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Phytochrome, Gibberellins, and Hypocotyl Growth

A Study Using the Cucumber (*Cucumis sativus* L.) *long hypocotyl* Mutant

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The possible involvement of gibberellins (GAs) in the regulation of hypocotyl elongation by phytochrome was examined. Under white light the tall *long hypocotyl* (*lh*) cucumber (*Cucumis sativus* L.) mutant, deficient in a type B-like phytochrome, shows an increased "responsiveness" (defined as response capability) to applied GA_4 (the main endogenous active GA) compared to the wild type. Supplementing far-red irradiation results in a similar increase in responsiveness in the wild type. Experiments involving application of the precursor GA_9 and of an inhibitor of GA_4 inactivation suggest that both the GA_4 activation and inactivation steps are phytochrome independent. Endogenous GA levels of whole seedlings were analyzed by combined gas chromatography-mass spectrometry using deuterated internal standards. The levels of GA_4 (and those of GA_{34}, the inactivated GA_4) were lower in the *lh* mutant under low-irradiance fluorescent light compared with the wild type, similar to wild type under higher irradiance light during the initial hypocotyl extension phase, and higher during the phase of sustained growth, in which extension involved an increase in the number of cells in the upper region. In all cases, growth of the *lh* mutant was more rapid than that of the wild type. It is proposed that GA_4 and phytochrome control cell elongation primarily through separate mechanisms that interact at a step close to the terminal response.

The shade-avoidance reaction is a complex developmental response in which plants perceive vegetational shade light signals and modify their growth habit by promotion of extension to outgrow competing neighbors (Smith, 1982; Ballaré et al., 1990). The light signal consists of an enrichment in the relative proportion of FR, which depletes active phys. Phys are a group of R-FR photoreversible biliproteins (Kendrick and Kronenberg, 1994), synthesized in an R-absorbing form (Pr). Upon light absorption, Pr is converted to Pfr, which is active in the majority of responses. The Pfr form can be inactivated by absorption of FR, which occurs to a greater extent in shade environments. The phys are encoded by a small divergent gene family (Quail, 1991), and their products have both discrete and overlapping functions.

Studies of the tall cucumber (*Cucumis sativus* L.) *lh* mutant, whose seedlings are insensitive to a pulse of FR at the end of each day (Adamse et al., 1988), have led to the observation that the mutant displays a constitutive shade-avoidance reaction (López-Juez et al., 1990) and lacks a PHYB-like protein (López-Juez et al., 1992). Therefore, it is proposed that phyB plays a dominant role in the shade-avoidance reaction in cucumber. Similar conclusions have been drawn from the study of the following mutants: *Arabidopsis thaliana phyB* (*hy3*, Nagatani et al., 1991; Somers et al., 1991), which is mutated in the *PHYB* gene itself (Reed et al., 1993); *Brassica rapa ein* (Devlin et al., 1992); *lv* of pea (Nagatani et al., 1990; J.L. Weller, personal communication); and transgenic *Arabidopsis* overexpressing *PHYA* or *PHYB* (McCormac et al., 1993). In addition, the tall, early-flowering ma_3R mutant of *Sorghum bicolor* shows deficiency in several phy-dependent responses and also lacks one type of phy that is abundant in green tissue (Childs et al., 1992).

Little is known about the mechanisms through which the phys control changes in elongation growth. Similarities between the action of GAs and of the photoreceptor were observed in early studies of phy and elongation (Downs et al., 1957). One explanation is that decreases in GA production or action might be part of the chain of events leading from phy action to reduced elongation. In the mutants mentioned above the lack of active phyB or phyB-like protein results, among other developmental responses, in a large increase in elongation growth (López-Juez et al., 1990). In fact, the *ein* mutant of *Brassica* (Rood et al., 1990a) and the ma_3R mutant of *Sorghum* (Beall et al., 1991) have been reported to contain higher amounts of GA_1, their endogenous active GA, than WT plants. Furthermore, the metabolism of GA has been shown to be affected by light exposure in dark-grown pea (Sponsel, 1986) or lettuce seedlings (Toyomasu et al., 1992). GA_1 biosynthesis has been indicated to be under phy control during de-etiolation in pea (Campell and Bonner, 1986) or after R or FR pulses to light-grown cowpea (García-Martínez et al., 1987; Martínez-García and García-Martínez, 1992a).

In contrast, early work showed that phy and GA acted in an additive, and therefore independent, fashion in the elongation of bean (Downs et al., 1957). In the *lv* pea mutant, no

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Abbreviations: DOCHC, 3,5-dioxo-4-propionylcyclohexanecarboxylic acid; FR, far-red light; HQI, metal halide (lamps); *lh*, long hypocotyl; phy, phytochrome; R, red light; WL, fluorescent white light; WT, wild type.

increase in active GA_1 was found, but the mutant was observed to be "GA hypersensitive" (Reid and Ross, 1988; Weller et al., 1994). In rice mesocotyls, phy activation decreases GA "sensitivity" (Nick and Furuya, 1993). In tomato, preliminary data from the photomorphogenic *au* mutant also suggest that an increase in response to GA correlates with its elongated phenotype (E. López-Juez and R.E. Kendrick, unpublished data). At this time it is not possible to draw clear conclusions concerning the involvement of GAs in phy action, and there is a possibility that the relationship is different for separate phy types or at different developmental stages. Physiological (Metzger, 1988) or genetic and physiological (Behringer et al., 1990) evidence has even suggested independent actions for phy and GAs.

Hypocotyl growth in the *lh* mutant offers several advantages as a system in which to study phy-GA interactions. The difference in elongation rate between the hypocotyl of the *lh* mutant and its WT under low FR-containing fluorescent light is dramatic, making the seedlings amenable to physiological analysis. It appears that phyA is unaffected by the *lh* mutation (López-Juez et al., 1992). The main endogenous active GA has been identified as GA_4 in cucumber (Nakayama et al., 1989, 1991), with the main GA active in other species, GA_1, also present at levels lower than GA_4 (Smith et al., 1991). Both GA_4 and GA_1 contain a 3β-hydroxyl group. We have used WT and the *lh* mutant to address the following questions: are the GA activation (3β-hydroxylation) and inactivation (2β-hydroxylation) steps affected by the lack of a type B-like phy? Are the endogenous GA levels altered? Is the response to GA changed? If alterations take place, is it possible to correlate them with a specific developmental phenomenon?

