

The Involvement of Gibberellin 20-Oxidase Genes in Phytochrome-Regulated Petiole Elongation of Arabidopsis

Tamotsu Hisamatsu, Rod W. King*, Chris A. Helliwell, and Masaji Koshioka

National Institute of Floricultural Science, Tsukuba 305–8519, Japan (T.H., M.K.); and Commonwealth Scientific and Industrial Research Organization, Plant Industry, Canberra 2601, Australian Capital Territory, Australia (T.H., R.W.K., C.A.H.)

Long day (LD) exposure of rosette plants causes rapid stem/petiole elongation, a more vertical growth habit, and flowering; all changes are suggestive of a role for the gibberellin (GA) plant growth regulators. For Arabidopsis (*Arabidopsis thaliana*) L. (Heynh), we show that enhancement of petiole elongation by a far-red (FR)-rich LD is mimicked by a brief (10 min) end-of-day (EOD) FR exposure in short day (SD). The EOD response shows red (R)/FR photoreversibility and is not affected in a phytochrome (PHY) A mutant so it is mediated by PHYB and related PHYs. FR photoconversion of PHYB to an inactive form activates a signaling pathway, leading to increased GA biosynthesis. Of 10 GA biosynthetic genes, expression of the 20-oxidase, *AtGA20ox2*, responded most to FR (up to a 40-fold increase within 3 h). *AtGA20ox1* also responded but to a lesser extent. Stimulation of petiole elongation by EOD FR is reduced in a transgenic *AtGA20ox2* hairpin gene silencing line. By contrast, it was only in SD that a T-DNA insertional mutant of *AtGA20ox1* (*ga5-3*) showed reduced response. Circadian entrainment to a daytime pattern provides an explanation for the SD expression of *AtGA20ox1*. Conversely, the strong EOD/LD FR responses of *AtGA20ox2* may reflect its independence of circadian regulation. While FR acting via PHYB increases expression of *AtGA20ox2*, other GA biosynthetic genes are known to respond to R rather than FR light and/or to other PHYs. Thus, there must be different signal transduction pathways, one at least showing a positive response to active PHYB and another showing a negative response.

The light environment of plants, particularly its quality and daily duration, regulates many aspects of plant development including seed germination, shoot elongation, shoot architecture, and flowering. Of the plant photoreceptors involved in such light responses, the phytochromes (PHYs), cryptochromes, and phototropins are most important (see Quail, 2002).

Typically, PHY acts by regulating the synthesis of the GA class of plant growth regulators. For example, a brief (5–10 min) exposure to PHY-active far-red (FR) light at the end-of-day (EOD) increases bioactive GA content in isolated, elongating epicotyls of cowpea (*Vigna unguiculata*; Martínez-García et al., 2000), a system in which applied GA stimulates elongation, as is also known for a number of rosette dicot species (for review, see Smith, 1995; García-Martínez and Gil, 2002). Similarly, a prolonged (>6 h) FR-rich long day (LD) exposure causes petiole and stem elongation and an increase in GA content in spinach (*Spinacia oleracea*; Wu et al., 1996), bean (*Phaseolus vulgaris*; Beall et al., 1996), and Arabidopsis (*Arabidopsis thaliana*; Xu et al., 1997; Gocal et al., 2001). In addition, FR-rich LD exposures increase the activity of a key group of GA biosynthetic enzymes, the 20-oxidases (Gilmour et al.,

1986), and there are matching increases in expression of 20-oxidase mRNAs (Wu et al., 1996; Xu et al., 1997; Lee and Zeevaart, 2002).

In contrast to the FR stimulation of 20-oxidase expression in shoots, in germinating seed (Toyomasu et al., 1993; Yamaguchi et al., 1998), and during seedling deetiolation (Reid et al., 2002), red (R) light acting via PHY may regulate expression of 3-oxidases, another class of GA biosynthetic gene. In germinating lettuce (*Lactuca sativa*) seed, for example, GA 3-oxidase expression and its biosynthetic product, GA₁, increase within 4 h of a brief R exposure (Toyomasu et al., 1993, 1998). Similarly, during Arabidopsis seed germination, expression of two 3-oxidase genes increases rapidly on exposure to R light (Yamaguchi et al., 1998). In a parallel manner, during deetiolation in pea, inhibition of stem elongation by R light involves a reduction in GA content and down-regulation of the expression of various GA biosynthetic genes (Reid et al., 2002). Interestingly, in germinating lettuce seed, R promotes the expression of a 3-oxidase during the early hours of germination but also inhibits the expression of two GA 20-oxidase genes, one of these showing an increase in FR (Toyomasu et al., 1998). Thus, depending on the physiological response and the wavelengths of light used, expression of any GA biosynthetic gene might increase or decrease.

