

# Changes in Gibberellin A<sub>1</sub> Levels and Response during De-Etiolation of Pea Seedlings<sup>1</sup>

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The level of gibberellin A<sub>1</sub> (GA<sub>1</sub>) in shoots of pea (*Pisum sativum*) dropped rapidly during the first 24 h of de-etiolation. The level then increased between 1 and 5 d after transfer to white light. Comparison of the metabolism of [<sup>13</sup>C<sup>3</sup>H] GA<sub>20</sub> suggested that the initial drop in GA<sub>1</sub> after transfer is mediated by a light-induced increase in the 2β-hydroxylation of GA<sub>1</sub> to GA<sub>8</sub>. A comparison of the elongation response to GA<sub>1</sub> at early and late stages of de-etiolation provided strong evidence for a change in GA<sub>1</sub> response during de-etiolation, coinciding with the return of GA<sub>1</sub> levels to the normal, homeostatic levels found in light- and dark-grown plants. The emerging picture of the control of shoot elongation by light involves an initial inhibition of elongation by a light-induced decrease in GA<sub>1</sub> levels, with continued inhibition mediated by a light-induced change in the plant's response to the endogenous level of GA<sub>1</sub>. Hence the plant uses a change in hormone level to respond to a change in the environment, but over time, homeostasis returns the level of the hormone to normal once the ongoing change in environment is accommodated by a change in the response of the plant to the hormone.

The inhibition of stem growth by light has been studied for decades. Pioneering work on pea (*Pisum sativum*; Lockhart, 1956; Kende and Lang, 1964) indicated that the inhibition could be reversed by the application of exogenous gibberellic acid (GA<sub>3</sub>), suggesting a role for GAs in mediating the light response. This work also suggested the involvement of phytochrome since the response to light was red (R)/far red (FR)-reversible (Lockhart, 1956).

Even at this early stage two main theories were postulated: (a) That photoreceptor-mediated changes in GA levels result in altered rates of elongation, or (b) that light reduces the sensitivity of the plant to endogenous levels of active GAs.

Recent research on a variety of plant species has provided evidence for both of these theories. Toyomasu et al. (1992, 1998) was successful in demonstrating that the levels of GA<sub>1</sub> in lettuce hypocotyls were controlled by light and suggested that light conditions control hypocotyl elongation via changes in the GA<sub>1</sub> levels in this tissue. This conclusion could be reached as the response of lettuce explants to applied GA<sub>1</sub> was similar both in the dark and in white light (WL).

Continuing work utilizing pea and sweet pea has produced evidence that in continuous light the responsiveness of the plant to the endogenous pool of GA<sub>1</sub> is reduced compared with dark-grown plants. Evidence that light affects the response to GA<sub>1</sub> came from the study of the elongated phyB-deficient mutant of pea, *lv* (Reid and Ross, 1988; Weller et al., 1995). The increased rates of elongation observed in light-grown *lv* plants were not the result of differ-

ences in GA<sub>1</sub> synthesis or metabolism. However, the *lv* mutant exhibited an increased response to all levels of applied GA<sub>1</sub> and this was suggested to be the cause of the elongated phenotype. That *lv* plants showed a reduced elongation response to both photoperiod extension with incandescent light, and to complete darkness suggested that these light treatments may act in a similar manner to the *lv* mutation (phy-B deficient), namely to increase the tissue response to the endogenous level of active GA<sub>1</sub>.

A comprehensive study by Weller et al. (1994) investigated the effects of light and phy B on GA levels and stem elongation. They examined the endogenous levels of GAs in the *lv* mutant (Reid and Ross, 1988) and the light response of the slender (*sln*) mutant, which possesses elevated levels of GA<sub>1</sub> at the seedling stage (Reid et al., 1992), as well as wild-type (WT) plants. Weller et al. (1994) found that dark-grown WT plants were approximately three times longer than light-grown plants in total length and internode length, yet in the apical portions there was no substantial difference in GA<sub>1</sub> levels. In comparison, light-grown *sln* plants had a GA<sub>1</sub> level more than five times that of relevant WT controls, but were still only around twice the height. Light-grown *lv* plants had reduced levels of GA<sub>1</sub> in leaf and upper internode tissue in comparison with *lv* plants, yet grew to twice the total height. These findings tend to cast significant doubt on the theory that the increased length of dark-grown plants relative to plants grown in continuous R or WL is attributable to an increase in the level of GA<sub>1</sub> (Campbell and Bonner, 1986), detectable in analysis of whole seedlings. However, Weller et al. (1994) did find a significant reduction in the level of GA<sub>20</sub> in dark-grown and *lv* plants, as in previous studies (Ross and Reid, 1989; Ross et al.,

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1992b), which may support the effect of light on GA<sub>20</sub> metabolism reported by Sponsel (1986).

