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Selective Deactivation of Gibberellins below the Shoot Apex is Critical to Flowering but Not to Stem Elongation of *Lolium*

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ABSTRACT Gibberellins (GAs) cause dramatic increases in plant height and a genetic block in the synthesis of GA_1 explains the dwarfing of Mendel's pea. For flowering, it is GA_5 which is important in the long-day (LD) responsive grass, *Lolium*. As we show here, GA_1 and GA_4 are restricted in their effectiveness for flowering because they are deactivated by C-2 hydroxylation below the shoot apex. In contrast, GA_5 is effective because of its structural protection at C-2. Excised vegetative shoot tips rapidly degrade [^{14}C]GA_1, [^{14}C]GA_4, and [^{14}C]GA_{20} (>80% in 6 h), but not [^{14}C]GA_5. Coincidentally, genes encoding two 2β-oxidases and a putative 16–17-epoxidase were most expressed just below the shoot apex (<3 mm). Further down the immature stem (>4 mm), expression of these GA deactivation genes is reduced, so allowing GA_1 and GA_4 to promote sub-apical stem elongation. Subsequently, GA degradation declines in florally induced shoot tips and these GAs can become active for floral development. Structural changes which stabilize GA_4 confirm the link between florigenicity and restricted GA 2β-hydroxylation (e.g. 2α-hydroxylation and C-2 di-methylation). Additionally, a 2-oxidase inhibitor (Trinexapac Ethyl) enhanced the activity of applied GA_4, as did limiting C-16,17 epoxidation in 16,17-dihydro GAs or after C-13 hydroxylation. Overall, deactivation of GA_1 and GA_4 just below the shoot apex effectively restricts their florigenicity in *Lolium* and, conversely, with GA_5, C-2 and C-13 protection against deactivation allows its high florigenicity. Speculatively, such differences in GA access to the shoot apex of grasses may be important for separating floral induction from inflorescence emergence and thus could influence their survival under conditions of herbivore predation.

INTRODUCTION

There are clear associations between applied and endogenous gibberellins (GAs) and flowering in dicotyledonous species including *Arabidopsis* (Wilson et al., 1992; Xu et al., 1997; Blázquez and Weigel, 2000; Eriksson et al., 2006), and flowering in monocotyledonous species including *Lolium* spp. (Evans, 1964; Pharis and King, 1985; King and Evans 2003; King et al., 2001, 2006; MacMillan et al., 2005). On transfer to a long day (LD), the levels of bioactive GAs (variously GA_1, GA_4, GA_5, and GA_6) increase rapidly in the leaf, petiole and shoot apex of both monocots and dicots (Metzger and Zeevaart, 1980; Talon and Zeevaart, 1990; Xu et al., 1997; Gocal et al., 1999, 2001; King et al., 2001, 2003, 2006; MacMillan et al., 2005).

Increase in endogenous levels of bioactive GAs is often associated with increased expression of 20-oxidase GA biosynthetic genes (Xu et al., 1997; Hisamatsu et al., 2005; MacMillan et al., 2005; King et al., 2006). However, a GA increase could also be brought about by a reduction in GA degradation. One group of such enzymes—the 2-oxidases—inactivate bioactive GA_1 and GA_4 by hydroxylation of Carbon-2 (C-2; see Figure 1)—an action demonstrated in a large number of species, including *Phaseolus coccineus* (Thomas et al., 1999), *Arabidopsis* (Thomas et al., 1999), *Pisum sativum* (Lester et al., 1999; Martin et al., 1999), and spinach (Lee and Zeevaart, 2002). As expected, GA content is elevated in the pea 2-oxidase mutant *sln* and shoot growth is enhanced (see ref. in Lester et al., 1999). The converse—reduced GA levels and dwarfing—result from 2-oxidase overexpression (Sakamoto et al., 2001;

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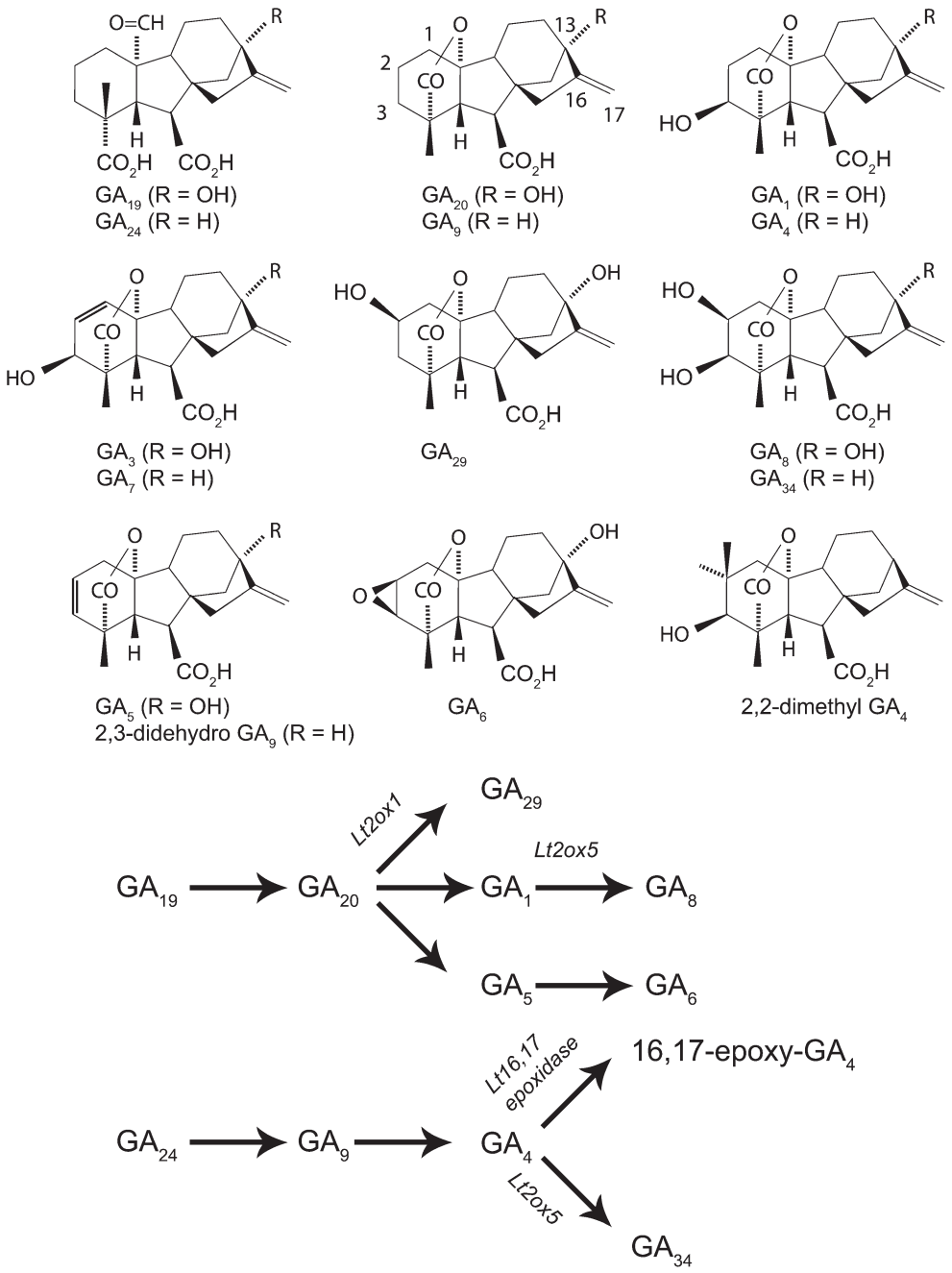

Figure 1. Structural Diagrams for the GAs Used Here and the Metabolic Pathway Covering the Last Steps of GA Biosynthesis and Inactivation.

