Flowering as metamorphosis: two sequential signals regulate floral initiation in *Lolium temulentum*

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INTRODUCTION

Forty years ago Lang (1957) demonstrated that gibberellin (GA), when applied to the leaves, led to flowering in several biennial and long-day (LD) plants. This observation has been extended to many LD plants, including *Lolium temulentum*, although many LD plants, as well as other flowering types, do not flower in response to applied GA (Evans, 1964; Lang, 1965; Zeevaart, 1983; Pharis and King, 1985). In several of the responsive plants, including *L. temulentum*, a transient rise in endogenous GAs has been observed following an inductive photoperiod (Jones and Zeevaart, 1980; Metzger and Zeevaart, 1980; Pharis et al., 1987) while application of GA inhibitors has reduced, stimulated, or had no effect on flowering depending upon many factors including when applied, photoperiodic response type, and species (Lang, 1965; Evans, 1969b; Zeevaart, 1983).

In LDs, application to short-day grown *L. temulentum* of an appropriate dose of GA to the leaves, or injection into the apex, causes all plants to initiate floral development (Evans, 1964, 1969b). Although many GAs are not as effective as a single LD in terms of the rate, and extent, of floral morphogenesis, more recent studies have identified highly florigenic GAs that elicit a response similar to that of a single LD (Evans et al., 1994). Evans (1969b) concluded that exogenous GAs do not play a role in floral evocation (i.e. induction of an apex into a florigen that40s allowed to grow vegetative tissues above the youngest expanding leaf primordium) to determine it for floral initiation. Then, GA elicits the expression of this florigenically determined state and the apex initiates inflorescence morphogenesis. In addition, we have analyzed the kinetics of floral initiation by leaf applied GA3 and provide data consistent with the interpretation that the leaf-applied gibberellin did not itself act on the apex to cause floral determination or initiation. Rather, the exogenous gibberellin appeared to stimulate the production of a signal in the leaves that then led to floral initiation.

Key words: floral determination, flowering, gibberellin, *Lolium temulentum*


**Photoperiodic induction and GA₃ application**

At 7- to 8-weeks old, plants were exposed to a single LD by extending the 8 hour day to 24 hours with light from incandescent lamps with a photon flux density of 400-500 μmol·m⁻²·sec at soil level. Seeds were planted in standard 10 cm plastic pots, four seeds per pot, containing Metro-Mix 250 (Scotts, Marysville, OH).

Plants were watered daily with tap water and fertilized weekly with Ra-pid-gro (23-19-17) (Ortho, San Ramon, CA).

**Shoot apex culture and floral score**

Apices from 7- to 8-week-old plants were excised and cultured as previously described (McDaniel et al., 1991). Briefly, apices (all initiated leaf primordia and tissues above the first upwardly growing leaf primordium) with the smallest over-arching leaf primordium left intact, were placed on about 1.5 ml of Linsmaier and Skoog medium (1965) at pH 5.5 with 0.7% agar, 5% sucrose and hormones, as indicated, in a 24-well culture plate. GA₃ was dissolved in absolute ethanol and subsequently diluted with distilled water to the appropriate concentration. From 6-6.5 hours after the beginning of the light period, a single 10 μl drop containing 50 μg of GA₃ was applied to the uppermost expanded leaf about 2 cm from the ligule such that the drop did not roll off the leaf or into the ligule. In leaf removal experiments, GA₃ was applied to the leaf below the uppermost expanded leaf. As did Pharis et al. (1987), we identified this concentration as optimal, and the time as one of high sensitivity.

