Long-Day Induction of Flowering in *Lolium temulentum* Involves Sequential Increases in Specific Gibberellins at the Shoot Apex[1]

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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_5 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 [$^2H_4$] 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 gibberellin (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; Blásquez 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; Blásquez 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,

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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 florally inductive 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 preinflorescence-stage apices, their content increasing later.

Of the seven GAs analyzed in these vegetative apices, GA_{19} 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_{19} was 96.3 $\pm$ 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 florally inductive 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

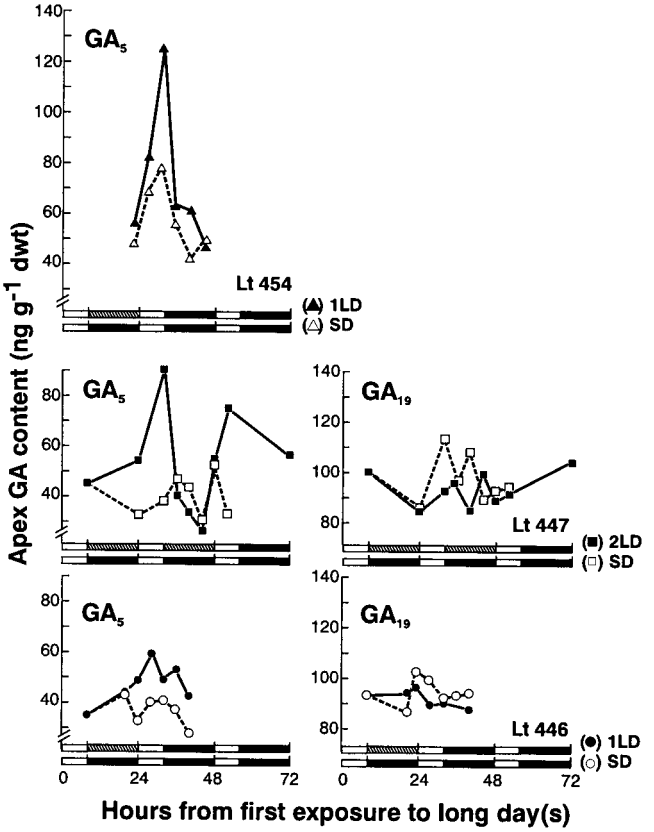

Figure 1. Diurnal changes in three separate experiments in the GA_5 and GA_{19} content (ng g^{-1} dry weight [dwt]) of the shoot apex of *L. temulentum* of vegetative plants in SD (white symbols) and for plants exposed to 1 or 2 florally inductive LD (black symbols). The LD low-irradiance day length extension is shown as a stippled bar, darkness as a black bar, and daylight as a white bar.