Some of the relevant enzymes are shown in italics.

Busov et al., 2003; Schomburg et al., 2003; Lee and Zeevaart, 2005; Curtis et al., 2005; Kloosterman et al., 2007). A second pathway for GA deactivation involves epoxidation at C-16, 17 (see Figure 1) and, in rice, overexpression of a GA 16α, 17-epoxidase caused dwarfing and a mutant (*eui*) showed enhanced stem elongation (Zhu et al., 2006).

In our initial studies with *Lolium temulentum* leaves, we found no decrease in 2-oxidase transcript when their GA content increased at the time of LD floral induction (King et al., 2006). However, deactivation of GAs could be important during their transport to the shoot apex—a suggestion supported by findings with rice, in which a 2-oxidase was found to express highly in sub-apical vascular bundles of vegetative plants and later to become undetectable when the inflorescence developed (Sakamoto et al., 2001). As an extension of this suggestion of tissue-localized effects, GA deactivation could also be selective, as the various enzymes show different substrate specificities (Thomas and Hedden, 2006).

In support of a model in which GAs are selectively excluded from the apex, GA_1 and GA_4 are absent from the vegetative shoot apex of *L. temulentum* but are present in leaves, while GA_5 is present in both tissues and increases significantly in LD (Gocal *et al.*, 1999; King et al., 2001, 2003, 2006). The structural differences between these three GAs, summarized in the GA biosynthetic pathway in Figure 1, highlight the potential for GA_5 to resist 2β-oxidation because of its C-2–3 double bond, while its 13-OH would tend to inhibit GA epoxidation at C-16,17 (see Zhu et al., 2006). Conversely, GA_1 and GA_4 would be inactivated by 2-oxidases (see Thomas and Hedden, 2006) and GA_4 could also be inactivated by 16,17-epoxidases (Zhu et al., 2006).

Here, to determine the possible role in flowering of both 2-oxidases and 16,17-epoxidases, we have examined their mRNA expression patterns near the shoot apex of *L. temulentum.* Enzyme activity has also been assessed based on metabolism of [^{14}C]-GAs supplied to isolated shoot tips. Lastly, we have confirmed the linkage between GA deactivation and flowering by examining the response to GAs structurally altered to give protection against GA deactivation at C-2, C-13, and C-16,17, and/or by applying Trinexapac Ethyl to inhibit 2-oxidases (see Rademacher, 2000).

RESULTS

Differences in GA Structure which Determine their Bioactivity

Previously, we suggested that effectiveness of GAs might reflect differences in their stability (Evans et al., 1990; King et al., 2001; King and Evans, 2003). As an example, GA_1 and GA_5 are likely to be more stable than GA_4 and they are more florigenic when applied once to the leaf of *L. temulentum* (Figure 2A and 2C), the untreated plants remaining vegetative in SD. The response to GA_5 best matches that to a LD because it causes flowering but with limited stem elongation, whereas GA_1 causes flowering but with excessive stem elongation (Figure 2D and see review in King and Evans, 2003).

GA_5 with its C-2,3 double bond (Figure 1) should be structurally protected against 2-oxidase catalysed hydroxylation at C-2. Likewise, 2-oxidase protection by the addition of C-2 methyl groups to GA_4 may account for the huge increase in florigenicity of 2,2-dimethyl GA_4 compared with GA_4 (Figure 2A and Evans et al., 1990, 1994).

Interestingly, for stem elongation of the same plants, the ranking of GAs changes. Although superior for flowering, GA_5 is least effective for stem elongation (Figure 2D). By implication, then, GA 2-oxidation is relatively less important in stem tissue—a conclusion also supported by the smaller differential seen in stem elongation induced by 2,2-dimethyl GA_4 relative to GA_4 (2,2-dimethyl GA_4 was ~100-fold more effective than GA_4 for stem elongation but ~5 000–10 000-fold more effective for flowering; Figure 1A vs 1B and c.f. Evans et al., 1994). Thus, not only is GA deactivation detrimental for flowering, but the structurally based differences in response to applied GA for stem elongation and flowering suggest that deactivation might be greater nearer the shoot apex. For this reason, we examined gene expression patterns in the shoot apex and, for comparative purposes, further down the shoot.

Localization of 2-oxidases and a Putative 16,17-epoxidase Just Below the *L. temulentum* Shoot Apex

To define the pattern of gene expression near the apex, we harvested 0.8-mm stem segments from all stem tissue in the top 7 mm of stem from the tip, as shown schematically in Figure 3. Harvests were made in the middle of the day for plants held continuously in SD with or without GA_1 application 3 d earlier and at the same time (3 d later) for plants exposed to a single LD. All plants were 8 weeks old at the time of harvest and the very lowest segments were discarded because they were not always present and encroached on the root zone (Figure 3). Further experiments confirmed the findings of this study.

LtGA2ox1 expression was greatest within 2–3 mm of the apex, but, further down the stem, it was less than 50% of the maximum (Figure 3A). A similar but much magnified pattern of *LtGA2ox1* expression in the lower stem is evident for shoots harvested 3 d after GA_1 was applied to the leaf or the leaf was exposed to a LD (Figure 3A). In these two treatments (LD; and SD + GA_1), expression of *LtGA2ox1* was lowest in the apical segment and indistinguishable from the low expression in the untreated SD apical segment to which all expression levels were normalized.

The high stem and sub-apical expression of *LtGA2ox1* (Figure 3A) is indicative of deactivation of GA_{20}—the immediate precursor of bioactive GA_1 and GA_5. Bioactive GA_1 and GA_4 themselves would be deactivated by LtGA2ox5 (see Figure 1). Based on the expression profiles of these two genes, their greatest action would be at or just below the shoot apex, their expression dropping to less than 20–30% of the maximum further down the young stem tissue (Figure 3A and 3C). In addition, transport of active GA_4 into the shoot apex would be affected by its inactivation by a putative LtGA16,17epoxidase (Figure 1). Later, we present evidence for endogenous epoxidation of GA_4 but the gene assayed for expression (Figure 3B) is no more than a putative epoxidase. The *L. temulentum* gene shows 97% amino acid identity with the *L. rigidum* cytochrome P450 gene, which, in turn, shows 41% identity with the rice epoxidase. We were unsuccessful with the yeast functional epoxidase assay used by Zhu et al. (2006).

A characteristic of some plant 2-oxidases is GA up-regulation of their expression (Thomas et al., 1999; and see review in Thomas and Hedden, 2006). Such feed-forward regulation of *LtGA2ox1* by GA_1 is shown in Figure 3A. The increase in endogenous GAs within hours of the LD exposure (King et al., 2001, 2006) would also account for LD up-regulation of *LtGA2ox1* (Figure 3A). Interestingly, *LtGA2ox5* expression was reduced dramatically for harvests 3 d after the LD, but LD had no effect on expression of the putative *LtGA16,17epoxidase* (Figure 3B and 3C).