**RESULTS**

Inflorescence development following LD induction

21 days after a single LD induction, apices on intact plants formed inflorescences with about 20 spikelets all of which had florets at, or beyond, stage 8. A hint of a gradient may be observed with the terminal and more apical spikelets being slightly more advanced than the most basal spikelets. As we reported (McDaniel et al., 1991), apices cultured onto GA₃ medium formed near normal inflorescences. A marked developmental gradient, however, was observed with the terminal and apical spikelets being more advanced and the basal most spikelet primordia appearing vegetative or at double ridges, stage 2. A more pronounced gradient as well as other subtle morphological variations were observed when apices were from plants that had been given marginal inductive conditions with water for each experiment. 7-week-old plants were watered exclusively for 6 days prior to induction and on Day 1 with a solution containing 20 mg/l ancymidol. In addition, plants were sprayed at the beginning, and the end, of the light period with the same solution until run-off from the leaves occurred, for the same 7-day period and on Day II. 48-52 hours after the beginning of the inductive LD, apices were excised onto medium containing kinetin and ancymidol (5 mg/l), or GA₃ and ancymidol. Apices from other untreated plants from the same population of plants were excised 48-52 hours after the beginning of the inductive period and placed on kinetin, or GA₃ medium. Apices on kinetin-ancymidol medium were transferred to fresh medium after 10 and 20 days. After 21 days, all apices on kinetin, GA₃ and GA₃-ancymidol media were dissected. Some apices on kinetin-ancymidol medium were dissected at 21 days while most were transferred a third time at 30 days onto either kinetin-ancymidol, or GA₃-ancymidol, medium and dissected after 20 days.

**Leaf removal**

Fully expanded leaves were removed by cutting the leaf sheath just below the ligule. When all leaves, or all leaves but the GA₃ treated leaf, were removed, the apical leaf blades were removed by cutting the plant just below the ligule of the uppermost expanded leaf. For night leaf removals, plants were removed from the dark growth room into very dim fluorescent light, leaves removed, and plants returned to the dark.

**Biological variation**

Growth conditions, plant age and other poorly characterized factors influence the floral response of *L. temulentum* (Evans, 1969a; Evans and King, 1985). Even under apparently uniform growth and culture conditions, we have observed considerable biological variability in the flowering response in vitro of *L. temulentum* in our laboratory (e.g. Table 1, Results Section). As a consequence, each experiment had internal control groups of plants, and comparisons of floral responses were made within a population of plants grown, and manipulated, at the same time. Table and figure legends give experiment numbers. For each experiment, except for the plant age experiment (Fig. 6), all of the plants, or apices, were taken from the same population of plants. For each experiment from 4 to 24 plants were in each of the following control groups, as appropriate: maintained under short-day conditions for 21 days and dissected (were always vegetative), induced and returned to short-day conditions for 21 days and then dissected (were always floral), apices from short-day grown plants excised and cultured on GA₃ medium for 21 days and then dissected (were always vegetative), and apices from induced plants excised and cultured on kinetin or GA₃ medium for 21 days and then dissected (response depended upon medium and time of excision).
or apices were cultured on medium lacking GA3. Although apices grew and initiated inflorescences on hormone-free medium, survival was generally substantially less than on kinetin or GA media. For this reason, apices were cultured onto kinetin or GA medium except where noted.

**Time-dependent gibberellin response**

More than a thousand apices from short-day-grown plants have been excised and cultured on GA3 medium. None has ever flowered in culture. When apices from induced plants were explanted between 32 and 36 hours after the beginning of the inductive LD onto medium containing kinetin, most did not flower, while when excised onto GA3-containing medium, most did flower (Table 1, Fig. 1). When the apices were explanted 47-51 hours after the beginning of the inductive LD, most of the apices flowered although more flowered on GA3 than on kinetin medium. Apices cultured on hormone-free medium gained the capacity to flower in culture several days after the beginning of the LD.

### Table 1. Variability in time-dependent, gibberellin-dependent flowering of cultured apices of *Lolium temulentum*, strain Ceres

<table>
<thead>
<tr>
<th>Time cultured (Hours after beginning of long day)</th>
<th>Medium*</th>
<th>% Floral±s.d. (range) (n)†</th>
<th>Floral scores±s.d. for floral apices (range) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32-36 Kinetin</td>
<td>35±28 (0-90) (22)</td>
<td>4.9±0.3 (4.3-5.3) (19)</td>
<td></td>
</tr>
<tr>
<td>32-36 GA3</td>
<td>88±15 (57-100) (19)</td>
<td>5.8±0.6 (4.3-6.8) (19)</td>
<td></td>
</tr>
<tr>
<td>47-51 Kinetin</td>
<td>67±24 (38-100) (9)</td>
<td>5.2±0.3 (4.5-5.6) (9)</td>
<td></td>
</tr>
<tr>
<td>47-51 GA3</td>
<td>99±2 (94-100) (8)</td>
<td>6.7±0.6 (5.7-7.4) (8)</td>
<td></td>
</tr>
</tbody>
</table>

