (μg) | Flowering Response (Shoot Apex Length) (mm) |
|-----------|---------------------------------------------|---------------------------------------------|
| 0         | 0                                           | 2.69 ± 0.07                                 |
| 1         | 2.3                                         | 2.73 ± 0.16                                 |
| 5         | 6.5                                         | 2.98 ± 0.21                                 |
| 25        | 9.5                                         | 3.85 ± 0.43                                 |
a distinction between the physiologically active and total pools of GA5 and \(^{2}\text{H}_4\)GA5.

A separate issue is that only 9.5 pg of the 25 \(\mu\)g of \(^{2}\text{H}_4\)GA5 applied to the leaf had reached the shoot apex 48 h later (Table I, Fig. 6), i.e. less than one part in 1 million applied. Such limited import agrees with our earlier studies in which comparable low proportions of leaf-applied radiolabeled sulfate or phosphate reached the shoot apex (Evans and Rijven, 1967). Such limited import no doubt reflects the minute size of the shoot apex as well as potential for compartmentalization and metabolism of a compound during its uptake and transport.

**DISCUSSION**

GAs as LD Floral Stimuli

We have detected a number of GAs in the minute shoot apex (about 5 \(\mu\)g dry weight) of the grass *L. temulentum* and established that a florally inductive
LD leads to an early doubling in GA5 levels and, several days later, to large increases in GA1 and GA4. Using highly sensitive and specific MS, we detected GAs at sub-picogram levels. Furthermore, our repeated checks of reliability of the analyses by spiking samples with the GA of interest, and our inclusion of deuterated internal standards for each GA, made these assays specific and reproducible across independent experiments. Previous GC-MS-based studies have not focused on the shoot apex alone, nor has it been considered that different GAs may participate at different stages of floral development. The most relevant report, that of Talon et al. (1991), was focused on the response of subapical tissue that comprised the major portion of their shoot tip samples. Our samples excluded nonapical tissue and were “apically enriched” in that the floral apex of this grass includes up to 24 spikelet sites.

GAs Associated with Floral Evocation

Our previous studies have provided considerable detail on the timing of floral evocation at the shoot apex of L. temulentum. After reaching the critical day length of approximately 16 h, the LD floral stimulus in L. temulentum is translocated down the leaf blade and sheath at 1 to 2 cm h−1 (Evans and Wardlaw, 1966) to reach the shoot apex early on the day after the LD. Experiments with shoot apices excised from such floral plants at various times (McDaniel et al., 1991; King et al., 1993) indicate that floral evocation is virtually complete by the end of that day.

Thus, the close matching in the timing of increase in GA5 that we see at the shoot apex makes it a strong candidate for the LD (photoperiodic) stimulus, a claim we base on the following grounds: (a) During the light period following the LD, the endogenous GA5 doubled at the apex; (b) the GA5 concentration was close to that required for flowering in vitro; (c) the timing of the doubling in endogenous GA5 coincides with the timing of floral evocation; (d) applied GA5 was transported intact from the leaf to the shoot apex (Fig. 6), where it then regulated flowering. This GA5 was imported in physiologically meaningful amounts as increasing leaf application led to parallel increases in both the amount of GA5 transported to the apex and in flowering (Table I); and (e) applied GA5 acts without causing extensive stem elongation, a characteristic of the response to a single LD but not of the response to multiple LD cycles or to other candidate GAs such as GA1, GA3, or GA4 (Evans et al., 1990, 1994a, 1994b).

Considering (a) and (b) above, a doubling in GA5 content of the shoot apex would be biologically important because floral response increased 3-fold with a 3-fold increase in the GA5 dose supplied to excised shoot apices cultured in vitro (King et al., 1993). We have not measured the GA content of cultured apices, but the shoot apex concentration of endogenous GA5 reached $6 \times 10^{-8}$ M, a value close to the $10^{-7}$ M GA5 threshold required for flowering of cultured apices (King et al., 1993). In addition, other endogenous GAs are likely contributors to bioactivity and our preliminary studies indicate a role for GA6. The latter GA is florally effective, causes flowering with little or no stem elongation, and the shoot apex con-

![Figure 5](image1.png)

**Figure 5.** Effect on flowering of a single application to the leaf of 25 µg of GA4 or GA5. The earliest treatment was 6 h after the start of the main light period prior to the single LD extension. Flowering was determined at 21 d and the values are the mean and se.