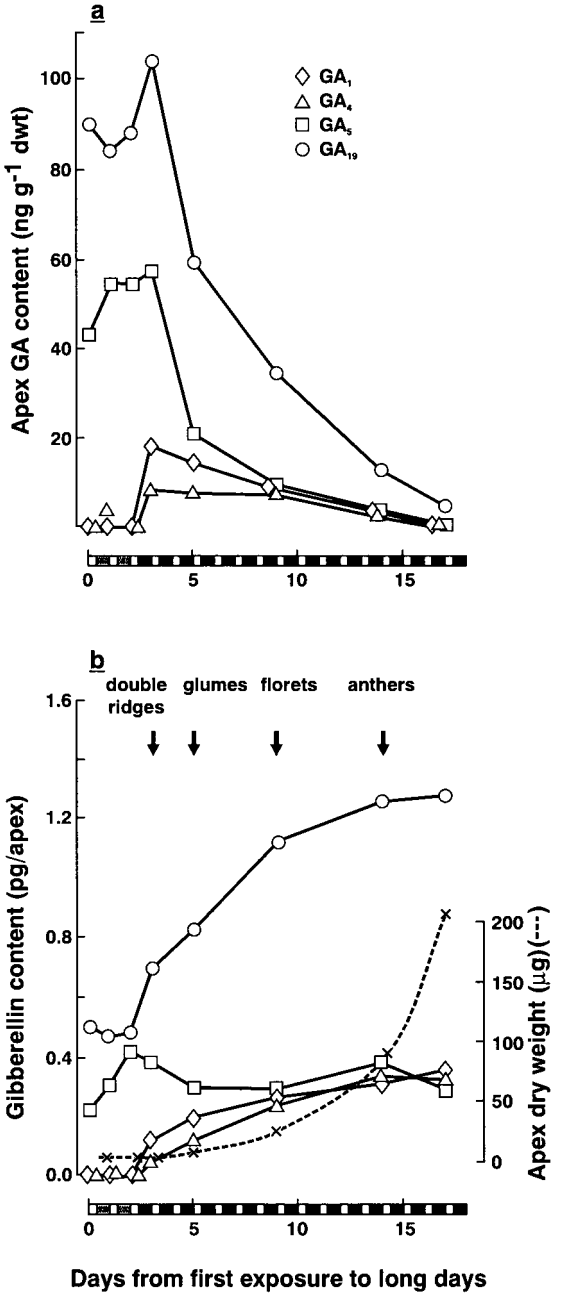

Figure 2. Changes during inflorescence development in the content of various GAs in the shoot apex of *L. temulentum* exposed to 2 LD (Expt. Lt 447). Harvests were at the start (8:30 AM) of each daily 8-h sunlight exposure. a, GA content is shown as ng g^{-1} dry weight; b, GA content is shown as ng per apex, together with apex dry weights (×).

about 6×10^{-8} M. This concentration approaches the threshold of 10^{-7} 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_5 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

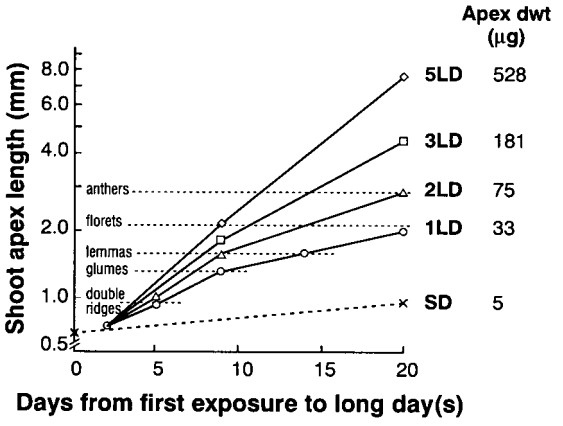

Figure 3. Effect of increasing numbers of LD on shoot apex length (mm) for *L. temulentum* (Lt449). Measurements continued until 21 d after the first exposure to up to 5 LD. The error bars were smaller than the symbols. For each daylength treatment, apex dry weight (μg) was determined at 21 d on batches of 20 apices. Plants held in SDs remained vegetative and apex dry weight and length increased relatively little over 3 weeks. These dry weights along with values at earlier harvests were used in deriving a relationship between apex length and weight (see "Materials and Methods").

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_1, 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 $\pm$ 4.3 ng g^{-1} dry weight), but GA_9 had increased 7-fold to 24.3 $\pm$ 7.0 ng g^{-1} dry weight, and GA_4 increased 23-fold to 9.3 $\pm$ 5.0 ng g^{-1} dry weight, whereas GA_{34} showed little change (2.3-fold increase to 25.9 $\pm$ 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_4 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 [$^2H_4$] 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 obtained when stem length was measured on the same plants (not shown). In parallel treatments but involving [$^2H_4$] 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 [$^2H_4$] GA_5 arrival at the apex. Nevertheless, the match in time between apex arrival of [$^2H_4$] GA_5 and leaf export of GA_5 indicates probable physiological relevance. We detected no [$^2H_4$] GA_6 at the apex although it is a logical metabolite of [$^2H_4$] 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 [$^2H_4$] 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 [$^2H_4$] 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 [$^2H_4$] 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 [$^2H_4$] GA_5 in the apex, there was apparently

Table I. *Effect on apex GA content and on flowering of various doses of [$^2H_4$] GA_5 applied once to the leaf blade 6 h after the start of a LD exposure*

The apex was harvested 48 h later.