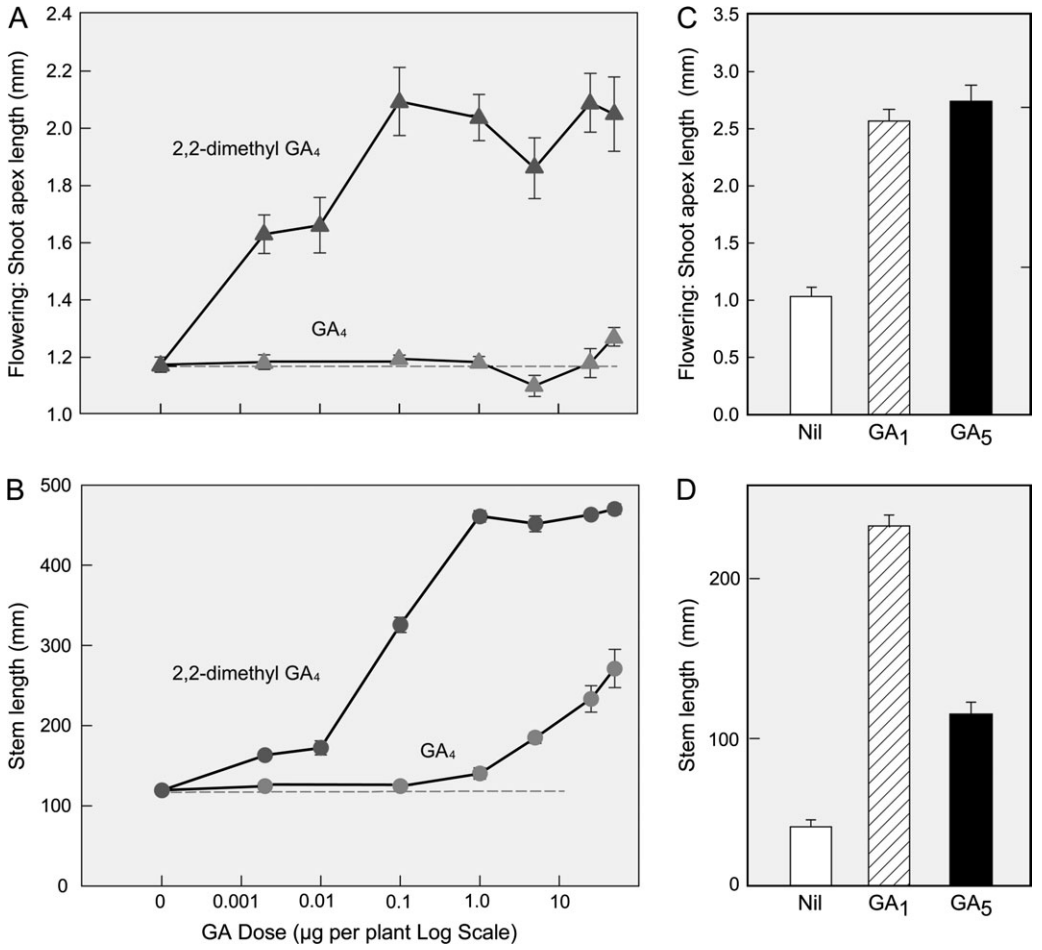

Figure 2. Flowering (mm Shoot Apex Length) and Stem Length (mm) for Plants of *L. temulentum* Treated Once with 2,2-dimethyl GA_4 or GA_4 at Various Doses (A, B) or with GA_1 or GA_5 at 25 µg per Plant (C, D).

The plants were 8 week old when treated and flowering and stem length were measured 3 weeks later, as in our previous studies (e.g. Evans et al., 1990). All GAs were applied to plants in SD but, for comparative purposes, in Figure 2A and 2B, a batch of untreated plants was exposed to one LD, which led to a floral shoot apex length of 2.04 ± 0.05 mm and a stem length of 140.9 ± 3.6 mm (not shown on figure). Elongation of the stem tissues below the shoot apex at a dose of 1 µg per plant indicates that GA_4 was taken up but it caused no flowering unless dimethylated at Carbon-2. Values are means ± S.E. (n = 14).

In rice, one 2-oxidase expresses in vascular bundles just below the shoot apex (Sakamoto et al., 2001) and, in *Arabidopsis*, a 2-oxidase reporter gene construct expresses highly but diffusely just below the shoot apex (Jasinski et al., 2005). Similarly, for *L. temulentum*, *LtGA2ox1* expression was localized to vascular parenchyma in stem cross-sections taken 1 mm below the shoot apex (Figure 4A). At a 4.5 greater magnification, a repeat hybridization with an adjacent section showed the same pattern (Figure 4B). At an equal concentration of riboprobe, the control (sense probe) barely hybridized (Figure 4C).

The increased *LtGA2ox1* expression with GA_1 or LD treatment (Figure 3) is associated with the onset of flowering of *L. temulentum*. To further examine this relationship between GA, flowering, and 2-oxidases, we applied various doses of GA_5 and GA_1 to plants in SD. These two GAs differ in effectiveness for flowering and stem elongation. GA_5 is more florally effective and GA_1 is more effective for stem elongation (Figure 5B and see also Figure 2C and 2D), as we reported previously (Evans et al., 1990; King and Evans, 2003). Of these two GAs, GA_5 was far less effective for inducing *LtGA2ox1* expression, especially at doses of 1–5 µg per plant (Figure 5A).

In a repeat study, effectiveness of GAs for inducing expression of *LtGA2ox1* was $GA_1 > GA_4 > GA_5$ at a dose of 5 µg per plant (Table 1) but effectiveness is reversed for flowering (i.e. $GA_5 > GA_1 \geqslant GA_4$; e.g. Figure 2 and see King and Evans, 2003). For stem elongation, the ranking of GAs is the same as for *LtGA2ox1* expression (data not shown). Although transcript of *LtGA2ox1* does not increase when GA_5 causes flowering (Figure 5), it increases in association with flowering induced by GA_1 (Figure 3). As we discuss later, these contradictory results could reflect differences in both GA deactivation and in receptor binding affinity.

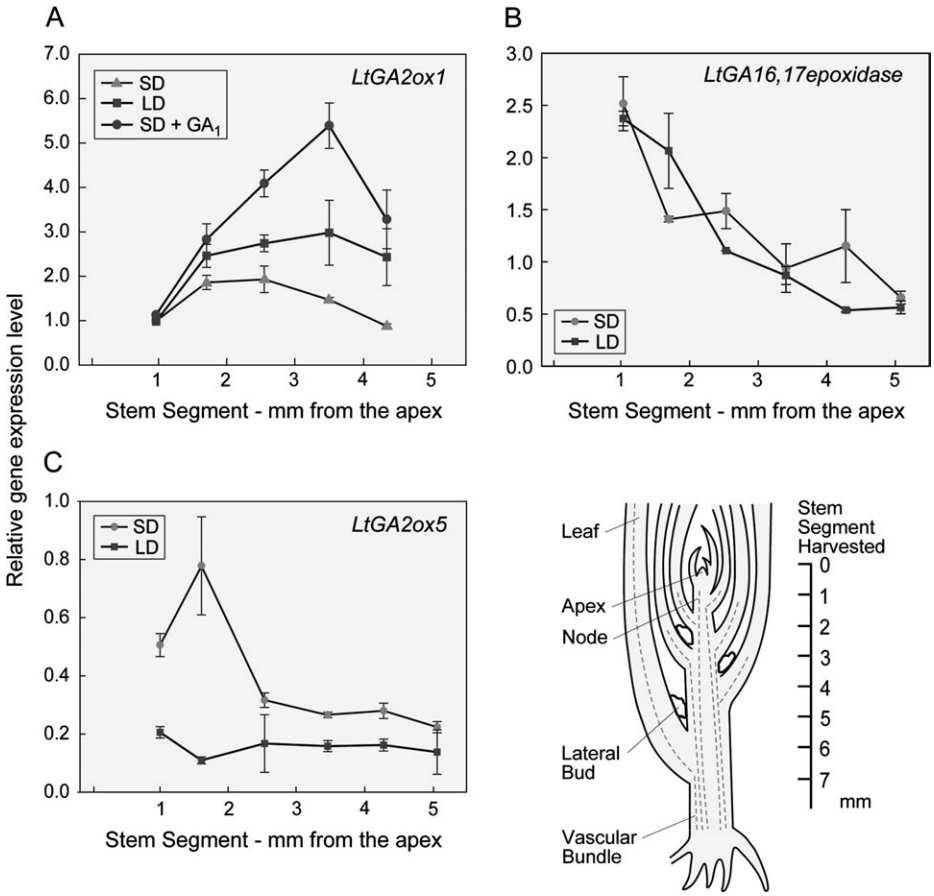

Figure 3. Expression of GA Deactivation Genes in Shoot Tips of *L. temulentum* for Plants Held in SD, Exposed to a Single LD, or Treated with GA_1 in SD.