Evidence from application studies and work with *sln* and severely GA<sub>1</sub>-deficient double mutant combinations has shown an additive relationship between the level of endogenous GA<sub>1</sub> and light (Weller et al., 1994). Furthermore, the fact that elongation responses to light treatments were maintained even at saturating doses of GA<sub>1</sub> suggests that the phy-B elongation response may operate partially or fully independently of changes in the level of GA<sub>1</sub>. The above evidence suggests that light acts by modifying some step in the GA<sub>1</sub> signal transduction pathway, in agreement with studies on rice (Nick and Furuya, 1993), cucumber (Lopez-Juez et al., 1995), and Arabidopsis (Reed et al., 1996). When the phenotypes of *lv* plants were compared with *Lv* plants with added exogenous GA<sub>1</sub>, it was found that the action of the *lv* mutation could not be directly mimicked by exogenous GA<sub>1</sub> application even though characteristics such as length and leaflet area were similar between the two genotypes (Weller et al., 1994).

As illustrated above, the majority of past work on pea supports the theory (Reid, 1988; Gawronska et al., 1995) that continuous light inhibits elongation by altering the response of plant tissue to the level of active GA. However, recent work by Ait-Ali et al. (1999) and Gil and Garcia-Martinez (1998) has provided strong evidence that in the short term, after transfer from darkness, light has a direct effect on the levels of endogenous GA<sub>1</sub> in elongating pea shoots. The regulation of the later stages of shoot GA biosynthesis were also investigated during the de-etiolation process. When WT etiolated seedlings (6 d of continuous darkness) were transferred to continuous WL, it was found that the level of GA<sub>1</sub> in shoots dropped dramatically within 2 h to 20% of the level found in dark-grown controls (Ait-Ali et al., 1999). The levels of immediate GA<sub>1</sub> precursors, namely GA<sub>19</sub> and GA<sub>20</sub>, were not markedly altered compared with control plants, suggesting that the reduction in the level of GA<sub>1</sub> during de-etiolation was not a result of reduced GA biosynthesis.

The work of Ait-Ali et al. (1999) leaves several important questions unanswered as the last time point for sampling in the data was 24 h after transfer to WL, and at this stage the endogenous level of GA<sub>1</sub> in the shoot was still significantly reduced compared with dark-grown controls. Studies comparing the GA<sub>1</sub> levels in expanding tissue of continuous WL- and dark-grown shoots have shown little difference (Weller et al., 1994). Therefore it might be expected that the GA<sub>1</sub> level in de-etiolating seedlings would increase over an extended time course to a level common to both light- and dark-grown plants. It is of crucial importance to find out what happens to the level of GA<sub>1</sub>, relative to light- and dark-grown plants, for an extended period of time after transfer to WL. If this low level is maintained throughout the de-

etiolation process, then it is probable that the inhibition of elongation by light is due to a direct effect on GA biosynthesis. If, however, the level of GA<sub>1</sub> increases after the initial drop while elongation remains inhibited, then a combination of light-induced changes to GA biosynthesis and light-induced variation in the response of the plant to the level of endogenous GA<sub>1</sub> may best explain the control of this developmental change.