[$^2H_4$] GA_5 Applied to the Leaf	[$^2H_4$] GA_5 Detected at the Apex	Flowering Response (Shoot Apex Length)
μg	*pg*	*mm*
0	0	2.69 $\pm$ 0.07
1	2.3	2.73 $\pm$ 0.16
5	6.5	2.98 $\pm$ 0.21
25	9.5	3.85 $\pm$ 0.43

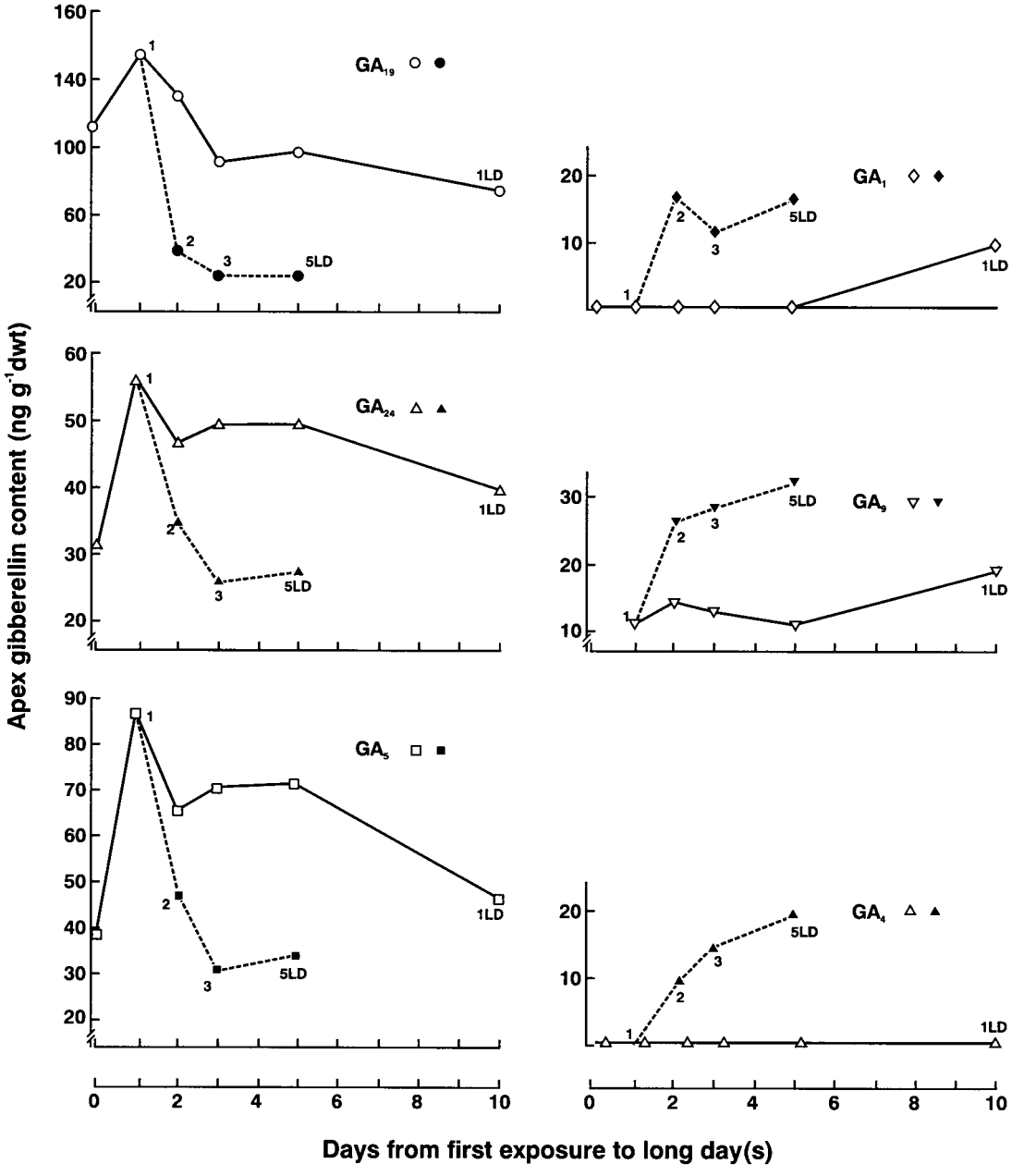

Figure 4. Effect on *L. temulentum* shoot apex GA content of exposure to a single LD or to up to 5 LD (Expt Lt 443). Apices were harvested at 8:30 AM each day for up to 10 d after the single LD or at 8:30 AM on the day of ending the exposure to 1, 2, 3, or 5 LD. Where multiple LD cycles were imposed prior to harvest (filled symbol), their number is shown next to the symbol.

a distinction between the physiologically active and total pools of GA_5 and [$^2H_4$] GA_5.