Genetic studies have provided clear evidence that the PHY family of photoreceptors are responsible for responses to brief R or FR exposures. In Arabidopsis,

* Corresponding author; e-mail rod.king@csiro.au; fax 62–262465275.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.059055.

PHYB, D, E, and possibly PHYC, act redundantly in such regulation of shoot elongation, leaf growth, and flowering (Franklin et al., 2003). R/FR photoreversibility is also diagnostic of this action of the PHYB-type genes whereby, in a sequence of brief exposures (i.e. 5–10 min), the effect of R can be fully reversed by a following FR exposure (see Borthwick et al., 1952; Smith, 1995). Unlike the other four PHY, Arabidopsis PHYA is unstable in light, it is important in responses involving prolonged exposure to FR light, it is considered to act during LD exposures (see Quail, 2002), and it might be active in the FR responsive, GA-regulated responses discussed above.

In our earlier studies with Arabidopsis, we showed that FR-rich light during an LD caused rapid increases (within 2 d) in both petiole elongation and their content of bioactive GAs (Gocal et al., 2001), while application of an inhibitor of GA biosynthesis abolished the LD stimulation of elongation. Thus, it was important to establish which photoreceptor(s) were active and on which GA biosynthetic gene(s). As a model plant, Arabidopsis provides the necessary molecular information and genetic tools for such a study and, here, we have analyzed GA biosynthetic gene expression and used mutants and gene silencing lines to examine the way light regulates plant growth. The four questions that provide a focus for this study are: (1) Which genes of GA metabolism show substantial increases in expression in response to FR exposure? (2) Does expression of GA biosynthetic genes oscillate in a circadian manner? (3) Does decreasing the expression of GA biosynthetic genes reduce petiole elongation? (4) Which photoreceptors are important in the stimulation of both petiole elongation and the expression of GA biosynthetic genes?

RESULTS

Enhanced Expression of GA Biosynthetic Genes with FR Exposure

Following exposure to a single low-irradiance FR-rich LD from incandescent lamps, Arabidopsis plants double their petiole elongation compared with plants kept in short day (SD; Fig. 1). Furthermore, since a 10-min EOD FR exposure in SD gave a comparable stimulation to that from a LD (Fig. 1), we can conclude that the enrichment for FR wavelengths in incandescent lamps accounts for their effectiveness. We return to this matter later but first examine the effect of LD/FR exposure on the expression of GA biosynthesis genes.

Using quantitative real time PCR (Q-PCR) and specific primers for 13 of the known GA biosynthetic genes, the effect of daylength on their mRNA expression level was examined in young elongating petioles of Arabidopsis (Fig. 2). We could detect little or no transcript of *AtGA3ox2* and *AtGA2ox1*, while *AtGA2ox3* was barely detectable and we could not detect expression of *AtGA2ox4* or *AtGA2ox5*. The *ACTIN* standard was

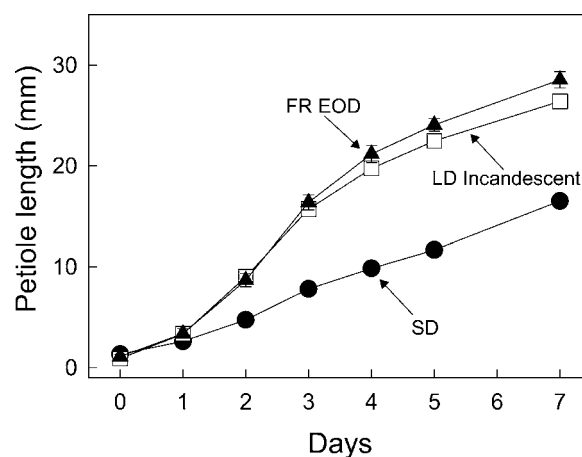


Figure 1. Effect of daylength (SD versus LD) or a brief EOD FR light exposure on elongation of young petioles of Arabidopsis. Plants of ecotype Columbia were 7 weeks old. The EOD FR exposure was for a 10-min duration at the end of the 8-h SD. The LD incandescent exposure was for 16 h at the end of the main 8-h day. Values in this and subsequent figures are means \pm SE, the latter shown as bars which, when not evident, were smaller than the symbol.

effectively detected in all samples, which excludes the possibility of failed assays. The lack of detection of *AtGA3ox2* may reflect differences in tissue specificity as it has only been detected previously in germinating seeds (Yamaguchi et al., 2001).