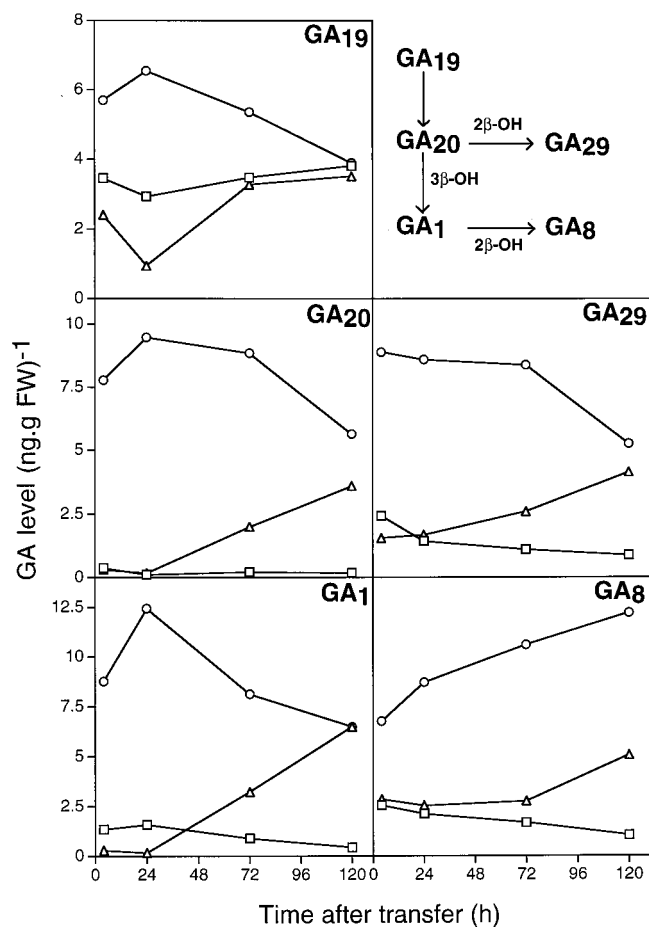
The current work was designed to answer these questions, with a range of experiments encompassing the measurement of endogenous GA levels, the metabolism of radiolabeled GAs, and the elongation response to GA<sub>1</sub> application, under differing light regimes. The combination of these techniques has given comprehensive insight into the effect of light on specific steps in the GA biosynthetic pathway, and provides evidence that the transfer from dark to light acts to first reduce the level of GA<sub>1</sub> and then the response of pea seedlings to endogenous levels of GA<sub>1</sub>. The resulting model invokes both of these processes in describing how the shoot elongation of pea seedlings is controlled by the light environment and draws together disparate views that have existed in the literature for 40 years.

## RESULTS

### Changes in GA Levels during De-Etiolation

Comparison of the GA<sub>1</sub> concentration in shoots of WT plants transferred from dark to light with dark-grown and WL-grown controls shows that transferred plants contained relatively little GA<sub>1</sub> 4 h after transfer, as found previously (Ait-Ali et al., 1999; Fig. 1). This is consistent with measurements that show that elongation in cv Torsdag is maximally inhibited about 3 h after exposure to continuous R light (Behringer et al., 1990). The reduction in shoot GA<sub>1</sub> concentration was maintained to the 24-h sample point, where the GA<sub>1</sub> level was 70 times less than found in WL-grown controls and more than eight times less than dark-grown controls (measured on a whole shoot basis). Between the 24- and 72-h sample points, the level of GA<sub>1</sub> increased, and this trend continued to 120 h, when the final measured concentration was comparable with WL-grown controls (6.5 ng g fresh weight<sup>-1</sup>) and significantly higher than dark-grown controls (0.4 ng g fresh weight<sup>-1</sup>, measured on a whole shoot basis).

On transfer to WL, GA<sub>20</sub> levels did not alter substantially, with similar GA<sub>20</sub> concentrations to dark-grown controls at the 4- and 24-h sample points (Fig. 1). Plants sampled at 72 and 120 h after transfer to WL showed a steady increase in GA<sub>20</sub> content, to a maximum of 3.6 ng g fresh weight<sup>-1</sup>, whereas dark-grown controls at these time points contained a steady low level. The GA<sub>8</sub> content of transferred plants became consistently higher than dark-grown controls as time passed and reached a maximum at



**Figure 1.** Comparison of the level of 13-hydroxylated GAs in continuously dark-grown ( $\square$ ), transferred ( $\Delta$ ), and continuously light-grown WT plants ( $\circ$ ) over a de-etiolation timecourse of 120 h. Transferred plants were grown in continuous darkness for a period of 7 d then subjected to continuous WL treatment for the time period shown. Continuous dark and light data represent the relevant GA level in these plants at the time of sampling transferred plants. 2 $\beta$ -OH, 2 $\beta$ -hydroxylation; 3 $\beta$ -OH, 3 $\beta$ -hydroxylation.

120 h after transfer of 5.1 ng g fresh weight<sup>-1</sup> (Fig. 1). In general, the concentrations of GA<sub>29</sub> in transferred plants (Fig. 1) were intermediate between dark-grown and WL-grown controls, with the GA<sub>29</sub> content of transferred plants late in de-etiolation approaching that found in continuous light-grown controls. Similar results were found for all GAs measured in a replicate experiment of dark-grown and transferred plants at 4 h and show that the drop in

the GA<sub>1</sub> level at 4 h after transfer to light is significant ( $P < 0.01$ ; Table I). The rise in GA<sub>1</sub> levels 120 h after transfer was also confirmed (see Fig. 2).