*Average survival on GA3 medium was 89% and on kinetin medium 85%.
†Range is the average for all apices in an experiment and n is the number of experiments evaluated.

Interestingly, when apices were excised between 32 and 36 hours after the beginning of the inductive LD onto kinetin medium and then, after several to many days, transferred to a medium containing GA3, the percentage that was floral was the same as if apices were initially cultured onto GA3 medium (Fig. 2). Although explanted apices continue to grow in culture, the most rapid leaf initiation occurred in the first 20 days of culture, even when subcultured every 10 days. Usually, most apices that were explanted at about hour 34 and subcultured at 10 and 20 days onto kinetin medium grew vegetatively for 30 days and initiated, on average, eight new leaf primordia and expanded six existing primordia (Fig. 3). When transferred to GA3 medium after the initial 30 day culture period, the upper eight spikelets of an inflorescence were produced by the cellular descendants of the shoot apical meristem present at the time of explanting (i.e. the cells above the youngest leaf primordium).

![Fig. 1. Percentage of *L. temulentum* apices explanted from long-day induced plants that flowered in culture as a function of excision time and culture medium.](image1.png)

![Fig. 2. Percentage of *L. temulentum* apices explanted from long-day induced plants that flowered in culture as a function of culture medium and time in culture.](image2.png)
2-4 days in order to exhibit an enhanced floral response whether they were exposed to GA3 at the beginning of the culture period or after being first on kinetin medium for some period of days (Fig. 4). In the two experiments depicted in Fig. 4 about 80% of the excised apices flowered when placed directly on GA3 medium. If apices were placed directly on GA3 medium, they had to be on the medium for about 4 days before 80% flowered after being returned to kinetin medium. Likewise, when cultured first on kinetin medium and then transferred onto GA3 medium, they had to be on GA3 medium for about 2 days before 80% flowered after being returned to kinetin medium.

Ancymidol inhibition of flowering

Plants were treated with ancymidol and cultured on media with, and without, ancymidol 48-52 hours after the beginning of the inductive LD. All of the kinetin and GA3 control apices flowered as expected (Fig. 5). Ancymidol treatment reduced flowering to below 50% at the terminal position and to under 25% for lateral positions for those apices cultured on kinetin-ancymidol medium. In contrast, all apices cultured on GA3-ancymidol medium flowered, forming spikelets at terminal and lateral positions. Apices transferred from kinetin-ancymidol medium after 30 days onto GA3-ancymidol medium all formed terminal spikelets, and over 90% of the apices formed lateral spikelets. In contrast, just over 60% of the apices transferred to kinetin-ancymidol medium formed terminal spikelets and just under 40% formed lateral spikelets.

Inflorescence development following GA3 leaf application

Floral initiation in response to applied GA led to morphological patterns different, in vivo and in vitro, from those observed after a single LD. For intact plants, Evans (1969b) reported inflorescences had an inverse gradient with the lowest spikelets being more advanced than the upper spikelets. He noted that sometimes the terminal spikelet was absent with the terminal meristem appearing vegetative. We observed these patterns as well as double gradients and empty places in an inflorescence where spikelets should have been. Apices explanted from SD grown plants treated with GA3 initiated inflorescences in culture that had the following patterns: a basepetal gradient of...
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SECOND, removal of the leaf to which GA3 was applied indicated that the GA3 leaf was required for about 1 day in order to get 90% flowering. Third, removal of all leaves demonstrated that at least some leaf blade was required for 4 days to get 70% flowering.