A separate issue is that only 9.5 pg of the 25 μg of [$^2H_4$] GA_5 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 μg dry weight) of the grass *L. temulentum* and established that a florally inductive

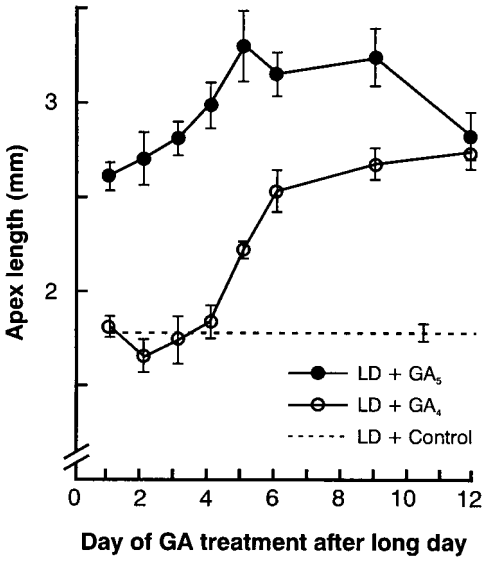

Figure 5. Effect on flowering of a single application to the leaf of 25 μg of GA_4 or GA_5. The earliest treatment was 6 h after the start of the main light period prior to the single LD extension. Flowering was determined at 21 d and the values are the mean and SE.

LD leads to an early doubling in GA_5 levels and, several days later, to large increases in GA_1 and GA_4.

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 GA_5 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 GA_5 doubled at the apex; (b) the GA_5 concentration was close to that required for flowering in vitro; (c) the timing of the doubling in endogenous GA_5 coincides with the timing of floral evocation; (d) applied GA_5 was transported intact from the leaf to the shoot apex (Fig. 6), where it then regulated flowering. This GA_5 was imported in physiologically meaningful amounts as increasing leaf application led to parallel increases in both the amount of GA_5 transported to the apex and in flowering (Table I); and (e) applied GA_5 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 GA_1, GA_3, or GA_4 (Evans et al., 1990, 1994a, 1994b).

Considering (a) and (b) above, a doubling in GA_5 content of the shoot apex would be biologically important because floral response increased 3-fold with a 3-fold increase in the GA_5 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 GA_5 reached 6×10^{-8} M, a value close to the 10^{-7} M GA_5 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 GA_6. The latter GA is florally effective, causes flowering with little or no stem elongation, and the shoot apex con-

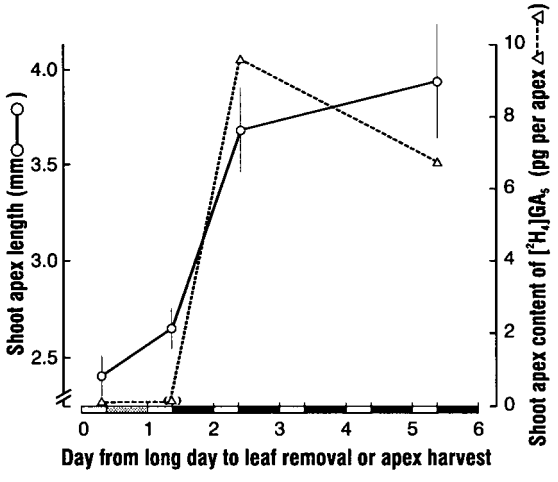

Figure 6. Timing of GA_5 export from a treated leaf to the shoot apex as shown either by increase in the floral response of the shoot apex with increasing time until removal of the GA-treated leaf blade, or by arrival at the shoot apex of [$^2H_4$] GA_5 following its application to the leaf. In both 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 GA_5 flowered to the same extent as those with the treated leaf removed at the start. There was only a trace of [$^2H_4$] GA_5 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 [$^2H_2$] GA_5 internal standard.

tent of GA_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_1, GA_3, or GA_4 in floral evocation can be excluded based on their inherent growth activity and the poor floral activity of GA_1 and GA_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 3β-hydroxylase enzyme did not inhibit flowering although stem elongation was inhibited (Evans et al., 1994a).