To obtain answers to these questions, experiments have been carried out in which different GAs and inhibitors of GA metabolism were applied. Endogenous levels of GAs have been measured and the cellular basis of elongation analyzed. This was done under two different light environments: a low-irradiance one (comprising both a low FR and a FR-enriched regime), in which cell elongation took place without any cell division during the growth of the *lh* mutant hypocotyl, and a high-irradiance one, in which it was found that cell division was probably also involved in the *lh* mutant hypocotyl growth.

MATERIALS AND METHODS

Plant Material

Seeds of the cucumber (*Cucumis sativus* L.) *lh* mutant and nearly isogenic WT were produced at Wageningen Agricultural University (Wageningen, The Netherlands). The mutant was described and characterized previously (Adamse et al., 1987; López-Juez et al., 1992).

Light Sources

For the experiments involving GA applications (Figs. 2–5) WL was obtained from Mitsubishi/Osram (Tokyo, Japan) FL 20SW fluorescent tubes, at an irradiance of 40 μmol m^{-2} s^{-1} at the plant level (400–700 nm, LI 185-B quantum sensor; Li-Cor Inc., Lincoln, NE). The WL plus FR was produced by using the same tubes together with Toshiba (Tokyo, Japan) FL 20S.FR74 tubes wrapped with red and blue acetate film (red No. 22, blue No. 72; Tokyo Butai Shomei, Tokyo, Japan). The addition of FR resulted in no detectable increase in PAR (400–700 nm). The HQI light was obtained from a combination of Toshiba DR400/T(L) and Mitsubishi MLRBOC 400F-U HQI lamps, providing 120 μmol m^{-2} s^{-1}. The spectral photon irradiances of these light sources, measured with an LI-1800 spectroradiometer (Li-Cor), are shown in Figure 1.

Growth of Plants and Application of GAs and GA Inhibitors

Seeds were soaked in water for 2 h and sown on wet filter paper in Plantcon plastic boxes (Flow Laboratories, McLean, VA). After 2 d in WL, the germinated seeds with an approximately 2-cm radicle were soaked for 1 to 2 h in a 1-μg mL^{-1} solution of analytical grade uniconazole (Sumitomo Chemical, Tokyo, Japan). This inhibited endogenous GA biosynthesis by preventing the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Izumi et al., 1985). After soaking and without rinsing, five seeds were planted in a 10-cm plastic pot with a 2:1 (v/v) mixture of vermiculite: potting soil (cucumber type; Kureha, Tokyo, Japan). After 6

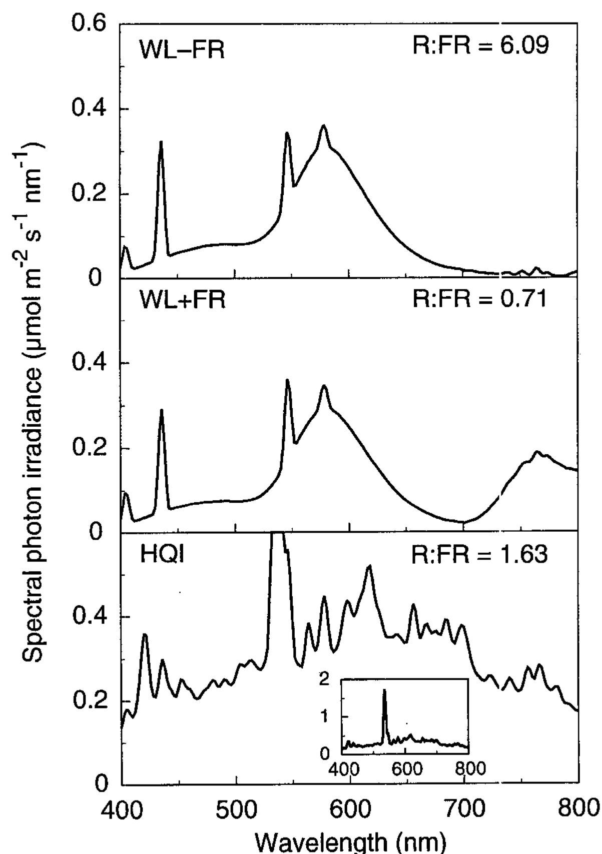

Figure 1. Absolute spectral photon irradiance of the light regimes used in this work. For each regime, the R:FR ratio, calculated for two 10-nm bands centered around 660 (R) and 730 (FR) nm, is indicated.

d in WL, the emerged hypocotyl was measured and a segment of approximately two-thirds of its length (10 mm for WT and 15 mm for *lh*) was labeled with a fine marker pen (Pilot namae-pen or DR-ink pen). No damage by either ink was visible. The different segment size of each genotype contained an identical number of epidermal cells (approximately 350/row). Various GAs were then applied in a 2-μL drop of 50% ethanol to the base of the cotyledon, close to but not on the apex. The drop was evaporated by gentle blowing. Plants to which no GA had been applied received an equivalent drop of solvent. The segments were measured again after 5 d. Application of DOCHC, an inhibitor of 2-oxoglutarate-dependent dioxygenases including the hydroxylases converting GA_9 to GA_4 and GA_4 to GA_{34} (Nakayama et al., 1990a, 1990b, 1991), was carried out in the same way as application of GAs but 12 h in advance. The plants were kept at 25°C. Data are presented as the means ± SE for 10 hypocotyls.