![Figure 6](image2.png)

**Figure 6.** Timing of GA5 export from a treated leaf to the shoot apex as shown either by increase in the floral response of the shoot apex with increasing time until removal of the GA-treated leaf blade, or by arrival at the shoot apex of [H4]GA5 following its application to the leaf. In both treatments, a dose of 25 µg of GA was applied to the leaf blade 6 h after the start of the main light period prior to the single LD extension. Values for floral apex length are the means and se. Plants not treated with GA5 flowered to the same extent as those with the treated leaf removed at the start. There was only a trace of [H4]GA5 detected at the apex after 24 h, but it is shown in brackets because there was uncertainty about quantitation of the gas chromatography (GC)-MS peak for the [H4]GA5 internal standard.
tent of GA\textsubscript{6} also doubles at the time of floral evocation (R.W. King, L.N. Mander, T. Moritz, R.P. Pharis, and L.T. Evans, unpublished data). As an aside, roles for GA\textsubscript{1}, GA\textsubscript{3}, or GA\textsubscript{4} in floral evocation can be excluded based on their inherent growth activity and the poor floral activity of GA\textsubscript{1} and GA\textsubscript{4} (Evans et al., 1990, 1994a, 1994b). Furthermore, for these latter two GAs, inhibitor studies also excluded any early role at the time of the LD. An inhibitor capable of blocking the 3\beta-hydroxylase enzyme did not inhibit flowering although stem elongation was inhibited (Evans et al., 1994a).

Although exogenously supplied GA\textsubscript{3} is transported from the leaf to the shoot apex of \textit{L. temulentum}, it remains to be shown that the leaf is the source of GA\textsubscript{3} for the shoot apex because detection of GA\textsubscript{3} in leaf extracts of \textit{L. temulentum} has proven technically difficult. The shoot apex itself could be the site of GA\textsubscript{3} formation via a single step conversion of GA\textsubscript{20} to GA\textsubscript{3} (Hedden and Kamiya, 1997). GA\textsubscript{3} being an endogenous transported form of GA as suggested for pea (\textit{Pisum sativum}; Proebsting et al., 1992). In support of this scenario, the content of GA\textsubscript{20} in leaves of \textit{L. temulentum} does increase with exposure to 2 LD (Gocal et al., 1999). However, when GA\textsubscript{20} was applied to the leaf of vegetative plants, it had little or no floral activity compared with GA\textsubscript{3} (Evans et al., 1990). Furthermore, the recent report of Sakamoto et al. (2001) of high levels of expression of a GA 2-oxidase gene in procambium at the base of the vegetative shoot apex of rice (\textit{Oryza sativa}), but not in florally evoked apices, suggests that prior to floral evocation there will be rapid catabolism of any GA\textsubscript{20} arriving at the shoot apex. Given such uncertainties, GA\textsubscript{3} is either a primary LD floral stimulus in \textit{L. temulentum} or its content increases directly at the shoot apex in response to a transported stimulus. Either way, it is GA\textsubscript{3} at the shoot apex that our findings suggest is important for LD-induced floral evocation.

GAs Associated with Inflorescence Development

For the first time, our study shows that GAs are important at the beginning of inflorescence differentiation. This claim is based on our evidence of a dramatic increase at inflorescence differentiation in the shoot apex content of the 3\beta-hydroxylated GA\textsubscript{1} and GA\textsubscript{4}. Furthermore, at this time applied GA\textsubscript{3} first becomes florally active (Fig. 5), being inactive for earlier floral evocation (Pharis et al., 1987; Evans et al., 1990). In addition, the involvement of GA\textsubscript{3} and GA\textsubscript{4} in inflorescence initiation is supported by our earlier studies with inhibitors of GA\textsubscript{20} 3\beta-hydroxylase. We found (Evans et al., 1994a) that several days after the inductive LD, flowering and stem elongation were both inhibited by application of Trinexapac-ethyl (ethyl-[3-oxido-4-cyclopropionyl-5-oxo]oxo-3-cyclohexenecarboxylate). With \textit{L. temulentum}, such acycliclohexanediones block the 3\beta-hydroxylation of GA\textsubscript{20} to GA\textsubscript{1} and cause GA\textsubscript{20} to accumulate (Junttila et al., 1997).