(A) *LtGA2ox1* expression.
(B) Putative *16,17-epoxidase* expression.
(C) *LtGA2ox5* expression.
Stem segments were harvested after 3 d and were ca. 0.8 mm thick. Their position is plotted as mm below the shoot apex and is also shown diagrammatically in the figure. Values are means ± S.E. (n = 3).

GA Degradation Studies

As an independent test of the relationship between flowering and GA deactivation near the shoot apex, we supplied [^{14}C]GAs to excised vegetative shoot apices which included up to 2 mm of apex/stem base (cf. diagram in Figure 3). Based on tissue extraction and radiocounting of HPLC fractions, within 6 h, [^{14}C]GA_{20} was converted almost completely to its 2β-hydroxylated product [^{14}C]GA_{29} (Figure 6). Previously, we had confirmed this product as [^{14}C]GA_{29} by GCMS after HPLC and also showed similar rapid and complete 2β-oxidation of GA_1 to GA_8 (Junttila et al., 1997).

Given their different substrate specificities in functional assays (see Materials and Methods), *LtGA2ox1* would regulate GA_{20} deactivation in the shoot tip and *LtGA2ox5* would metabolize GA_1 and GA_4. Previously, we showed that shoot tips rapidly converted all applied GA_1 to its 2-hydroxlated product, GA_8 (Junttila et al 1997). Here, when [17-^{14}C]GA_4 was applied to shoot tips, the most abundant metabolite chromatographed with GA_{34}, the expected 2-hydroxylated metabolite of GA_4 (Figure 6). A more polar peak (fraction 20–21) chromatographed where we would expect to find [17-^{14}C]GA_{34} catabolite (Pearce et al., 2002). The most polar peak chromatographed with a retention time on C-18 reverse phase HPLC, which is characteristic for a 16,17-epoxide (S. Yamaguchi, personal communication). Our GCMS examination of this peak showed the presence of a product with the same GC retention time as GA_4 16,17-diol, with a strong ion at *m/z* 493 (M^+-17-CH_2OTMS; cf. Zhu et al., 2006, for GCMS information) and which, because of the fragmentation pattern during MS, is characteristic for both [17-^{14}C] labelled and endogenous [^{12}C] GA_4 substrate. The 16,17-diol analyzed here is formed rapidly when the epoxide is exposed to low concentrations of acetic acid, as employed during HPLC (see evidence in Zhu et al., 2006). Thus, we infer from these data that the 16,17-epoxide is one GA_4 metabolite and that both 2-oxidases and 16,17-epoxidases contribute to GA_4 deactivation in and just below the shoot tip.

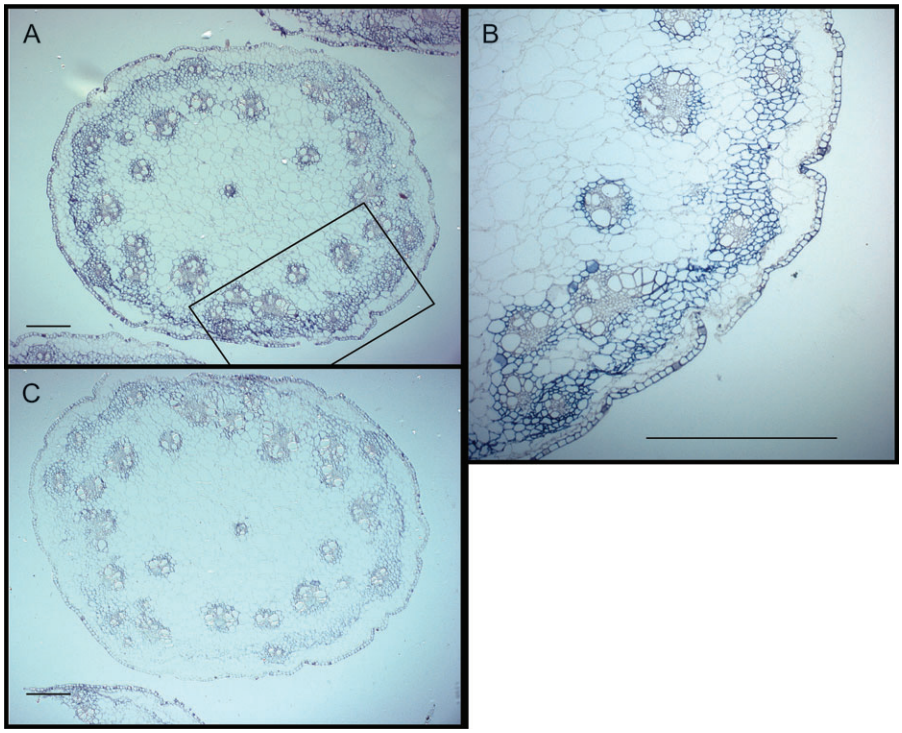

Figure 4. Expression of *LtGA2ox1* by In-Situ Hybridization for Stem Cross-Sections Taken 1.0 mm Below the Shoot Apex of Vegetative Plants of *L. temulentum*.

Near-to-adjacent sections were hybridized together with antisense riboprobe (A) or with sense probe (C). In (B), the boxed area in (A) is shown at a 4.5× magnification but from an adjacent section from a separate hybridization with the antisense probe. The scale bars are 150 μm.

Despite the rapid metabolism of [^{14}C]GA_{20} (Figure 6) to its 3β-hydroxylated product GA_{29}, in the same experiment, [^{14}C]GA_5 was hardly catabolised (Figure 6). The small amount of a GA_6-like product in fraction 18 fits with our previous in-vitro evidence that GA_5 is converted by a 3-oxidase to GA_6 (King et al., 2004). As GA_5 and GA_{20} only differ structurally in their C-2,3 bond (see Figure 1), it is presumably the double bond of GA_5 which protects it from 2β-oxidation.

To examine the effects of LD on GA deactivation, we supplied [^{14}C]GA_{20} or [^{14}C]GA_4 for 6 h to batches of shoot tips isolated in SD or at various times after a single florally inductive LD. We report deactivation as percent remaining substrate, since multiple metabolites may form in this assay (see Figure 6). Initially, metabolism was rapid in shoot tips from vegetative plants or for plants exposed to a LD. After 6 h, only 15–30% of extractable counts remained as non-metabolized [^{14}C]GA_{20} or [^{14}C]GA_4 (Table 2). Two or more days after the LD, the rate of metabolism had dropped by ~30%, which matches the relatively low-level *LtGA2ox5* expression seen 3 d after the LD (Figure 3). The late decrease in [^{14}C]GA_4 metabolism (Table 2) may reflect its potential for 16,17-epoxidation as well as 2-oxidation, but the spread of metabolites detected on HPLC was too great to allow us to make this conclusion with any confidence.

Endogenous GA_1 and GA_4 become detectable in the *L. temulentum* shoot apex several days after a single LD (King et al., 2001), which coincides with decreased metabolism of GA_4 and GA_{20} by this time (Table 2). Additional assays with [^{14}C]GA_4 (but in duplicate, not triplicate) confirmed these findings and its metabolism was further reduced following repeated LD exposures (data not shown). Such repetition of the LD causes both more rapid flowering and an earlier and more dramatic increase (by up to 10-fold) in shoot apex GA_1 and GA_4 content (King et al., 2001).