### Spatial Distribution of GA<sub>1</sub>

Analysis of the distribution of GA<sub>1</sub> in 12-d-old peas grown under differing light regimes yielded some novel results. Figure 2 shows the distribution of GA<sub>1</sub> in the apical, upper internode and lower internode portions of the whole shoot under each light treatment. The most striking result was the distribution of GA<sub>1</sub> in dark-grown plants, which exhibit an extremely strong concentration gradient down the shoot to the extent that basal portions contained no measurable amounts of GA<sub>1</sub> on a nanogram per gram fresh weight basis (Fig. 2). In plants transferred 5 d previously from darkness the highest GA<sub>1</sub> concentration was in the upper internodes, not in the apical portion. The GA<sub>1</sub> level of 12.2 ng g fresh weight<sup>-1</sup> in the upper internodes of transferred plants was higher than the same tissue in light-grown plants (6.7 ng g fresh weight<sup>-1</sup>) and only slightly less than in the apical portion of light-grown plants (15.0 ng g fresh weight<sup>-1</sup>). This shows that 5 d after transfer from darkness, the level of GA<sub>1</sub> in the elongating internodes of transferred plants is at least comparable with the level found in continuous light-grown plants.

The level of GA<sub>1</sub> in the apical portion of dark-grown plants was similar to the same section of light-grown plants and plants transferred to WL for 5 d (Fig. 2). This result illustrates the problems with comparing the GA<sub>1</sub> content of dark-grown plants at the whole shoot level, as the high proportion of lower internode tissue with reduced levels of GA<sub>1</sub> serves to dilute the upper internode tissue containing high levels of this GA. This phenomenon produces significant differences in the overall GA<sub>1</sub> content at the whole shoot level when comparing dark-grown and light-grown plants (see Fig. 1), even though the levels in the growing apical region are similar.

### GA<sub>20</sub> Metabolism during De-Etiolation

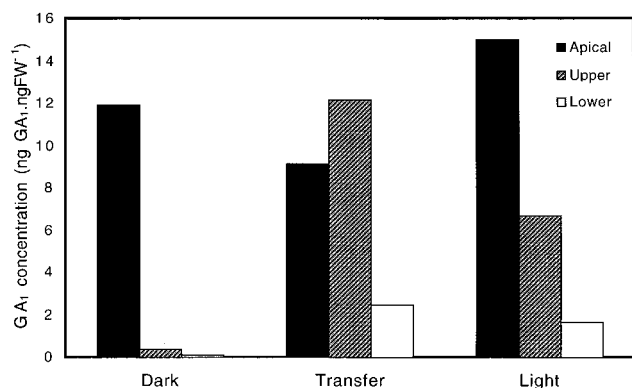
From the data collected by HPLC-radiocounting, it is clear that the change in light conditions from continuous darkness to WL has a marked effect on the metabolism of labeled GA<sub>20</sub> in the upper expanding internodes of pea (Fig. 3). The effect on GA<sub>20</sub> metab-

**Table I.** Gibberellin levels in dark and transferred WT seedlings

The transferred seedlings were transferred to white light from the dark 4 h before harvest. Seedlings were harvested at soil level. Means  $\pm$  SE of two replicates are shown.

	GA <sub>20</sub>	GA <sub>1</sub>	GA <sub>29</sub>	GA <sub>8</sub>
	ng g <sup>-1</sup> fresh wt			
Transferred	0.21 $\pm$ 0.11	0.19 $\pm$ 0.09	1.4 $\pm$ 0.18	2.8 $\pm$ 0.03
Dark	0.29 $\pm$ 0.09	1.4 $\pm$ 0.03	1.8 $\pm$ 0.67	2.3 $\pm$ 0.28





**Figure 2.** The spatial distribution of GA<sub>1</sub> in the apical, upper internode (upper) and lower internode (lower) tissue of WT peas grown for 12 d in continuous WL (light), continuous darkness (dark), or grown for 7 d in darkness then transferred to WL for 5 d (transfer). GA<sub>1</sub> concentration is based on a grouped sample of 15 plants.