Although exogenously supplied GA_5 is transported from the leaf to the shoot apex of *L. temulentum*, it remains to be shown that the leaf is the source of GA_5 for the shoot apex because detection of GA_5 in leaf extracts of *L. temulentum* has proven technically difficult. The shoot apex itself could be the site of GA_5 formation via a single step conversion of GA_{20} to GA_5 (Hedden and Kamiya, 1997), GA_{20} being an endogenous transported form of GA as suggested for pea (*Pisium sativum*; Proebsting et al., 1992). In support of this scenario, the content of GA_{20} in leaves of *L. temulentum* does increase with exposure to 2 LD (Gocal et al., 1999). However, when GA_{20} was applied to the leaf of vegetative plants, it had little or no floral activity compared with GA_5 (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 (*Oryza sativa*), but not in florally evoked apices, suggests that prior to floral evocation there will be rapid catabolism of any GA_{20} arriving at the shoot apex. Given such uncertainties, GA_5 is either a primary LD floral stimulus in *L. temulentum* or its content increases directly at the shoot apex in response to a transported stimulus. Either way, it is GA_5 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 3β-hydroxylated GA_1 and GA_4. Furthermore, at this time applied GA_4 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_1 and GA_4 in inflorescence initiation is supported by our earlier studies with inhibitors of GA_{20} 3β-hydroxylation. 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 *L. temulentum*, such acylcyclohexanediones block the 3β-hydroxylation of GA_{20} to GA_1 and cause GA_{20} to accumulate (Junttila et al., 1997).

For both the L. temulentum shoot apex (this study) and the leaf (Gocal et al., 1999), exposure to multiple LD causes increases in GA_1 and GA_4 levels. By contrast, despite the increase in the shoot apex content of GA_5 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_1 and GA_4 which are readily 2β-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_1 and GA_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_5 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 *L. temulentum*, it is the late changes in GA_1 and GA_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_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 *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_1 or GA_4.

Overall, despite recent speculative claims to the contrary (Colasanti and Sunderasan, 2000; Samach and Coupland, 2000), our studies show that, for *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 *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 μmol m^{-2} s^{-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_5-treated leaf blade was cut off at various times after application. Some metabolism/transport studies with GA_5 utilized a sample of [15,15,17,17-$^2H_4$] GA_5 and, on GC-MS, this form was readily distinguished from a deutero [17,17-$^2H_2$] GA_5 internal standard as well as from GA_5 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 and 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-$^2H_2$] GAs added as internal standards to give a deutero:protio 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 (Sorbent 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-trimethylsilyltrifluoroacetamide at 70° 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° 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_1E_1-B_2E_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.

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

Ions used for HR-SIM measurements and detected transitions for SRM measurements are shown. The specific ions were detected in the time range when the specific GA eluted on the GC.

GA	Ions HR-SIM	SRM
GA_1	506.2520; 448.2101	506–448
[$^2H_2$]-GA_1	508.2645; 450.2227	506–207
GA_3	504.2364; 370.1961	504–370
[$^2H_2$]-GA_3	506.2489; 372.2086	506–372
GA_4	284.1776; 224.1565	418–390
[$^2H_2$]-GA_4	286.1902; 226.1691	420–392
GA_5	416.2020; 299.1645	416–299
[$^2H_2$]-GA_5	418.2145; 301.1770	418–301
GA_6	432.1968; 303.1417	432–303
[$^2H_2$]-GA_6	434.2091; 305.1540	432–207
GA_9	298.1570; 270.1620	298–270
[$^2H_2$]-GA_9	300.1690; 272.1740	300–272
GA_{19}	434.2489; 375.2355	434–375
[$^2H_2$]-GA_{19}	436.2614; 377.2481	436–377
GA_{20}	418.2176; 375.2355	418–375
[$^2H_2$]-GA_{20}	420.2301; 377.2481	420–377
GA_{24}	314.1789; 286.1930	314–286
[$^2H_2$]-GA_{24}	316.1914; 288.2055	316–288
GA_{34}	506.2520; 289.1440	506–289
[$^2H_2$]-GA_{34}	508.2645; 291.1565	508–291

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 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-$^2H_2$]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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