Studies with an Inhibitor of GA Metabolism and with GA Structural Variants

The evidence above of differences in metabolism of GAs (GA_4 and $GA_{20} > > > GA_5$; Figure 6) and of distinctive effects of GA structure on florigenic activity (Figure 2) highlights the possibility of using differences in GA structure to assess the relationship between GA deactivation and flowering. Therefore, we examined effects of further structural changes to GAs along with application of Trinexapac Ethyl (TNE)—a 2-oxidase inhibitor (Rademacher, 2000).

TNE and GA_4 caused flowering when applied together to the leaf of *Lolium perenne* in SD (Figure 7). Given individually, neither TNE nor GA_4 had any effect on flowering. Thus, their effectiveness, when applied together, indicates specificity of TNE as an inhibitor of 2-oxidases and, potentially, with no side effects, of TNE. Consistent with this conclusion, there was no

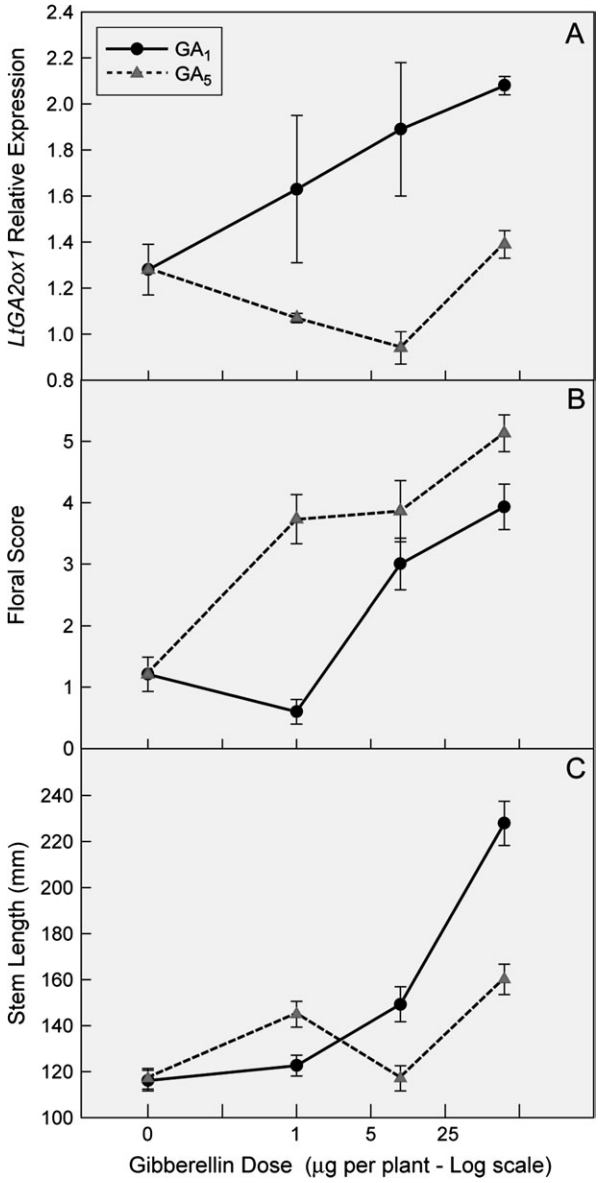

Figure 5. Effect of the DOSE of Leaf Applied GA_1 (●) or GA_5 (Δ) (25 μg per plant) on Expression of *LtGA2ox1* in the Top 3.5 mm of the Shoot Tip of *L. temulentum*.

Plants were maintained under 8 h SD and harvests were 3 d after GA treatment. Flowering and stem elongation are shown for the same experiment. Without GA application, the plants continued to grow vegetatively. Values are means ± S.E. (n = 3 for (A) and 14 for (B) and (C)).

additional flowering in response to TNE applied with 2,2-dimethyl GA_4—a finding we expected because this synthetic GA is already protected against 2-oxidation (Figure 7). However, this latter evidence is not compelling because the dose of 2,2-dimethyl GA_4 may have been saturating and this may have hidden any effect of TNE.

In similar studies with *L. temulentum*, TNE applied simultaneously with GA_1 or GA_4 increased their florigenicity (Figure 8).

Table 1. Effect of different applied GAs (5 μg per plant) on expression of *LtGA2ox1* in the shoot tip of *L. temulentum* (top 3.5 mm).

	Effect of applied GA on gene expression (all data relative to untreated LD control plants)			
	LD	GA_1	GA_4	GA_5
LtGA2ox1	1.00	2.67 ± 0.31	2.30 ± 0.13	1.65 ± 0.27

Tissue was harvested 3 d after GA application. All plants were exposed to a single LD at the start of the treatment. Values are means ± S.E. (n = 3).

Untreated plants were vegetative in SD and TNE alone caused no flowering. Only plants with a score of 2 and above are considered floral (cf. McDaniel et al., 1991) so GA_4 was only florigenic when both 2-oxidation was restricted by co-application of TNE and its epoxidation was hindered, as in 16,17-dihydro GA_4, or by adding a 13-OH group, as in GA_1 (Figure 8). Protection against 16,17 epoxidation by C-13 hydroxylation was clear in the study of Zhu et al. (2006) and this protection may explain the greater florigenicity of GA_1 relative to GA_4, the latter GA lacking a C-13 hydroxyl (Figure 1). Structural change at C-16,17 (as in 16,17-dihydro GAs) had less of an effect on florigenicity than the presence of a C-13 hydroxyl.

To help localize the site of action of 2-oxidases, TNE was injected in water into the air space above the shoot apex of *L. temulentum* and, at the same time, GA_4 was applied in ethanol to the leaf. These plants were also exposed to a single LD to allow better visualization of the weak response to GA_4. When given alone, neither applied chemical had any effect on flowering relative to an untreated control (mm floral shoot apex length: LD, 2.1 ± 0.08; LD plus GA_4, 2.23 ± 0.07; LD plus TNE, 2.17 ± 0.07; n = 14). However, flowering was promoted following co-application of GA_4 to the leaf and TNE to the apex (mm floral shoot apex length: 2.68 ± 0.11). This result is consistent with TNE acting at the shoot tip to restrict 2β-oxidation and inactivation of GA_4 during its transport from the leaf to the shoot apex.

The importance of protection against epoxidation and 2-oxidation was further supported by our application of structural variants of GA_1 and GA_4. Presence or absence of a C-13 hydroxyl was combined with an α- or β-hydroxyl at C-2. The presence of the C-13 hydroxyl gave consistently better flowering in paired comparisons across three GAs (Figure 9A). Also, the addition of a 2β hydroxyl depressed GA activity, ($GA_8 < GA_1$ or $GA_{34} \leqslant GA_4$) but more so for stem elongation than for flowering (Figure 9). Most striking is the greater flowering in response to 2α-hydroxylation, as in GA_{56} and GA_{47} (Figure 9A), but their florigenicity is no better than for GA_5 (data not shown).