For endogenous GA measurements, after imbibition in water for 2 h, seeds were planted directly in the vermiculite:soil mixture, in 35-cm square plastic trays for 4-d-old seedlings, 10-cm plastic pots, at two seedlings per pot (WL and WL plus FR), or 8-cm pots, individually (HQI light).

To analyze GA response under HQI light, seedlings were grown in 8-cm pots. Uniconazole was applied at a final concentration in the pots of 0.1 μg mL^{-1}. The GA_4 was applied as a 2-μL drop of 50% methanol to the cotyledon petiole. Controls received a 2-μL drop of 50% methanol. An identical 15-mm segment was labeled in both the WT and *lh* at the time of GA_4 application and 2 d later with a ruler. Times of application were as follows: uniconazole at 2.5 d and GA_4 at 3.5 d; uniconazole at 3 d and GA_4 at 10 d.

Isolation and Quantitation of Endogenous GAs

Plants were harvested, weighed, frozen in liquid nitrogen, and stored at −80°C until extraction. Endogenous GAs were purified as previously reported (Kobayashi et al., 1993), with the addition of a gel permeation HPLC step (Kobayashi et al., 1989). The purification involved extraction twice in 80% methanol, solvent partitioning yielding an acidic ethyl acetate fraction, Bond Elut (Varian, Harbor City, CA) C_{18} and DEA cartridge purification, HPLC purification through a gel permeation column (Shodex HF-2001; Showa-densho, Tokyo, Japan), a C_{18} reverse-phase column (ODS-1100N; Senshu Pak, Tokyo, Japan), and a $N(CH_3)_2$ ion-exchange column (Nucleosil; Macherey-Nagel, Duren, Germany), followed by trimethylsilyl derivatization and GC-MS analysis with a TSQ 700 mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a 3400 gas chromatograph (Varian), in either the full-scan- or selected-ion-monitoring mode. In experiments in which GA_{24} was analyzed, derivatization of this sample was by methylation. For quantitation, 10 ng each of [2,2,3,6-$^2H_4$]GA_4 and [15,17,17-$^2H_3$]GA_9 were added to the initial extract as internal standards, in the case of samples of 50 g fresh weight. For 10-g samples, 5 ng of each standard were used. In some experiments 10 ng of [1,2,2,3,6-$^2H_5$] GA_1 was also added as a standard. The GA_{34} was quantified against the [$^2H_4$]GA_4 standard in the same GC injection, so the values should be taken as approximate, although valid for comparisons.

Measurements of Zonal Growth and Cell Size and Number

Ten hypocotyls were labeled into six identical portions on d 4, with a fine permanent marker as above. The position of the marks (length of the segments) was measured daily.

For epidermal cells, a transparent nail varnish was applied to the hypocotyl. The dry print was peeled off with cellophane tape, and cell length was measured in one of two ways: either by measuring a number of consecutive cells directly under the microscope with a micrometric ruler or by photographing the preparation under the microscope and later measuring individual cells on the projected slide image, with a microscope grid image as a reference. Cells of a specific type were chosen, namely those that were outside "fields" of stomata and that were not in contact with hairs. Cell size was determined in the uppermost, middle, and lowest portions of the hypocotyl for WT and in two extra, intermediate positions for *lh*, 30 to 60 cells being measured at each position. Data are the means ± SE for three hypocotyls.

Total DNA was extracted from two hypocotyls per sample and measured according to the method of Baër et al. (1982). Calf thymus DNA (Pharmacia, Uppsala, Sweden) was used as a standard. To calculate the multiples of the basic diploid (2C) nuclear DNA content per hypocotyl, a value of diploid DNA content per cell of 0.76 pg was used (Arugumanathan and Earle, 1991). Data are presented as the means ± SE for two samples (four hypocotyls).

RESULTS AND DISCUSSION

PhyB and GA 3β-Hydroxylation

The first experiments of GA application and endogenous GA analysis were performed under low-irradiance WL. This allowed comparison between the WT and the *lh* mutant, as well as enabled the investigation of their response to supplementary FR.

Reports concerning pea (Campell and Bonner, 1986) and cowpea (García-Martínez et al., 1987) have suggested a basic action of phy through regulation of GA 3β-hydroxylation, namely in the conversion of inactive GA_{20} into active GA_1. In cucumber, the most abundant (and most active when applied) 3β-hydroxylated GA is GA_4 (Nakayama et al., 1989), which is biosynthesized from GA_9 by 3β-hydroxylation. Although GA_1 is also present, the absolute level is only one-fifth of that of GA_4 (Smith et al., 1991). Therefore, we first tested the action of GA_4 and its precursor, GA_9, on seedlings whose endogenous GA levels had been depleted with the GA biosynthesis inhibitor uniconazole. Seedlings from uniconazole-pretreated germinating seeds were treated with GAs and their effect on elongation of an upper labeled hypocotyl segment was measured. The results (Fig. 2) indicate a greater response to either GA_4 or GA_9 in the *lh* mutant than in WT. The response of WT to both GA_4 and GA_9 was also increased to a similar extent by FR treatment. The ratio of the response