The major effect of LD is to increase expression of *AtGA20ox2* in elongating petioles, especially after the second LD exposure (Fig. 2). These findings fit with earlier evidence that GA 20-oxidases are important for the LD-regulated increases in GA biosynthesis that are required for increases in petiole and stem elongation (Gilmour et al., 1986; Xu et al., 1997; Lee and Zeevaart, 2002). However, it appears that not all 20-oxidases show such a large LD response. For *AtGA20ox1*, there was only a limited effect of LD on its expression (Fig. 2).

Despite the fact that the *AtGA3ox* genes are important for increased GA biosynthesis during R-light-regulated seed germination in Arabidopsis (Yamaguchi et al., 1998), our study shows that LD had no effect on expression of *AtGA3ox1* in petioles (Fig. 2). Also, there is no change in expression in response to EOD FR treatments (Fig. 7) or in stems of Arabidopsis plants exposed to up to eight cycles of LD (Xu et al., 1997).

Does Expression of GA Biosynthetic Genes Oscillate in a Circadian Manner?

Given the clear diurnal oscillations we observed in expression of at least one 20-oxidase gene and possibly some of the genes for early biosynthetic steps (Fig. 2), control by a circadian oscillator is likely. Therefore, some 7-week-old plants held in 8-h SD from high intensity light were transferred to continuous high intensity light (LL) and at the same constant temperature. Young petioles were harvested every 4 h and, as shown