Our observations raise questions about the extent that 2β-oxidation inactivates a GA (reviewed in Thomas and Hedden, 2006). For stem elongation of *L. temulentum*, the 2β-oxidation products of GA_1 and GA_4 (i.e. GA_8 and GA_{34}, respectively), were not completely inactive (Figure 9). It is also not clear why GA_{56} had such a weak effect on stem elongation (Figure

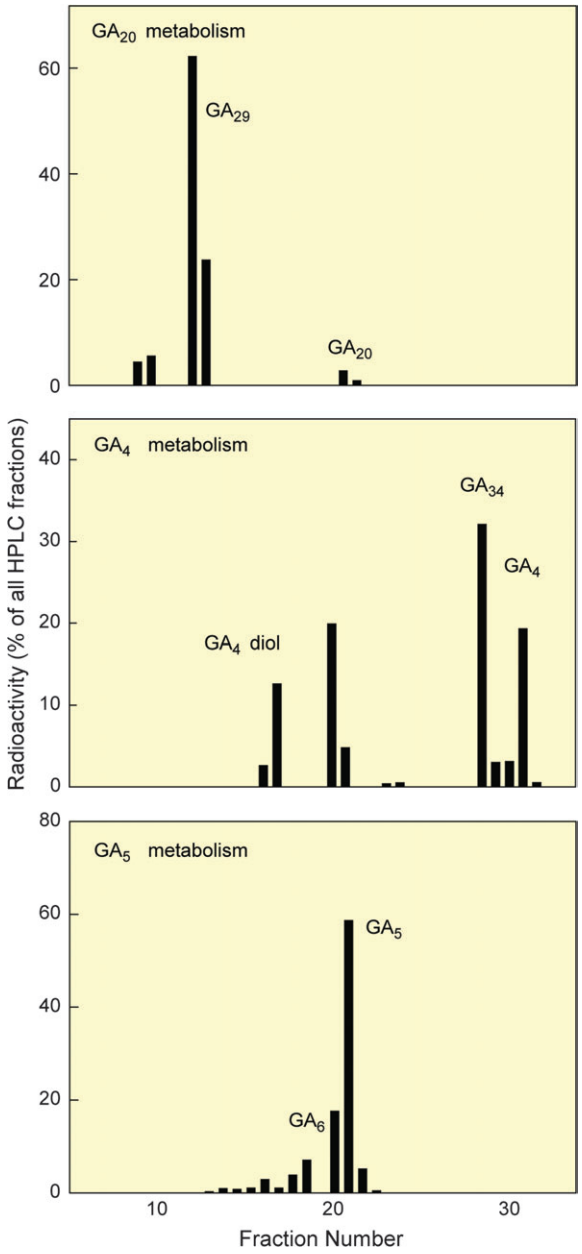

Figure 6. Metabolism of Radiolabelled GAs as Shown by C-18 Reversed Phase HPLC of Methanolic Extracts of Isolated Shoot Tips of *L. temulentum* Harvested 6 h after Incubation on Agar Containing [^{14}C]labelled GA.

HPLC fractions of 1 mL were collected every minute and counted in a liquid scintillation spectrometer. The named peaks relate to elution times for standards. The values on the histograms are averages of at least three separate extractions and HPLC runs.

9B), but the same result was found in a repeat experiment. Nevertheless, when taken together, these results show that protection against deactivation at C-2 by a 2α hydroxyl in combination with protection at C-13 creates a more florigenic GA, but also that, for stem elongation, such protection is less important.

Table 2. Metabolism of [^{14}C]GA_4 or [^{14}C]GA_{20} Supplied to Isolated Shoot Tips Incubated on Agar for 6 h at Various Times before (0 d), during (1 d), or after (2–4 d) Exposure of *L. temulentum* to a Single LD.

	Non-metabolized GA substrate (% of total extracted counts)				
Substrate supplied	Day from start of LD				
	0	1	2	3	4
[^{14}C]GA_{20}	20.5 ± 2.9	15.1 ± 2.7	56.5 ± 6.1	41.8 ± 6.1	50.9 ± 9.4
[^{14}C]GA_4	29.8 ± 0.3	19.1 ± 1.1	16.7 ± 1.5	20.6 ± 3.8	42.8 ± 2.9

Counts are expressed as percent remaining substrate of either GA_4 or GA_{20} (i.e. counts remaining as substrate as percent of total radioactivity). Values are means ± S.E. (n = 3).

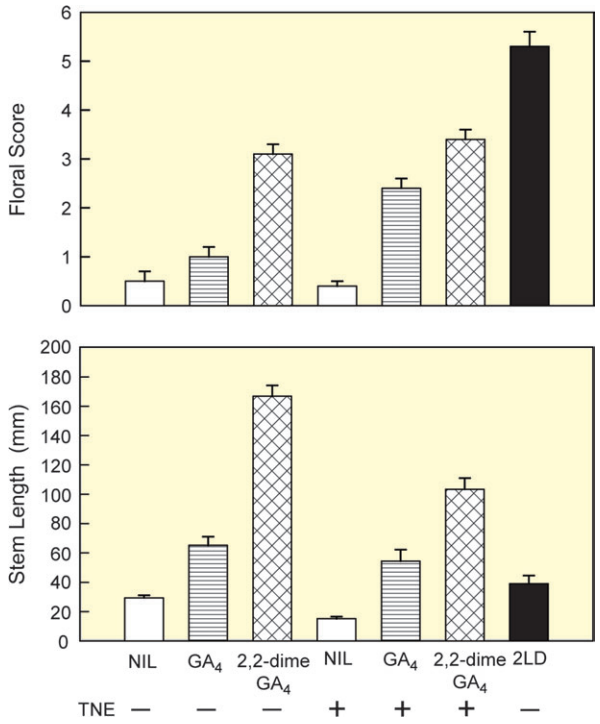

Figure 7. Applied GAs (25 μg per plant) Can Replace the Need for LD in the Induction of Flowering and Enhance Stem Elongation of Vernalized Plants of *Lolium perenne* Held in SD.

GA_4 became active when applied simultaneously with TNE (25 μg per plant), which would inhibit 2-oxidation. For comparative purposes, the response to 2 LD is also shown. Floral Scores of 2 or more indicate a floral apex. Values are means ± S.E. (n = 14).

DISCUSSION

GA synthesis, deactivation, pool sizes, receptor affinities, and specificities as substrate for deactivation enzymes will all determine their effectiveness for flowering. Here, we show that GA deactivation is important in GA-regulated flowering. The two 2β-hydroxylases we examined and a putative 16,17 epoxidase would all deactivate bioactive GAs and they express most highly in or just below the shoot apex and probably in the vascular tissue, where GAs would be unloaded in their transport

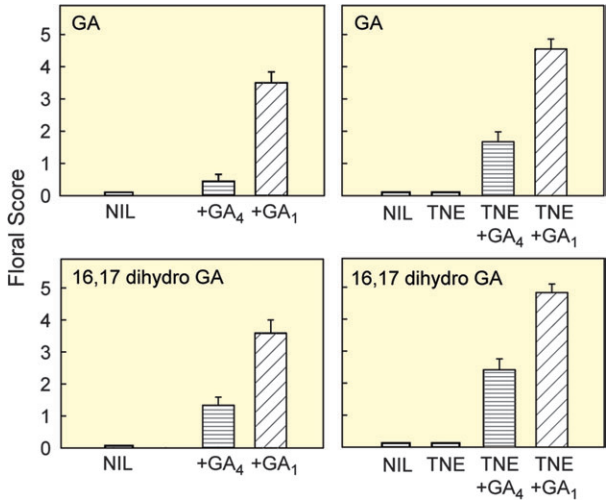

Figure 8. Effect of GA_1 and GA_4 on Flowering (Floral Score). of *L. temulentum* in SD in Association with Structural Hindrance at C-16,17 or with a Co-application of Trinexapac Ethyl (TNE) to Inhibit 2-oxidation.

All chemicals applied at 25 μg per plant. Values are means ± S.E. (n = 12).