olism was evident after only 4 h of transfer to WL. In comparison with dark-grown controls the ratio of the percentage of label in the GA<sub>1</sub>-like peak to the GA<sub>8</sub>-like peak was reduced (0.8:1 in transferred plants compared with 2.4:1 in dark-grown plants; Fig. 3). On the other hand, there was no evidence that the step from GA<sub>20</sub> to GA<sub>29</sub> was promoted by transfer to light. At 24 h after transfer a very similar picture unfolds. Again the ratio of the GA<sub>1</sub>-like peak to the GA<sub>8</sub>-like peak was reduced in transferred plants compared with dark-grown controls (Fig. 3); in fact, the light-mediated change in GA<sub>1</sub> metabolism seems more pronounced at the 24-h sample point. The 24-h transfer experiment was replicated with very similar findings; the ratio of GA<sub>1</sub> to GA<sub>8</sub> across the two replicates was  $0.16 \pm 0.005$  for the transferred plants and  $0.67 \pm 0.07$  for the dark grown plants.

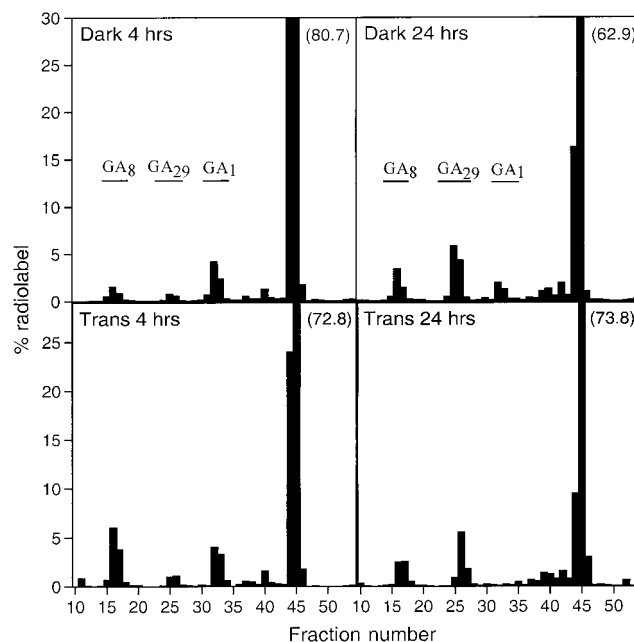
#### Variation in GA<sub>1</sub> Response during De-Etiolation

To investigate the effect of light on GA<sub>1</sub> responsiveness, plants of genotype *na* (extreme dwarf) were sown at the same time and then grown either in continuous darkness, continuous light, or transferred from darkness to light at two different times. In each case, one-half of the plants were treated with GA<sub>1</sub> 6 d after sowing. Total plant height was measured at 1, 2, and 3 d after GA<sub>1</sub> application (Table II). Continuous dark-grown plants showed the greatest response to GA<sub>1</sub> (i.e. difference between elongation of control and GA<sub>1</sub>-treated plants). Plants transferred to light 1 d after GA<sub>1</sub> treatment showed a smaller response to GA<sub>1</sub> than continuous dark-grown plants (Table II;  $P < 0.001$  for d 2–3). The response was smaller still in plants that had been transferred to light 1 d before GA<sub>1</sub> treatment ( $P < 0.05$  for d 2–3). The least response to GA<sub>1</sub> was observed in plants grown in continuous light. These data strongly indicate that as the extent of de-etiolation, or length of exposure to

light increases, the elongation response to GA<sub>1</sub> decreases.

#### DISCUSSION

Quantification of GA<sub>1</sub> levels during de-etiolation has revealed that the drop in GA<sub>1</sub> in plants during the early stages of de-etiolation is a short-term phenomenon. This study successfully replicated the findings of Ait-Ali et al. (1999) and Gil and Garcia-Martinez (1998) by demonstrating a significant reduction in the GA<sub>1</sub> content of seedlings harvested 4 and 24 h after transfer to WL (Fig. 1). However, continued measurement reveals a subsequent increase in the level of GA<sub>1</sub> to a point 120 h after transfer where the GA<sub>1</sub> content of transferred plants was comparable with light-grown controls at the same time point (Figs. 1 and 2). This is consistent with the results of Weller et al. (1994) and shows that the continued inhibition of elongation after transfer to WL cannot be attributed to a continued reduction in the GA<sub>1</sub> content of transferred plants, relative to WL-grown controls. The reduction in GA<sub>1</sub> content is limited to a period between the 4- and 72-h sample points after transfer (Fig. 1), yet the inhibition of elongation in expanding internodes continues past the 120-h sample point. Indeed, studies of the elongation of pea under different light regimes (Weller et al., 1994) suggest this inhibition of elongation by WL occurs throughout seedling elongation. Therefore the



**Figure 3.** Analysis by HPLC-radiocounting of the methyl esters of extracts from WT pea seedlings fed [<sup>13</sup>C<sup>3</sup>H]GA<sub>20</sub>. All hormone applications occurred after 7 d of growth in continuous darkness. Dark, Plants kept in continuous darkness; Trans, plants transferred to WL after hormone application. Data are shown as the percentage of total radioactivity in the HPLC run. The retention times of authentic GA standards are indicated, as is the percentage of radioactivity for GA<sub>20</sub> since these are off scale.