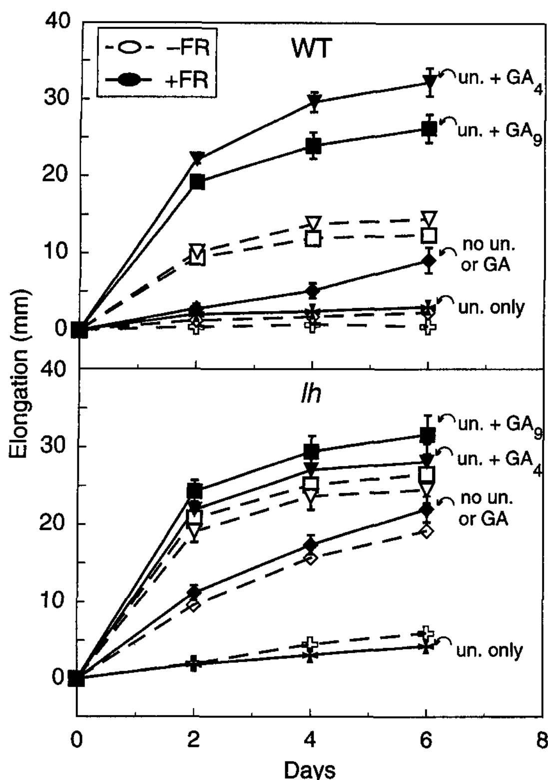

Figure 2. Response of the labeled segments of WT and *lh* mutant hypocotyls to the application of GA_9 (squares) or GA_4 (triangles). All seedlings, except those indicated (no uniconazole or GA, diamonds), had been pretreated with the GA biosynthesis inhibitor uniconazole (un.) and later treated without GA (crosses) or with 0.1 μg per seedling of GA_9 or GA_4. Control seedlings received neither uniconazole nor exogenous GA. Light regimes were WL minus FR (open symbols, dashed line) and WL plus FR (closed symbols, continuous line).

in the presence and the absence of extra FR was identical for both GAs. Since GA_9 is only active in cucumber because it is converted by 3β-hydroxylation to GA_4 (Nakayama et al., 1991) and because seedlings respond to GA_9 to the same extent as they do to GA_4 in WL minus FR and WL plus FR, 3β-hydroxylation must occur whether phy is predominantly in the Pr or Pfr form. Results qualitatively similar to those of Figure 2, in which 0.1 μg of the GA was applied, were obtained when a 0.5-μg dose was used. An increase of the response to both GAs was observed in WT when Pfr was depleted by FR, whereas the response was high in *lh* even in the absence of FR. The segments of WT and *lh* were of different initial lengths (equal number of epidermal cells), but the response of *lh* was also greater than that of WT on a relative length basis. This increase in the response to GAs by depletion of Pfr has also been shown in pea (Reid and Ross, 1988; Nagatani et al., 1990) and cowpea (Martínez-García and García-Martínez, 1992b) for GA_1 and in the rice mesocotyl (Nick and Furuya, 1993) for GA_3.

Seedlings treated with the GA biosynthesis inhibitor uniconazole and in which GA_4 or GA_9 was not supplied externally failed to elongate even in FR-enriched light (Fig. 2), showing that biosynthesis of GAs is required for the expression of the phy response.

PhyB and GA Response

We further investigated the inverse relationship between Pfr level and GA response by determining a dose-response curve to GA_4 under conditions of reduced endogenous GAs. This kind of physiological analysis provides some indication of the molecular mechanism underlying the difference in response to the application of a hormone (Firn, 1986). In particular, a shift of the response curve toward lower doses, while maintaining the shape, would mean an increase in "affinity," or strength of hormone binding to its receptors, presumably by a modification of the receptor. In contrast, a higher "receptivity" or increased number of receptors would result in a proportional increase of the response for every given dose. A change in "responsiveness" (not defined by Firn) refers to an alteration in a characteristic of the cells that is unrelated to hormone reception but that determines the extent of the response of the tissue, i.e. the response capability. An altered responsiveness would lead to a modification of the curve indistinguishable from that resulting from a change in receptivity. The dose-response curve (Fig. 3) shows a change in either receptivity or responsiveness in the WT after illumination with FR-rich light and a constitutive high receptivity or responsiveness in the *lh* mutant. It is unlikely that the altered response was due to differences in GA uptake between the two genotypes, since the difference appeared

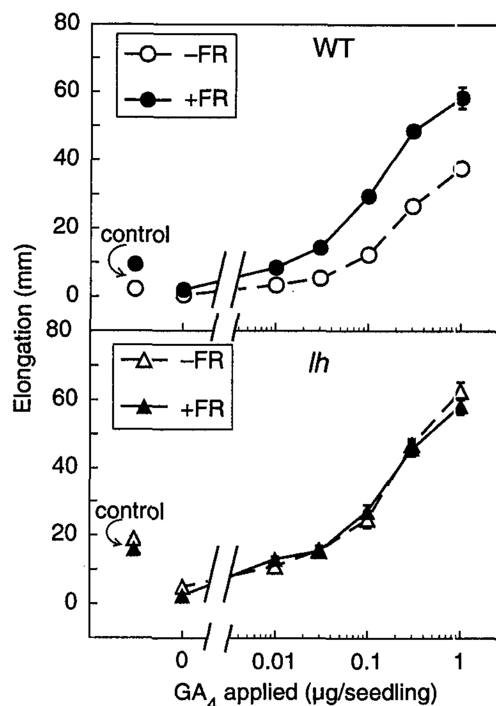

Figure 3. Response of the labeled segments of WT and *lh* mutant hypocotyls of uniconazole-pretreated seedlings to the application of different doses of GA_4. Control seedlings were not treated with uniconazole. Light regimes as in Figure 2.

not only between the *lh* mutant and WT but also in the WT through the action of supplementary FR (a difference in GA uptake might explain the fact that in Fig. 2 the response of the *lh* mutant to GA_4 was slightly higher, whereas that of the WT to GA_9 was consistently slightly higher).

The highest dose of GA_4 applied in this experiment was 1 μg per plant. At this dose we failed to observe a complete saturation of the response. Nevertheless, a leveling off of the curves toward the highest dose was observed. Moreover, data from the application of 0.1 and 0.5 μg of GA_4 in the experiment shown in Figure 2 indicated that the greater response in the latter case was partly due to an extension of the time during which growth occurred (results not shown). Nakayama et al. (1991) showed a saturated response for 1 μg of applied GA_4 in comparable cucumber seedlings. Consequently, the highest doses used in the experiments shown in Figure 3 in part simply result in GA_4 acting for a longer time. These data, therefore, indicate that a response to a decrease in Pfr may be observed, even in the presence of GA levels that are already close to saturation. Again this argues against phy controlling hypocotyl growth through simply an increase in the level of endogenous GAs.