For both the \textit{L. temulentum} shoot apex (this study) and the leaf (Gocal et al., 1999), exposure to multiple LD causes increases in GA\textsubscript{1} and GA\textsubscript{4} levels. By contrast, despite the increase in the shoot apex content of GA\textsubscript{3} after a single LD, its content in the apex declining with exposure to multiple LD. Why the spectrum of GAs at the apex shifts over time is a matter for speculation but, for GA\textsubscript{1} and GA\textsubscript{4} which are readily 2\beta-hydroxylated, a reduced expression of GA 2-oxidase activity at inflorescence differentiation, as reported in rice (Sakamoto et al., 2001), would allow an increase in apical GA\textsubscript{1} and GA\textsubscript{4} at this time. In a converse manner, in the vegetative shoot apex and at floral evocation these GAs would be degraded but there could be a buildup of GA\textsubscript{3} because of its lesser susceptibility to 2-oxidase activity due to its ring A C2-3 double bond.

Of the two actions of GAs proposed to control flowering of \textit{L. temulentum}, it is the late changes in GA\textsubscript{1} and GA\textsubscript{4} at floral differentiation that are most similar to GA changes that occur just before the onset of rapid stem elongation (bolting) of dicots where GA\textsubscript{1} levels can increase dramatically in shoots and petioles (Talon and Zeevaart, 1990; Talon et al., 1991; Zeevaart et al., 1993; Zanewich and Rood, 1995; for review, see Metzger, 1995). However, unlike dicots where GA biosynthesis inhibitors may block bolting but not flowering (Cleland and Zeevaart, 1970), with \textit{L. temulentum} GA biosynthesis inhibitors inhibit stem elongation but may either promote or inhibit flowering (Evans et al., 1994a). Given our evidence of complexity not only in the spectrum of active GAs, but in their timing of action and in the response of flowering to inhibitors of GA biosynthesis, it would be interesting to examine for dicots early shoot apex changes during flowering and for GAs other than GA\textsubscript{1} or GA\textsubscript{4}.

Overall, despite recent speculative claims to the contrary (Colasanti and Sundersan, 2000; Samach and Coupland, 2000), our studies show that, for \textit{L. temulentum} at least, GAs may serve as LD flowering signals. Also, our findings provide a novel and more dynamic view than has been considered previously to explain the role of GAs in floral evocation and inflorescence differentiation.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Plants of \textit{Lolium temulentum} strain Ceres were grown vegetatively in 8-h SDs in sunlit controlled-environment cabinets as described previously (Evans et al., 1990). Floral induction by LD involved one or more exposures to 16-h extensions of the 8-h day using light from incandescent lamps at a low photon flux density (10 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1}). Three weeks later, the flowering response was scored for both stage of morphogenesis and apex length, these two measures being closely related (Evans et al., 1990). At this time stem length was also measured. Timing from the LD
is taken from the start of the 8-h main photoperiod of the first LD.

All GA applications were made to the uppermost expanded leaf blade in a 10-μL drop of 95% (v/v) ethanol: water, the controls being treated with 95% (v/v) ethanol: water. The GAs were pure samples supplied by Prof. Lewis Mander (Research School of Chemistry, Australian National University, Canberra). In one study, to assess export, the GA$_3$-treated leaf blade was cut off at various times after application. Some metabolism/transport studies with GA$_5$, utilized a sample of [15,15,17-2H$_3$] GA$_3$, and, on GC-MS, this form was readily distinguished from a deuterio [17,17-2H$_2$] GA$_5$ internal standard as well as from GA$_3$, but all were matched for relevant ions and their abundance.

GA Analysis

The first four experiments were carried out at 1-month intervals as duplicate pairs, and 2 years later a fifth set of harvests was made. Shoot apices were harvested as batches of 40 for each sampling time/treatment and, over all experiments, 4,320 apices were used. At dissection, they were immediately frozen in a microcentrifuge tube and each batch of 40 apices was freeze dried. To account for differences in apex weight especially over time, we derived apex dry weights from measured apex lengths based on a linear relationship that exists after log transformation of length vs. dry weight (r$^2$ = 0.988; n = 14). The flowering response to LD exposure was similar for all experiments, and particularly for each of the paired duplicates in the four initial experiments (data not shown). Plants held in SD remained vegetative.

For GA assay, homogenized tissue was extracted with 500 μL of 80% (v/v) aqueous MeOH at 4°C, with 30 pg of [17,17-2H$_3$] GAs added as internal standards to give a deuterio:proton ratio close to 1.0. The GAs were partitioned 3× at pH 2.8 into an equal volume of ethyl acetate (EtOAc). The extract was applied in EtOAc to a pre-equilibrated 100-mg aminopropyl Isolute cartridge (Sorbent AB, Västra Frölunda, Sweden). The cartridge was washed with 3 mL of EtOAc, then eluted with 5 mL of 0.2 M formic acid that was run directly onto a pre-equilibrated 100-mg C$_18$ Isolute cartridge (Sorbert AB) and the GAs were then eluted with 2 mL of 80% (v/v) MeOH.