**Table II.** Comparative effect of exogenous GA<sub>1</sub> application on the rate of elongation of *na* plants

The plants were either grown in continuous dark, WL, or transferred from continuous darkness to WL conditions. GA<sub>1</sub> was applied directly to internode 2-3, 6 d after planting. Late transfer plants were transferred to WL 1 d after GA<sub>1</sub> application. Early transfer plants were transferred 1 d before GA<sub>1</sub> application. The rate of elongation was calculated by measuring the change in total shoot length after a 24 h period.  $n \geq 14$ .

Treatment	Rate of Elongation (mm d <sup>-1</sup> )	
	D 1-2	D 2-3
Continuous dark control	4.0 ± 0.6	5.2 ± 0.5
Continuous dark applied	30.2 ± 1.3	25.3 ± 1.2
Difference	26.2	20.1
Late transfer control	2.1 ± 0.3	1.5 ± 0.2
Late transfer applied	9.6 ± 0.6	14.2 ± 1.7
Difference	7.5	12.7
Early transfer control	1.7 ± 0.1	1.9 ± 0.3
Early transfer applied	8.5 ± 0.6	10.1 ± 1.5
Difference	6.8	8.2
Continuous light control	3.3 ± 0.2	3.3 ± 0.2
Continuous light applied	9.4 ± 0.8	10.5 ± 1.2
Difference	6.1	7.2

current work, while confirming that GA<sub>1</sub> levels and elongation drop on transfer to WL, casts significant doubt on the theory that shoot elongation is controlled solely by variation in the endogenous level of shoot GA<sub>1</sub>.

The endogenous level of GA<sub>1</sub> precursors and catabolites were also measured during de-etiolation to investigate whether the levels of these GAs suggest likely mechanisms for both the sharp drop and subsequent increase in shoot GA<sub>1</sub> levels. The most likely steps controlling the initial drop in GA<sub>1</sub> levels are the 3β-hydroxylation of GA<sub>20</sub> to GA<sub>1</sub>, and the 2β-hydroxylation of GA<sub>1</sub> to GA<sub>8</sub>. There was little variation in the GA<sub>20</sub> content of transferred plants at 4 and 24 h after transfer to WL (Fig. 1), which suggests that a light-induced change in the rate of GA<sub>20</sub> biosynthesis is not significant. It is interesting that the level of GA<sub>8</sub> in plants sampled 4 and 24 h after transfer was consistently higher than dark-grown controls (Fig. 1), which would be expected if the drop in GA<sub>1</sub> was mediated, at least in part, by an increase in 2β-hydroxylation to GA<sub>8</sub>.

The subsequent increase in GA<sub>1</sub> levels is preceded by an increase in GA 20-oxidase and GA 3β-hydroxylase transcript levels (Ait-Ali et al., 1999), as well as an increase in endogenous GA<sub>20</sub> levels (Fig. 1). A possible explanation is that the drop in GA<sub>1</sub> may act as a signal to up-regulate these transcript levels (perhaps via feedback regulation; Ait-Ali et al., 1999; Ross et al., 1999), which in turn acts to increase the endogenous level of GA<sub>1</sub>. The increase in GA<sub>1</sub> may also be attributed to a down-regulation of the 2β-hydroxylation of GA<sub>1</sub>, although no subsequent

decrease in the level of GA<sub>8</sub> was recorded to coincide with the subsequent increase in GA<sub>1</sub>.

A more definitive explanation of the mechanisms controlling the light-mediated drop in GA<sub>1</sub> levels came from the investigation of GA<sub>20</sub> metabolism during de-etiolation (Fig. 3). It strongly indicates that a light-mediated increase in shoot 2β-hydroxylation of GA<sub>1</sub> to GA<sub>8</sub> is at least part of the mechanism controlling the decrease in GA<sub>1</sub>. This is consistent with the results of Kamiya and Garcia-Martinez (1999). This finding suggests that a change in the light stimulus can have a direct effect on shoot GA<sub>1</sub> deactivation. It is clear that at both the 4- and 24-h sample points there were differences in the metabolism of labeled GA<sub>20</sub> between the two light treatments.