PhyB and GA 2β-Hydroxylation

It is possible that a change in response to GAs could in fact be caused by a difference in the turnover of the applied GA. If the applied GA remains active for a longer period, the response to any dose will be enhanced. Removal of active GAs takes place primarily through 2β-hydroxylation, and secondarily through conjugation (Fujioka et al., 1990). We tested the action of DOCHC, an inhibitor of both 3β- and 2β-hydroxylation, to determine whether it increased the response of WT to GA_4 under WL to the level observed under WL plus FR (Fig. 4). The application of DOCHC, as previously observed (Nakayama et al., 1991), increased the response to GA_4, but the increase was independent of the phy status. The fact that DOCHC strongly reduced the response to GA_9 (Fig. 4, inset) demonstrates that it effectively inhibited 3β-hydroxylation at the highest concentration used.

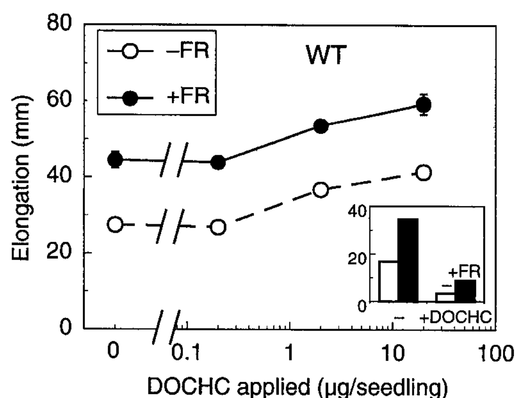

Figure 4. Response of labeled segments of hypocotyls of WT seedlings treated with 0.3 μg of GA_4 to the preceding application of varying doses of DOCHC to inhibit GA hydroxylases. Light regimes as in Figure 2. Inset, Seedlings did or did not receive the application of 20 μg of DOCHC and were then treated with 0.3 μg of GA_9 (as a control for the action of the chemical). Open bars, WL minus FR. Filled bars, WL plus FR.

To test further the possibility that phy control of 2β-hydroxylation is responsible for the change in response to GA_4, we performed experiments with GA_3. The GA_3 molecule possesses an extra double bond, which prevents it from being 2β-hydroxylated (Nakayama et al., 1990b). However, the phy status still modified the response to GA_3, to an extent larger than to GA_4 (data not shown). This further rules out the involvement of 2β-hydroxylation in the phy action observed on GA_4 response of the cucumber hypocotyls. The response to applied GA_3 was one-third to one-sixth of the response to GA_4.

PhyB and the Endogenous Level of GAs

The above experiments indicate that neither the last activation (3β-hydroxylation) nor the primary inactivation step of GA_4 metabolism (2β-hydroxylation) appears to be under phy control. We have analyzed the endogenous levels of the main active GA (GA_4), as well as its immediate precursor (GA_9) and 2β-hydroxylated metabolite (GA_{34}). In the first experiments, GA_1 was also investigated. Whole seedlings were used for extraction, to provide enough material at three times during growth. Particularly at the later stages, the hypocotyl contributes the majority of the weight of the seedling. Although the hypocotyl is the elongating organ, light perception by the cotyledons also affects hypocotyl extension, and the cotyledons mediate a differential growth response to light (simulated phototropism), which is absent in the *lh* mutant (Adamse et al., 1987). In lettuce seedlings, Toyomasu et al. (1992) showed that the pattern of change in GA levels during de-etiolation, as estimated by radioimmunoassay, is similar in hypocotyls and cotyledons.

The results for GA_4 under these light regimes (Table I) showed a gradual decrease in the concentration of active GA during seedling development, probably because of dilution with growth of the tissues containing the higher GA levels (the shoot tip in pea [Smith et al., 1992] or *Brassica* [Zanewich and Rood, 1993]). At d 4, 1 d after emergence of the seedlings from the soil (Fig. 5), elongation growth was very rapid, and the rates were different depending on the phy status (relative growth of 34% for WT without FR, 57% with FR, and 55% in the *lh* mutant, for the following 24 h). In this experiment, GA_4 levels were comparable or relatively lower (half) in the WT without FR than with supplementary FR at different times, independent of the rate of hypocotyl growth (Table I; Fig. 5). At the first two times in this experiment the content of GA_4 was lower in the *lh* mutant.

Quantitative analysis of GA_9 and GA_{34} (Table I) and of GA_1 and GA_{24} was also performed. The results provided no consistent evidence of phy control of GA interconversion. At d 8, when the difference in GA_4 was most evident, the ratio of GA_9 to GA_4 was similar in WT with and without supplementary FR. The apparent conversion of GA_4 to GA_{34} decreased (the ratio increased) with FR, but

Table I. *Endogenous levels of GA_9, GA_4, and GA_{34} for whole seedlings of WT and lh mutant grown under 40 μmol m^{-2} s^{-1} WL, without (−FR) or with (+FR) supplementary FR*

The values for GA_9 were corrected for the recovery of the [^{2}H]GA_9 internal standard. Those for GA_4 and GA_{34} were corrected for the recovery of [^{2}H]GA_4 (because they co-purified until the GC separation) and are, therefore, approximate for GA_{34}. For GA_{24} and GA_1, see text.