When apices are cultured at various times after the application of GA3, apices placed on GA3 medium expressed the capacity to initiate floral morphogenesis several days sooner, and ultimately reach a more advanced stage of floral morphogenesis, than apices cultured on kinetin medium (Fig. 9).

DISCUSSION

Although it has been known for a long time that GAs cause some plant species to flower, it has been unclear in most cases, how or if, the exogenous hormone intervenes in evocation, floral initiation, and floral morphogenesis (Evans, 1964, 1969b; Lang, 1965; Zeevaart, 1983; King et al., 1993). Our analysis of the role of GA3 in flowering of L. temulentum, strain Ceres, has provided evidence that GAs play separate, and distinct, roles in the apex and in the leaves. In the case of photoperiodically controlled floral initiation, the inductive photoperiod leads to the activation of two signals. The first signal, from the leaves, causes the shoot apex to acquire the capacity to respond to GA. This first signal is, within hours, followed by an endogenous increase of GAs at the apex (Pharis et al., 1987) which elicits floral morphogenesis. That is, the first signal evokes the apex into a florally determined state and the second signal, GA, acts uniquely on the various cells/tissues of the apex to elicit the expression of the determined state thereby forming an inflorescence. Leaf-applied GA3 appears to act on the leaf, very much like the LD photoperiod, causing processes in the leaves that result in a signal being sent to the apex which determines the shoot apex for floral morphogenesis. This determined apex subsequently initiates floral morphogenesis perhaps in response to endogenous GAs.

GIBBERELLINS AT THE APEX ELICIT FLORAL MORPHOGENESIS

Our earlier study established that most L. temulentum apices became florally determined on the day after the LD (i.e., Day II) so long as they were cultured in the presence of GA (McDaniel et al., 1991). It also established that floral determination in a population of plants occurred over a period of about 12 hours, usually beginning sometime early in Day II. In an earlier study, Pharis et al. (1987) established that during Day II a transient burst of GAs could be detected in L. temulentum apices. We hypothesized that most apices excised on Day II had been acted upon by a photoperiodically induced leaf signal but not by GA. This hypothesis is supported by the observation that apices excised onto kinetin medium just after the end of the light period of Day II grew vegetatively for many days, but when exposed to GA3, they initiated floral morphogenesis (Fig. 2). The transient requirement for GA is supported by the observation that excised apices only needed to be exposed to GA3 in the medium for several days to elicit floral initiation (Fig. 4). The strongest evidence for GAs eliciting the expression of floral morphogenesis from a florally determined apex came from treating plants, and culturing apices, with ancymidol which inhibits the oxidation of ent-kaurene to ent-kaurenoic acid, thereby reducing GA synthesis (Sponsel, 1987). Ancymidol treatment substantially reduced floral initiation in apices cultured in the absence of GA3. If GA3 was in the medium, ancymidol had no influence on floral initiation (Fig. 5). Our interpretation of ancymidol inhibition of floral initiation is simple and logical. Alternatively, in light of the varied responses to GA synthesis inhibitors administered around the time of evocation (Lang, 1965; Evans, 1969a,b; Zeevaart, 1983; Pharis and King, 1985), it is possible that ancymidol may be acting in other ways that blocking floral initiation. It is then, however, more difficult to explain why GA3 totally overrides the ancymidol inhibition of floral initiation.

In a detailed analysis of in vitro flowering of L. temulentum apices King et al. (1993) proposed three explanations for the relationship between the LD stimulus and the GAs: "1. that the LD stimulus in L. temulentum is GA; 2. that the LD stimulus translocated from the leaves to the shoot apex consists of two components, a ‘floral stimulus’ and a florigenic GA; 3. that GAs are not a component of the LD stimulus but interact synergistically with it to enhance the flowering response.” They did not favor the first hypothesis, nor do we think it is just GA. Our data provide strong evidence for the second hypothesis if one assumes that the transient burst of GAs reported by Pharis et al. (1987) comes from the leaves. They favored the third hypothesis because their experiments tended to concentrate on floral morphogenesis and their results indicated GAs played an important role in floral morphogenesis but not in evocation.