Taken together, these results support a model whereby the transfer from continuous darkness to continuous WL decreases GA<sub>1</sub> levels, at least in part by increasing the 2β-hydroxylation of GA<sub>1</sub> to GA<sub>8</sub>, a step that deactivates the bioactive GA in shoot elongation, GA<sub>1</sub> (Ingram et al., 1984; Ross and Reid, 1989; Ross et al., 1992a). Although this result sets a precedent for the study of GA<sub>1</sub> metabolism in pea, study of cowpea epicotyls (*Vigna sinensis* L.) suggests this is not the first time a change in light treatment has resulted in a direct effect on GA<sub>1</sub> metabolism. Martinez-Garcia and Garcia-Martinez (1992) suggested that exposure to FR light has an effect on both GA 2β- and 3β-hydroxylase activity in addition to altering the sensitivity of tissues to GAs. This work was continued (Martinez-Garcia and Garcia-Martinez, 1995) with convincing evidence that FR exposure reduces the 2β-hydroxylation of GA<sub>1</sub> to GA<sub>8</sub>.

It can be seen that the quantification of endogenous GAs and the investigation of GA<sub>20</sub> metabolism during de-etiolation have outlined some putative mechanisms for a light-mediated drop in endogenous GA<sub>1</sub> in the shoots of pea. The results of GA application at different points during the de-etiolation process (Table II) provide evidence that a combination of fluctuation in the level of shoot bioactive GA and modification of the plants' response to the level of this bioactive GA are the mechanisms by which light controls changes in elongation during de-etiolation. The most logical explanation for the patterns of variation in GA<sub>1</sub> levels is that the initial inhibition of elongation upon transfer to WL conditions is mediated by the drop in GA<sub>1</sub> concentration (Fig. 1), but with a subsequent increase in endogenous GA<sub>1</sub> levels the response of the plant to the bio-active GA<sub>1</sub> must be reduced, otherwise a subsequent increase in elongation rates would be observed. This results in a continued inhibition of shoot elongation in transferred plants. This suggests that short-term responses to changes in the environment are made, at least in part via changes in the level of active hormone. Due to homeostatic mechanisms such as feedback the plant may then re-establish normal levels of the hormone, with the longer term changes being mediated

by ongoing alterations in the plants' response to the hormone.

A reduction in the level of GA<sub>1</sub> in pea shoots would lead to a reduction in elongation of expanding internodes upon the plants' perception of the WL signal. This is consistent with the phenotypes of dark-grown WT GA-deficient plants. For example the growth rate of *na* (GA-deficient) plants in the dark is less than 20% of WT plants (Behringer et al., 1990). Brassinosteroid-deficient mutants of pea also have reduced growth rates in the dark (Behringer et al., 1990; Nomura et al., 1999). However, there is no evidence of a decrease in brassinosteroid levels after de-etiolation (L. Shultz, A. Symons, D. Gregory, and J. Reid, unpublished results). The role of the photoreceptors in the de-etiolation process also requires examination. This would add to work on the *lv-5* phy B-deficient mutant of pea (Weller et al., 1995), which suggests that phyB may be at least partly responsible for mediating the inhibition of elongation by WL, based on comparisons of the elongation of WT and *lv-5* plants. The cloning of genes for GA 3 $\beta$ -hydroxylation (Lester et al., 1997) and 2 $\beta$ -hydroxylation (Lester et al., 1999) should permit a molecular analysis of how these steps are regulated by light.

## MATERIALS AND METHODS

### Plant Material

The plant material used was the WT tall line 107 (derived from cv Torsdag) and line 1766 (*na*, nana). The *na* mutation blocks the GA pathway before GA<sub>12</sub>-aldehyde (Ingram and Reid, 1987). A small portion of the testa was removed with a razor blade before sowing. Plants were grown in a 1:1 (v/v) mixture of vermiculite and 10 mm dolerite chips topped with 4 cm of pasteurized peat/sand potting mixture in 140 mm slimline pots at a density of three plants per pot or in 400 mm  $\times$  300 mm boxes at a density of 30 per box.