Day	Plant Type/ Treatment	GA_9 →	GA_4 →	GA_{34}
			ng GA g^{-1} fresh wt (ng GA seedling $^{-1}$)	
4	WT −FR	1.20 (0.30)	2.18 (0.55)	3.40 (0.85)
	WT +FR	0.60 (0.19)	2.03 (0.64)	4.99 (1.56)
	lh −FR	1.08 (0.37)	0.49 (0.17)	1.32 (0.46)
8	WT −FR	0.23 (0.12)	0.39 (0.20)	3.27 (1.66)
	WT +FR	0.37 (0.25)	0.92 (0.62)	2.07 (1.40)
	lh −FR	0.16 (0.11)	0.05 (0.03)	0.66 (0.45)
14	WT −FR	0.26 (0.23)	0.40 (0.35)	3.08 (2.71)
	WT +FR	0.23 (0.25)	0.46 (0.50)	2.25 (2.45)
	lh −FR	0.32 (0.22)	0.49 (0.34)	2.05 (1.42)

the value of GA_{34} was relatively constant overall with or without supplementary FR. At 4 and 14 d, GA_{24}, the precursor of GA_9, was analyzed and could be detected in all samples but at levels too low for accurate quantitation. More important, GA_1 could not be detected in samples of 4-d-old seedlings, when other GAs were at their highest levels. The [^{2}H]GA_1 internal standard could be detected readily in this case, when included at a dose identical with those of other internal standards. Smith et al. (1991) detected only GA_1 in cucumber after immunochromatographic purification, at levels approximately one-fifth of those of GA_4. Taking into account this and the fact that exogenously applied GA_4 is far more active than GA_1 in promoting cucumber hypocotyl elongation (Brian et al., 1967), we did not continue the analysis of GA_1 in subsequent samples.

The data in Table I and Figure 5 show that a phy-dependent difference in growth rate can appear without any increase of endogenous GA_4 (WT with or without FR at d 4, WT and *lh* overall). To understand the significance of the values for GA_4 in Table I, it is possible to calculate approximately what level of GA_4 would be necessary to elongate a WT hypocotyl as much as that of an *lh* mutant. This can be done approximately from the data in Figure 3 since the segments contain approximately equal numbers of cells. According to those data, after treatment of the WT with uniconazole, it is necessary to apply approximately 10 ng of GA_4 to restore the growth to the level of the controls without GA biosynthesis inhibitor. To increase growth of the WT to the level of the *lh* mutant, about 4 times as much GA_4 would have to be supplied in the presence of FR and about 12 times as much in the absence of FR. That is a consequence of the approximately logarithmic relationship between GA dose and elongation (Weller et al., 1994). This further argues against the enhanced growth of *lh* mutant hypocotyls being explained by increased GA_4 levels.

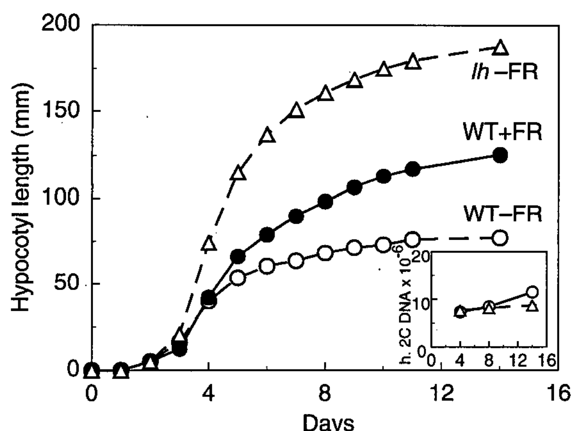

Figure 5. Hypocotyl growth curve of seedlings of WT and *lh* mutant grown under WL or WT grown under WL + supplementary FR (seedlings as in Table I). Inset, Total amount of hypocotyl (h.) DNA, for WT and *lh* grown under WL, expressed as multiples of the diploid nuclear quantity (2C).

Endogenous Level of GAs under HQI Light

Surprisingly, the trend observed in the GA_4 levels in Table I for the WT treated with supplementary FR was opposite of that in the *lh* mutant. In fact, although the development of the WT with or without supplementary FR was similar, the development of the *lh* mutant appeared to be delayed under the low-fluence WL conditions. Expansion of the first leaf of the *lh* mutant took place about 3 d later than in the WT (10-mm long young leaf on d 7 for WT and on d 10 for *lh*). The final area of the *lh* mutant leaf was 40% of that of the WT − FR (data not shown). Therefore, we decided to compare the contents of endogenous GAs under different conditions (higher irradiance and multidirectional illumination from lamps with a more uniform spectral distribution: HQI light). Under this regime development of WT and the *lh* mutant proceeded similarly.

In the HQI regime the level of GA_4 in the *lh* mutant (Table II) was comparable to that in the WT (slightly lower in experiment 1 or higher in experiment 2) at 4 d, 1 d after emergence, with very active hypocotyl extension in the mutant (Fig. 6). The level of GA_{34}, all of which was previ-

Table II. *Endogenous levels of GA_9, GA_4, and GA_{34} for whole seedlings of WT and lh mutant grown under 120 μmol m^{-2} s^{-1} of HQI light*
The values for each GA were quantified as in Table I.

Day	Plant Type	GA_9 →	GA_4 →	GA_{34}
			ng GA g^{-1} fresh wt (ng GA seedling^{-1})	
Experiment 1				
4	WT	0.29 (0.06)	0.79 (0.17)	2.46 (0.54)
	lh	0.77 (0.20)	0.48 (0.12)	0.84 (0.21)
7	WT	0.16 (0.13)	0.43 (0.34)	0.54 (0.42)
	lh	0.22 (0.24)	1.93 (2.09)	4.12 (4.46)
11	WT	n.d.[a]	0.16 (0.37)	1.12 (2.58)
	lh	n.d.[a]	0.51 (1.44)	2.15 (6.07)
Experiment 2				
4	WT	<1.09 (<0.33)[b]	0.28 (0.08)	4.2 (1.30)
	lh	1.31 (0.43)	0.67 (0.22)	1.3 (0.42)
7	WT	<0.41 (<0.32)[b]	0.26 (0.20)	2.5 (2.0)
	lh	0.91 (0.94)	0.48 (0.50)	12.1 (12.5)
11	WT	0.72 (1.40)	0.21 (0.42)	4.3 (8.4)
	lh	1.87 (4.90)	0.41 (1.13)	5.1 (13.4)

[a] n.d., Not determined. [b] Because of the presence of impurities, the value is overestimated.

ously active GA_4, was lower for the *lh* mutant in both experiments at this stage. At later stages, however, concentrations of GA_4 were higher at 7 d (2–5 times) and possibly 11 d (2–3 times) when *lh* mutant hypocotyls continued to elongate actively. After emergence, WT hypocotyls elongated only slightly after d 11, when they were first shaded by their first true leaves. Again, the values for GA_{34} tended to correlate with those of GA_4, being higher in the *lh* mutant in both experiments, particularly at 7 d. As before (Table I) the changes in GA_4 level were nevertheless not sufficient to explain the differences in growth if we assume a dose-response relationship to GA_4 similar to that in Figure 3. The values of GA_9 varied less but also tended to be similar or higher in the *lh* mutant than the WT.