The recent study of King et al. (1993) also reported that when apices were explanted from plants grown in short days for 8 weeks in high irradiance, they would initiate floral morphogenesis on medium lacking GA, while apices from younger
plants required not only GA in the medium but also a LD prior to explanting in order to flower. Their results established that, although apices on 8-week plants would have grown vegetatively for a number of weeks in vivo, they were florally determined and expressed this state when removed from the plant. That is, \textit{L. temulentum} eventually flowers under short days with time to flowering being positively correlated with increasing irradiance (Evans and King, 1985). For example, under our short-day growth room conditions \textit{Lolium} plants initiate floral morphogenesis between 4 and 5 months of age. The activation order of the processes that are responsible for flowering in short days is not known, whereas LD activation order has been characterized in earlier studies (Evans and King, 1985; McDaniel et al., 1991) and in more detail above (i.e., LD acts on competent leaves, LD leaf signal reaches apex, apex evoked, GA level rises in apex, and florally determined state expressed). The King et al. (1993) result indicates that flowering of \textit{Lolium} in short days is prevented in vivo by more than a lack of the two signals that cause flowering after photoperiodic induction. Perhaps the inhibitor reported by Evans (1960b) might be responsible. Their result is not unique as expression of the florally determined state in sunflower and \textit{Bougainvillea} apices is inhibited in vivo but expressed in vitro (Habermann and Sekulow, 1972; Steffen et al., 1988a,b; see McDaniel et al., 1992 for a discussion).

**Floral initiation by leaf applied GA\textsubscript{3}**

The simplest explanation would be that leaf applied GA is simply translocated to the apex where it evokes floral morphogenesis. Evans has not favored this explanation for many reasons (Evans, 1969b; King et al., 1993). Our data also are not consistent with this explanation. First, in intact plants, although LD and GA\textsubscript{3} sensitivities increase with plant age, the LD response saturates sooner, and more quickly, than the GA\textsubscript{3} sensitivity (Fig. 6). Second, under our growth conditions, although 7- to 8-week-old plants are fully responsive to leaf applied GA\textsubscript{3}, excised apices are incapable of responding to GA\textsubscript{3} unless they have been acted upon by the photoperiodically induced leaf signal. Third, all of the leaves must remain on the plant for about 4 days to obtain a high percentage flowering, while the GA\textsubscript{3} leaf alone can give a two step process. First, in response to the leaf applied GA, a leaf signal induces the apex into a florally determined state and then, endogenous GAs elicit floral morphogenesis.

**The role of gibberellins in flowering**

In \textit{Bryophyllum daigremontianum}, a long-short-day plant, exogenous GA\textsubscript{3} applied to plants in short-days causes the leaves to send a floral stimulus to the apex (Zeevaart, 1969). LDs appear to enable the leaves to produce endogenous GA that in short days causes the floral stimulus to be produced (Zeevaart and Lang, 1962, 1963). \textit{Lolium} leaves appear to respond to exogenous GA\textsubscript{3} in a manner similar to the response reported for \textit{Bryophyllum} leaves. Analysis of several GA mutants in \textit{Arabidopsis thaliana} have established that GAs are involved in flower initiation (Wilson et al., 1992). The responses characterized, however, were too complex to establish the exact role played by GA. The varied responses of different species of angiosperms to exogenous GA (Lang, 1965; Evans, 1969b; Zeevaart, 1983; Pharis and King, 1985) are consistent with the interpretation that floral initiation is regulated by controlling different signals, and processes, in different species (McDaniel, 1984; McDaniel et al., 1992). Thus, GAs may play a particular regulatory role in one species but not in another. This interpretation of the flowering literature indicates that a species, or genotype, must be physiologically and developmentally, characterized before its regulatory control can be placed in some mechanistically meaningful category. For example, the classic flowering types (e.g. long-day, short-day, day neutral) do not necessarily categorize plants as to mechanism of floral initiation control. As we have discussed, the developmental processes involved in floral initiation may be common to all angiosperms and categorization of flowering types as a function of the developmental mechanism(s) employed to regulate floral initiation may be relatively simple and informative (McDaniel, 1992, 1996).

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