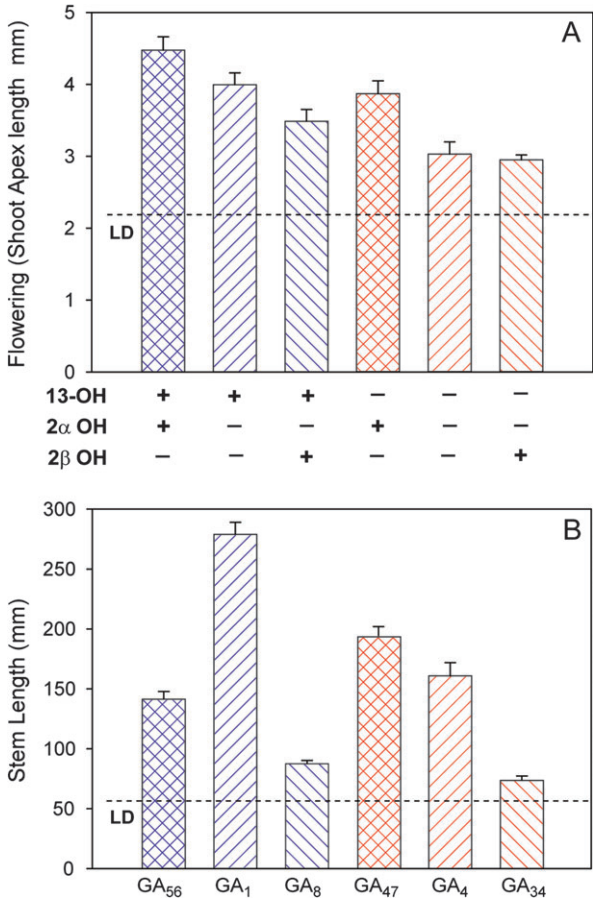

Figure 9. Effect of Modifying the Functional Groups at Carbon-2 and 13 on the Efficacy of Gibberellins for Flowering of *L. temulentum* (Shoot Apex Length: mm) or for Stem Elongation (mm).

All treatments involving C-13 hydroxylated GAs are shown in blue. GAs (25 μg per plant) were applied to the leaf blade prior to exposure to a single LD. LD control plants were treated with the same solvent. Means ± S.E. (n = 14).

from the leaf to the shoot apex. We show how differences in substrate preference of these enzymes make various GAs more or less stable and, so, more or less florigenic. Ready deactivation of GA_1 and GA_4 in isolated shoot tips but not of structurally protected GA_5 shows why GA_5 is an effective endogenous LD floral signal in the grass *Lolium*. Later, during floral development, we note that GA deactivation is reduced and then GA_1 and GA_4 can take on a regulatory role in flowering.

Localized Enzyme Expression and Substrate Preferences

Of the enzymes examined here, *LtGA2ox5* with its high activity in SD would rapidly degrade GA_1 and GA_4 and so interfere with their transport into the vegetative shoot apex. The supply of GA_{20} would be affected by *LtGA2ox1* and more so in LD than in SD. Additionally, the 16,17-epoxidases would inactivate GA_4 and, if our putative *Lolium* gene is a true epoxidase, its expression indicates activity in all day lengths. Further enhancing selectivity for deactivation, GA_1 and GA_4 induce 2-oxidase transcription in shoot tips (cf. Figures 2 and 5, Table 1 and evidence cited in Thomas and Hedden, 2006) but GA_5 causes less feed-forward induction of GA 2-oxidase (Figure 5, Table 1). A further class of GA deactivation enzymes were reported recently for *Arabidopsis*—the GA-methylases (Varbanova et al., 2007). However, the published Northern blot assays show that their expression is restricted to siliques, so they may not be relevant in the control of GA import into the shoot apex.

The importance of localized, sub-apical deactivation of GA_1/GA_4 but not of GA_5 in vegetative plants is shown by three independent lines of our evidence. First, during the 2 d after a florally-inductive LD, expression of GA deactivation genes is greater in the sub-apical vasculature than it is some millimetres further down the stem. This expression analysis confirms and considerably extends in-situ expression studies with rice (Sakamoto et al., 2001) and 2-oxidase promoter-GUS expression studies in *Arabidopsis* (Jasinski et al., 2005). Second, our activity assays with isolated shoot tips showed stability of GA_5 but rapid deactivation of GA_1, GA_4, or GA_{20} (Figure 6, and see Junttila et al., 1997). Third, access of endogenous GAs to the shoot apex matches their stability. GA_5 is present in vegetative shoot apices but GA_1 and GA_4 are absent or barely detectable (King et al., 2001). Similarly, the shoot apex content of GA_{20}, which is the precursor of GA_1, is less than 10% of that in sub-apical stem tissue (GA_{20} 1.0 ± 0.1 ng g^{-1} dry weight in the shoot apex but 13.9 ± 2.2 ng g^{-1} dry weight 3–6 mm below the shoot apex; King and Moritz, unpublished data). Thus, we now have a basis for understanding why GA_5 is most effective for flowering of *L. temulentum*.

GA Deactivation and Flowering

A number of our findings address the question of the relationship between sub-apical GA deactivation and flowering. First,

GAs susceptible to deactivation—GA_1 and GA_4—are weakly florigenic (Evans et al., 1990) and not detected in the shoot apex of vegetative plants or during the first days after exposure to a florally inductive LD. In contrast, GA_5 is not deactivated and, when applied, it is strongly florigenic. Furthermore, following the rapid ($<$4 h) increase in GA_5 in a leaf exposed to a florally inductive LD (King et al., 2006), its content in the shoot apex doubles within hours (King et al., 2001). Second, structural change to protect GA_1 and GA_4 at sites crucial for attack by 2-oxidases and 16, 17-epoxidases (C-2; C-13 and C-16,17; see Figure 1) enhances their florigenicity and this is especially apparent for 2,2-dimethyl GA_4—a GA form which is not metabolized in vitro by a plant 2-oxidase (Yamauchi et al., 2007). Less dramatic but similar protection at C-2 is evident from the increased florigenicity of the 2α-hydroxylated derivatives, GA_{56} and GA_{47}: both of these GAs are more active than their C-2 β-hydroxylated forms, GA_8 and GA_{34}, respectively (Figure 9). This set of structural comparisons (Figure 9) also highlights the value of the C-13 hydroxyl—an effect attributable to protection against C-16,17-epoxidases (Zhu et al., 2006) by reduced nucleophilicity at C-16,17 associated with electron withdrawal. Third, GA_4 becomes florigenic when 2-oxidase activity is blocked by simultaneous application of the 2-oxidase inhibitor, TNE (Figures 7 and 8). This interaction was apparently near the shoot apex because TNE applied to the shoot tip enhanced the effectiveness of GA_4 applied to the leaf. Thus, sensitivity/insensitivity to metabolism is central for florigenicity of a GA.

Taken together, selective deactivation of GA_1/GA_4 limits their florigenicity. In contrast, the greater stability of GA_5 and GA_6, endogenous GAs of *Lolium* (see King et al., 2001, 2003) allows them to act 'by default', as LD up-regulated transported signals that control early events of floral initiation of *Lolium* (see King et al., 2003, 2006). This 'by default' early action of GA_5 is consistent with the evidence that GA_4 and GA_1 can be intrinsically florigenic if protected against deactivation (Figures 2, 6, 7 and 8). It is also supported by our evidence that GA_4 becomes florigenic later in floral development (King et al., 2001). At this time, there is an associated reduction of $\sim$30% in the rate of GA_4 metabolism (Table 2) and endogenous GA_1 and GA_4 become detectable in the shoot apex (King et al., 2001). Interestingly, a rice 2-oxidase studied by Sakamoto et al. (2001) shows reduced sub-apical expression at this time and, in a preliminary experiment, we have detected a similar reduction in expression of *LtGA2ox1* (King, unpublished).