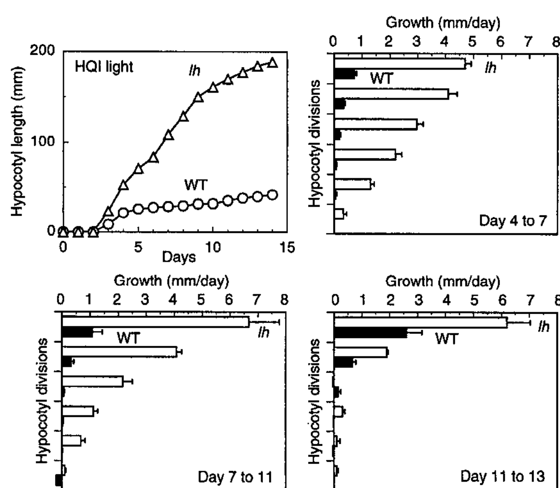

Figure 6. Hypocotyl growth curve of WT and *lh* mutant seedlings under HQI light (seedlings as in Table II). Bar graphs, Distribution of growth along the hypocotyl during the periods indicated. Hypocotyls were labeled at upper and lower ends and five intermediate positions at identical distances, growth at each position was recorded at the initial and final day of the period, and the mean daily value was calculated.

A comparison of GA levels between Tables I and II shows for the WT that at higher fluence rate of light (HQI) the levels of GA_4 were lower than at low fluence rate (WL). This may be of interest because a higher fluence rate also inhibits elongation. The significance of the change is nevertheless unclear, since it was hardly visible at the intermediate stages, when growth differences still occurred and was not supported by reproducible changes in the level of GA_{34}.

Cellular Basis of Hypocotyl and Internode Growth

The above data show that in the *lh* mutant a deficiency in phy may lead to an increase in elongation, without any concomitant increase in the concentration of active GA_4. This behavior has also been demonstrated for elongating internodes of the tall *lv* mutant of pea (Reid et al., 1983; Weller et al., 1994). However, our results show that an increase of active GA_4 (and its metabolite GA_{34}) can also accompany the tall phenotype of *lh* at late stages of hypocotyl growth. At this time the *lh* mutant can be compared to *ein* of *Brassica* (Rood et al., 1990a), ma_3R of *Sorghum* (Beall et al., 1991), or the hypocotyls of dark-grown lettuce (Toyomasu et al., 1992).

It is possible that, although GA levels appear not to mediate the elongation response to alterations in phy status, they may at some stage be under phy control. One possible explanation for the apparent discrepancy is that active GAs may be involved in a separate phy-dependent response or in one of the individual phenomena taking place during the elongation response under the control of phy. To search for such a specific phenomenon, we examined the extension of the hypocotyl under HQI light in some detail.

Growth was not distributed homogeneously along the hypocotyl but took place more actively in the upper region (Fig. 6). The upper region contained younger cells capable of growth. During development of the hypocotyl, exten-

sion seemed to be more and more restricted to this region (Fig. 6, compare d 4–7 with d 11–13).

We measured the individual cell length and the change in cell number and compared them to the increased growth of the *lh* mutant (Fig. 7). For epidermal cells we found that most of the response correlated with increased individual cell length (96–240% higher in the *lh* mutant, increasing with time). There was simultaneously an increased number of epidermal cells per row (between 21 and 55%, as deduced from Fig. 7). This extra phenomenon seemed to be of little significance at d 4 but became more relevant later. In *Arabidopsis* leaf epidermal cells it has been found that an increase in total DNA content reflects increases in the level of both ploidy of cells, endomitosis, and cell division, true mitosis (Melaragno et al., 1993). Therefore, we examined total hypocotyl DNA, and the results (Fig. 7, expressed as multiples of the nuclear diploid quantity) also suggested the occurrence of cell division. Epidermal cell lengths at upper, middle, and lower portions of the hypocotyl (Fig. 7) indicated that, although cells were always longer in the rapidly growing *lh* mutant, the average size of the uppermost cells changed relatively little; therefore a continued division of cells in this upper region in the *lh* mutant must have approximately balanced elongation. This phenomenon possibly contributed new small cells able to enter the elongation process, equivalent to the pool of precursor cells in the leaf epidermis (Melaragno et al., 1993). A similar response during shoot elongation has been observed previously (Reid et al., 1983; Martínez-García and García-Martínez, 1992b; Weller et al., 1994). In our case it correlated in time with the period of hypocotyl extension when GAs appeared to be under phy control.

Therefore, we re-examined the basis of growth of WT and the *lh* mutant under the lower fluence rate WL (Fig. 5), at which GA_4 levels were not elevated. Interestingly, total hypocotyl DNA amount increased much less, with higher growth in the *lh* mutant not being accompanied by a greater DNA quantity (Fig. 5, inset). For epidermal cells the differences were also small, the number in a row at 14 d being 425 ± 13 for WT and 478 ± 26 for the *lh* mutant. It was possible for an individual *lh* mutant epidermal cell to be up to 0.7 mm long under this regime.