### Growing Conditions

Growth of all plants in controlled conditions occurred at 20°C unless otherwise stated. Dark-grown plants were grown in a dark room for a total period of 12 d. WL-grown plants were placed in a controlled environment chamber (Convicon, Winnipeg, Manitoba, Canada) directly after sowing, in which a 24-h photoperiod of continuous WL was maintained by a bank of eight very high output fluorescent tubes (115 W F48T12/CW/VHO cool-white, Sylvania, Danvers, MA) and four incandescent globes (60 W Pearl, Thorn, Melbourne, Australia) delivering approximately 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at the plant apex. The shoots of WL-grown plants emerged from the growing medium approximately 5 d after sowing. In de-etiolation experiments plants were grown in the dark for 7 d and then transferred to the light. Operations on dark-grown plants were performed under a green safelight, which consisted of a 40 W

fluorescent tube (L40 W/20S cool-white, Osram, Germany) covered in alternate layers of blue and yellow plastic.

### Substrate Application

For GA<sub>20</sub> metabolism experiments, the substrate was [<sup>13</sup>C<sup>3</sup>H]GA<sub>20</sub> (15 mCi/mmol; Ingram et al., 1984). The hormone was applied at a rate of 10,000 dpm plant<sup>-1</sup> in 2  $\mu\text{L}$  of ethanol to internode tissue directly below the apical hook. Substrate was applied after 7 d of growth in continuous darkness. Immediately after application, 15 treated plants were transferred to the continuous WL conditions described above, while the remaining 15 treated plants were maintained in continuous dark conditions.

### Harvest Procedure

For determination of endogenous GA levels, shoots were harvested whole by excising at the soil surface. To investigate the spatial distribution of GAs, plants were subdivided into apical, upper internode, and basal internode portions. In WL-grown and transferred plants the harvested apical portion consisted of the apical bud and surrounding stipules, and the upper internode portion consisted of the next two uppermost internodes and accompanying petioles and leaf tissue, whereas the basal portion was the remaining internode tissue down to the soil surface. In dark-grown plants the apical portion consisted of the apical hook and 3 to 4 mm of the uppermost internode tissue. The remaining stem was split into upper and lower halves. Harvested tissue (6–20 g) was placed immediately into cold (–20°C) methanol (approximately 5 mL g<sup>-1</sup>).

For GA<sub>20</sub> metabolism experiments, whole shoots were harvested at the soil surface, inverted, and dipped in distilled water to remove excess labeled GA<sub>20</sub> that had not been absorbed into the plant tissue. Shoots were then divided into upper and lower halves based on shoot length; roots and cotyledons were also harvested at the 24-h time point.

### GA<sub>1</sub> Application Experiment

To investigate the GA<sub>1</sub> response in various light regimes, plants of genotype *na* were grown. The dose of GA<sub>1</sub> was 10  $\mu\text{g}$  in 2  $\mu\text{L}$  of ethanol applied to the internode between nodes 2 and 3. Control plants received ethanol only.

### Hormone Extraction, Purification, and Quantification

To begin the extraction, the concentration of methanol was reduced to 80% (v/v) and the sample finely homogenized. The extracts were then held at 4°C for 24 h. The extract was filtered through filter paper (no. 1, Whatman, Clifton, NJ) with the use of a Buchner apparatus, followed by three separate washes of the sample beaker for recovery of trace GAs. The volume of total extract was recorded in all experiments (data not shown), and labeled internal



standards were added in amounts specific to the type of tissue being extracted.

Extracts were reduced under vacuum at 35°C to 40°C to a small volume (<1 mL). A C<sub>18</sub> Sep-Pak cartridge (Waters, Milford, MA) was then preconditioned with 10 mL of methanol and 10 mL of 0.4% (v/v) acetic acid in distilled water. The reduced extract was transferred to the glass syringe in 1 mL of 1% (v/v) acetic acid, followed by 2 × 1 mL 0.4% (v/v) acetic acid washes. The extract was then forced through the Sep-Pak, followed with a 2-mL wash of 0.4% (v/v) acetic acid. GAs were then eluted from the Sep-Pak into a round-bottomed flask with 12 mL of 70% (v/v) methanol in 0.4% (v/v) acetic acid. Endogenous GAs were separated by HPLC and quantified by gas chromatography-selected ion monitoring with internal standards as described previously (Ross et al., 1995; Ross, 1998). [<sup>13</sup>C<sup>3</sup>H]GA<sub>20</sub> metabolites were analyzed by HPLC as the methyl esters (Ross et al., 1995).

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