Although not central to our focus on flowering, GA_1 and GA_4 stimulate stem elongation much more effectively than GA_5 (e.g. Figures 2 and 5). This is consistent with the shoot tip tissues showing a dominant effect involving protection against deactivation. Further from the shoot apex (Figure 3: basal stem segments 4–5 mm below the apex and just above the zone of lateral root formation), reduced deactivation of GA_1 and GA_4 would allow them to dominate by virtue of their greater receptor-binding effectiveness. This claim is supported by evidence that GA_1 and GA_4 are100–500 times more effective than GA_5 for amylase production by barley half-seeds, where neither GA synthesis nor degradation are likely to be important (King et al., 2004 and references therein). Overall, reduced GA_1 and GA_4 deactivation allows them to act on stem elongation and as the most important effectors because of their high receptor-binding capacity. By contrast, in the same plant, GA_5 can dominate shoot apex responses because it resists degradation and so is effective despite its potentially low receptor-binding capacity.

Unlike *L. temulentum*, in which only endogenous GA_5 and GA_6 increase at the shoot apex to regulate its flowering, with *Arabidopsis*, GA_1 and GA_4 increase in its shoot tip when it flowers in SD (Eriksson et al., 2006) and LD increases GA_4 levels in the shoot (Gocal et al., 2001). Also, unlike *Lolium*, in which applied GA_4 is ineffective for flowering (Figures 7 and 8), in SD, it causes flowering of *Arabidopsis* (Xu et al., 1997; Gocal et al., 2001; Eriksson et al., 2006) and of other LD-responsive species, including *Matthiola incana* (Hisamatsu et al., 1998) and *Thlaspi arvense* (Metzger, 1990). Thus, GA deactivation is apparently unimportant for flowering of these other LD species. As a further contrast, some annual dicots, including *Pisum sativum* (Reid et al., 1977) and *Sinapis alba*, do not require GA for their flowering and, not surprisingly, there is no evidence for a florigenic role for GA_5 (Corbesier et al., 2004). For another LD dicot—*Fuchsia hybrida*—GAs actually inhibit flowering, but this involves a unique response to GA associated with an over-stimulation of stem elongation and a diversion of photosynthate away from the shoot apex (King and Ben-Tal., 2001).

Overall, while GAs are clearly not a universal florigen, they play an important role in the flowering of a wide range of higher plant species (Pharis and King, 1985; King and Evans, 2003; Eriksson et al., 2006). Our focus here on their deactivation has provided a more substantial but more complex understanding and one which may be more relevant to flowering of grasses and cereals. The scenario of a localized deactivation of the most growth-active GAs allows for GA regulation of flowering while restricting/delaying stem elongation. Such a mechanism could be critical for grasses to survive extreme winter cold and grazing by animals. Interestingly, the late attenuation of GA_4 deactivation is consistent with enhanced stem elongation later in floral development. In contrast, dicot annuals, including *Arabidopsis*, which would have evolved without this selection pressure, flower in response to GA_4 and bolt at the same time as they initiate flowers. Unlike the grasses, herbaceous dicots may not have needed to acquire a mechanism for restricting access of highly growth-active GAs to the shoot apex.

METHODS

Plant Material and Growing Conditions

As described previously (MacMillan et al., 2005; King et al., 2006), plants of *Lolium temulentum* L. strain Ceres and a clone of *Lolium perenne*, CPM-1, were grown vegetatively in 8-h, short day (SD) photoperiods in sunlit controlled environment

cabinets. Both species remained vegetative in SD and could be induced to flower by a single LD for *L. temulentum* or by two LD for *L. perenne* after it had been vernalized at 8°C for 8 weeks in SD. We report flowering as either shoot apex length or floral score because these measures are equivalent (see McDaniel et al., 1991). There were 10–14 replicates per treatment and values for flowering and stem length are the mean ± S.E.

Leaf applications of GAs or TNE, Trinexapac-ethyl (Primo ^{TM}), ethyl[4-(1′-cyclopropyl{1′-hydroxy}methylene)-3,5-dioxocyclohexane-1-carboxylate], were in 95% (v/v) ethanol/water, as outlined previously (King et al., 2006). The culturing of excised shoot apices for analysis of GA metabolism was described in Junttila et al. (1997).

Expression of *L. temulentum* GA-2-oxidases and a 16,17-epoxidase

Common steps of GA deactivation often involve enzymes from conserved, multigene families but with differing substrate specificities (e.g. Thomas et al., 1999; Lester et al., 1999; Spielmeyer et al., 2004; Thomas and Hedden, 2006). Thus, *Lolium* likely has several GA 2-oxidases and we have examined two of these. *LtGA2ox1* will metabolize GA_{20} to GA_{29} but not GA_1 to GA_8 (King et al., (2006). The second gene, *LtGA2ox5* (GenBank # EF687858), was identified in a *L. perenne* EST collection using a barley 2-oxidase (*Hv2ox5*: AY551433) as a query sequence in a Blastn search. As for *Hv2ox5*, when transiently expressed in a reticulocyte assay (Spielmeyer et al., 2004), in 2 h, the protein of *LpGA2ox5* almost completely converted [^{14}C]GA_1 to its 2β-hydroxylated product [^{14}C]GA_8. [^{14}C]GA_4 and [^{14}C]GA_{20} were also metabolized by this enzyme (results not shown).

To analyze expression of a *L. temulentum* GA 16,17-epoxidase, we began with a *L. rigidum* cytochrome P450 gene, *Lol-31-j* (GenBank # AF321861; Fischer et al., 2001). This gene shows 41% amino acid homology to a rice GA 16,17-epoxidase, CYP714D1 (Zhu et al., 2006). Primers based on *L. rigidum* sequence were able to amplify the predicted epoxidase product from *L. temulentum* cDNA and there was 98% amino acid identity for a 197-bp product. Similarly, genes from *L. temulentum* and *L perenne* show very high amino acid identity (e.g. 97% for *GA20oxidase1* and *CONSTANS*: King et al., 2006, and 97% between *LtGA2ox5* and *LpGA2ox5* (not shown)).

The primer pairs used for *LtGA2ox1* were as described before (King et al., 2006); those for *LtGA2ox5* and *LtGA16, 17epoxidase* studies were:

LtGA2ox5: Forward: CAGGGCTTCTTCAAGGTGAC; Rev: GTGAGGCAGAGCAGGAGGTA

Putative LtGA16,17epoxidase: Forward: GATGGAGGAAGCAGGGTGTA; Rev:CAAGCAAGTGAGGGAAGAGG

For gene expression studies, hand-cut stem cross-sections 0.83 ± 0.05 mm thick, excluding leaf primordia, were collected sequentially from the shoot apex down and immediately frozen in tubes held on dry ice and combined with successive harvests of matching sections from 10–14 plants. The bulked samples were stored at –80°C until ground for RNA extraction. In related expression studies, the shoot tip (0–3.5 mm) was taken. Total RNA was isolated using an RNeasy mini kit (Qiagen USA, Valencia, CA).

The conditions for quantitative PCR assays were as detailed in King et al. (2006). For normalization, we included a commercially available mouse liver RNA. The Q-PCR analysis was performed using the 'Comparative Quantification' method with Rotogene 4.5 or 5.0 Software (Corbett Research).

To examine localization below the apex of *LtGA2ox1* mRNA, 10-μm-thick cross-sections were prepared from fixed, paraffin-embedded shoot tips. In vitro transcribed DIG-labelled riboprobes were synthesized for a 3′ gene-specific 360-bp fragment (nucleotide 1118–1477). Using *Eco*RI:*Hin*dIII to excise the nucleotide fragment inserted in pBluescript SK(+) or pBluescript KS(+), sense and antisense riboprobes were generated from the T7 promoter. Hybridizations were at a high stringency, with an equivalent sense or anti-sense probe concentration and were performed simultaneously on each tissue set. The basic in-situ hybridization protocol was as described in Ferrándiz et al. (2000).

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