The samples were methylated with ethereal diazomethane and, after evaporation, dissolved in MeOH and loaded onto a 100-mg Isolute aminopropyl cartridge. The methylated GAs were eluted with 3 mL MeOH, which was reduced to dryness and trimethylsilylated in 10 μL pyridine and 10 μL N-methyl-N-trimethylsilyl trifluoroacetamide at 70°C for 30 min. Samples were injected in dichloromethane in the splitless mode into a 5890 GC (Hewlett-Packard, Sydney) equipped with a 30-m × 0.25-mm i.d. fused silica capillary column with a chemically bonded 0.25 mm DB-5 MS stationary phase (J&W Scientific, Folsom, CA). The injector temperature was 270°C. The column temperature was held at 50°C for 2 min, then increased by 20°C min$^{-1}$ to 220°C, and by 4°C min$^{-1}$ to 270°C. The column effluent was introduced into the ion source of a JEOL JMS-SX/SX102A four-sector tandem mass spectrometer of B$_2$E$_1$B$_2$E$_2$ geometry (JEOL, Tokyo). The interface and the ion source temperatures were 270°C and 250°C, respectively. Ions were generated with 70 eV at an ionization current of 600 μA. Measurements involving HR-SIM were performed with an accelerating voltage switching from 10 KV and a resolution of 7,000 to 10,000.

Perfluorokerosene was used as a reference compound after choosing a suitable lock mass. The dwell time was 50 ms. For each GA, the ions recorded for HR-MS and SRM-MS are shown in Table II along with their deuterated analogs. In the SRM mode, the acceleration voltage was 10 KV and the precursor ions were selected by magnetic switching. The daughter ions formed in the first field-free switching. The daughter ions formed in the first field-free region were detected by switching the magnetic field and the electrostatic field simultaneously. The dwell time was 100 ms, and specific reactions for the different GAs were recorded according to Moritz and Olsen (1995).

Reliability of analysis was checked on several occasions by adding GAs-MeTMS or [17,17-2H$_3$]GAs-MeTMS to previously analyzed samples, and then re-analyzing the samples. Increased intensity of the GA peak at the right retention time, and lack of chromatography changes at the GA peak, indicated reliability of the analysis.

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**Table II. Ions detected for quantification of specific GAs and the deuterated analogues used as internal standards**

| GA   | Ions HR-SIM | SRM   |
|------|-------------|-------|
| GA$_1$ | 506.2520; 448.2101 | 506–448 |
| [17H$_2$]-GA$_1$ | 508.2645; 450.2227 | 506–207 |
| GA$_2$ | 504.2364; 370.1961 | 504–370 |
| [17H$_2$]-GA$_2$ | 506.2489; 372.2086 | 506–372 |
| GA$_3$ | 284.1776; 224.1565 | 418–390 |
| [17H$_2$]-GA$_3$ | 286.1902; 226.1691 | 420–392 |
| GA$_4$ | 416.2020; 299.1645 | 416–299 |
| [17H$_2$]-GA$_4$ | 418.2145; 301.1770 | 418–301 |
| GA$_5$ | 432.1968; 313.1417 | 432–303 |
| [17H$_2$]-GA$_5$ | 434.2091; 315.1540 | 432–207 |
| GA$_6$ | 298.1570; 270.1620 | 298–270 |
| [17H$_2$]-GA$_6$ | 300.1690; 272.1740 | 300–272 |
| GA$_8$ | 438.2689; 375.2355 | 343–375 |
| [17H$_2$]-GA$_8$ | 436.2614; 377.2481 | 436–377 |
| GA$_9$ | 482.1576; 420.2301 | 482–377 |
| [17H$_2$]-GA$_9$ | 484.1602; 422.2428 | 484–378 |
| GA$_10$ | 314.1789; 286.1902 | 314–286 |
| [17H$_2$]-GA$_10$ | 316.1914; 288.2055 | 316–288 |
| GA$_14$ | 506.2520; 289.1440 | 506–289 |
| [17H$_2$]-GA$_14$ | 508.2645; 291.1565 | 508–291 |
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