GENERAL DISCUSSION

The slow growth of seedlings whose endogenous GA biosynthesis is inhibited by uniconazole and the differential response of those seedlings to the application of exogenous GAs in the presence or absence of active phy are best described as an altered GA responsiveness controlled by phy. This means that GAs must be present to drive cell elongation and that the extent of such GA-driven growth is modulated by the current phy status (Fig. 8). The interaction may take place at a late step, possibly close to the mechanisms that are directly involved in cell wall extension (Cosgrove, 1993) rather than at an early point in the transduction of the GA signal. Indirect evidence suggests that this is the case. The response in 2 d of uniconazole-treated seedlings under HQI light to a 100-ng application of GA_4 per plant decreased in the WT from 23 mm at approximately d 4 to 5 mm at approximately d 11 and for *lh* from 49 to 18 mm at the same times. This reflects a gradual loss of extensibility. Similarly, the difference in GA response varied when comparing different organs (hypocotyl or petiole extension, data not shown). At the biophysical level, it has been shown that GA_3 affects growth of cucumber hypocotyls by increasing their mechanical plasticity and altering the biochemical properties of the cell walls so that

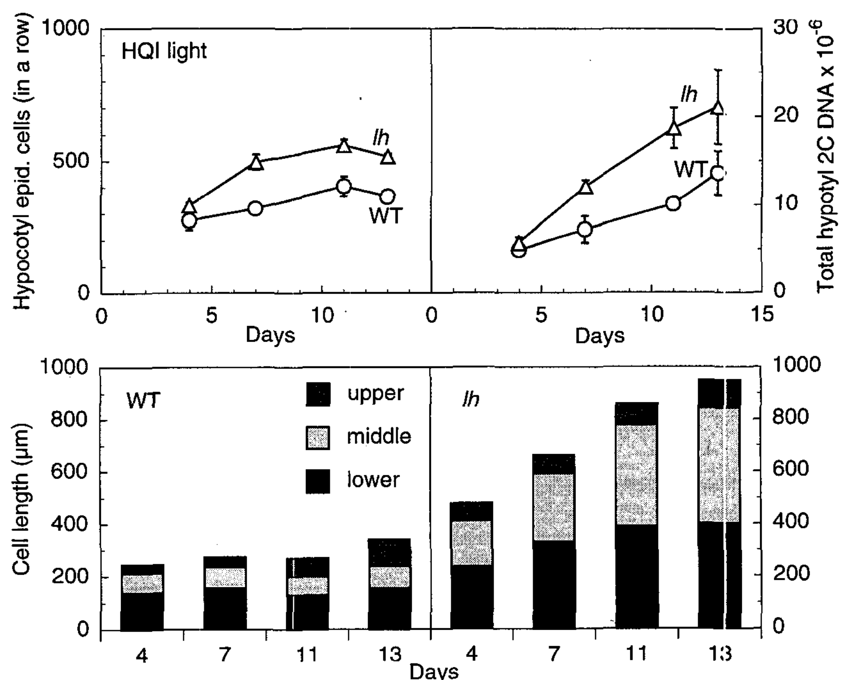

Figure 7. Top panels, Calculated number of WT and *lh* mutant hypocotyl epidermal cells in one row (hypocotyl length divided by average epidermal cell length) and total hypocotyl DNA amount (as multiples of the diploid nuclear quantity, 2C), of WT and *lh* mutant seedlings under HQI light. Bottom panels, Average length of individual epidermal cells at three different positions along the hypocotyl.

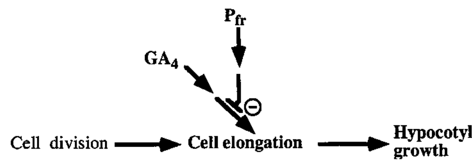

Figure 8. Model to explain the observed interaction between phy and GA_4 on cucumber hypocotyl growth. The model explains the proposed basic interaction taking place at any stage. Arrows represent sequential processes or promotive effects. The flat end line and (−) represent inhibitory action.

the wall yield coefficient is increased and the yield threshold reduced (Taylor and Cosgrove, 1989). In peas, GA_3-dependent growth is brought about by a similar biophysical mechanism (Cosgrove and Sovonick-Dunford, 1989), whereas R lowers the wall yield coefficient alone (Kigel and Cosgrove, 1991).

In such a scenario it is easy to understand the results presented above, in which GAs acted as a driving force for elongation, and Pfr could impose a brake on it. The behavior of the hypersensitive pea *lv* mutant plants (Reid and Ross, 1988; Weller et al., 1994), cowpea explants (Martínez-García and García-Martínez, 1992b), and rice mesocotyls (Nick and Furuya, 1993) can also be viewed in this way. Similarly, this explains why *Arabidopsis* seedlings overexpressing phyB show very limited response to GA_3 application (Wagner et al., 1991). In fact it has been recently observed that *phyB* mutants of *Arabidopsis* also show increased response compared to the WT to any dose of applied GA above the threshold, even to doses that are saturating for the WT (J.W. Reed, personal communication). Of all of these cases, only the results for rice mesocotyls, which exhibit a shift in the GA dose-response curve (change in affinity) in addition to an altered response to GA after R similar to the one we observed (Nick and Furuya, 1993), may indicate a more direct interaction between phy and GA signal transduction. Certainly the mechanism leading from phy action to the "brake" in GA_4-dependent elongation remains to be elucidated (Fig. 8). It is possible that such a mechanism involves changes in the local levels of another growth regulator at some point; in pea there is evidence suggesting that this might be auxin (Behringer et al., 1992).

Together with the basic mode of interaction described, the above data indicate that it is possible to observe phy actions on the level of endogenous GA_4. As discussed above, these changes in level are not sufficient to explain the difference in growth between the WT and the *lh* mutant (Fig. 3; Tables I and II). The fact that the changes in levels correlate in stage and light regime with changes in cell numbers rather than length, and that they seem not to be an absolute requirement for phy control of elongation, may help explain the anomaly that some but not all phyB-related mutants have shown increased GAs in previous studies. In fact the *Brassica ein* mutant also appears to contain normal (7 d) or elevated (14 d) GA levels, depending on the growth stage (Rood et al., 1990b). In the *lh* mutant, we have failed to identify any GA metabolic step as a possible candidate for phy control. If this holds true, early steps in GA biosynthesis should be examined (Talón et al., 1991).

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