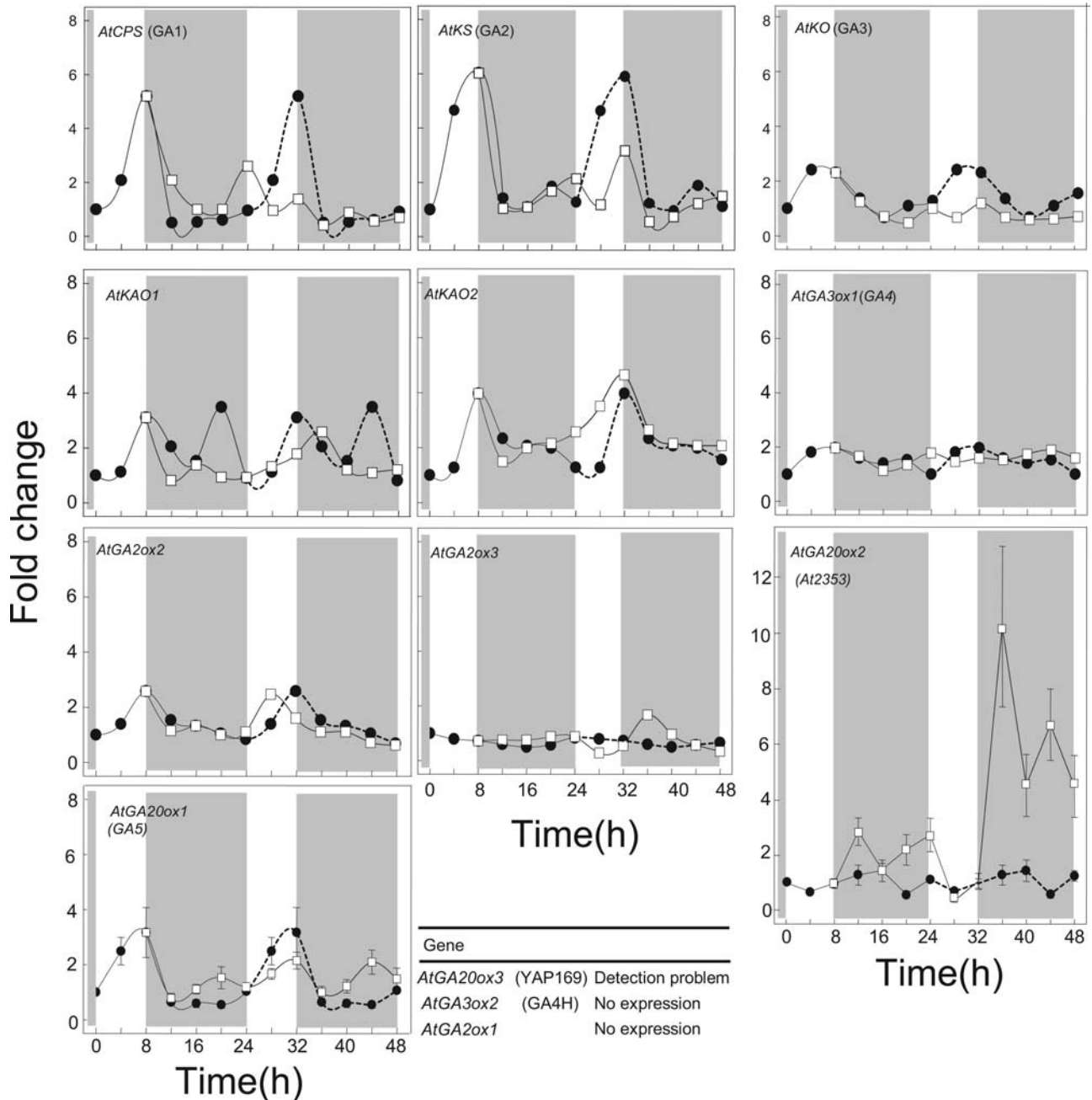


Figure 2. Effect of daylength on the pattern of expression in petioles of some GA biosynthetic genes. Arabidopsis plants were grown in SD and then either maintained in SD (●) or exposed to two LD cycles (□). The shaded areas on each graph indicate the 16-h period of either darkness in SD or low intensity ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) light from incandescent lamps for LD treatments. Changes in gene expression are shown as fold increases relative to the time zero value, which, generally, was the lowest value. The dashed line on the second day is the SD samples from the day before, although a repeat of this SD sampling to cover 2 SD (Fig. 3) shows that there will be small variations from day to day. Other conditions as in Figure 1.

in Figure 3, in constant conditions, expression of *AtGA20ox1* clearly oscillates with a circadian periodicity (approximately 24 h). The phase over the first 2 d was the same as for plants harvested at the same time but kept in SD (Figs. 2 and 3). There was little or no diurnal oscillation in expression of the second 20-oxidase, *AtGA20ox2* (Figs. 2 and 3), and it did not

show any circadian rhythmicity in constant conditions (Fig. 3). Rather, in SD, there was a noncircadian cycling in expression of *AtGA20ox2* and this was reproduced in both the experiments shown (Figs. 2 and 3). Expression of some 20-oxidases is down-regulated by bioactive GA products (see Phillips et al., 1995), which may explain this noncircadian cycling of *AtGA20ox2*.

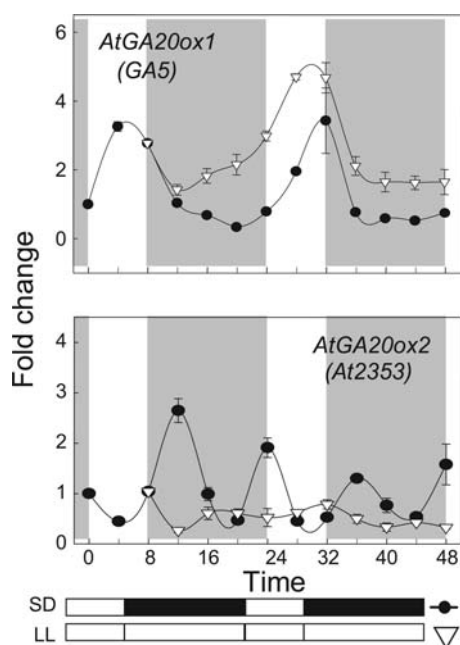


Figure 3. Oscillation in 20-oxidase gene expression in petioles of plants of Columbia transferred from SD cycles (8:16 h light:dark; ●) to constant LL (continuous fluorescent light at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 22°C ; ▽). Other conditions as in Figure 2.

Based on our spectrophotometric measurements, input RNA amounts were equal and the constancy of the *ACTIN* assays supports this claim. For the three replicates of all 23 samples in Figure 3, the average *ACTIN* expression was 0.90 ± 0.06 , after normalization across assays to the value of the first SD sample taken as 1.0. Thus, there is little variation in the loading control and the circadian oscillation must be in the expression of *AtGA20ox1*. Furthermore, the absence of any cycling in *AtGA20ox2* expression in the same RNA samples supports this argument. Previously, Blazquez et al. (2002) failed to find evidence of oscillations in expression of *AtGA20ox1* with seedlings of Columbia grown in almost identical conditions, but their use of total shoot tissue could account for this difference.

To restate the above findings, *AtGA20ox1* is entrained by the circadian clock and expresses more during the 8-h main SD-light period (Figs. 2 and 3). By contrast, expression of *AtGA20ox2* is not entrained by the circadian clock, it expresses at low levels in darkness or during any R-rich fluorescent exposure (Figs. 2 and 3), and it expresses most during the FR-rich LD, increasing up to 7-fold, whereas expression of *AtGA20ox1* barely doubled in LD (Fig. 2). To emphasize these differences between *AtGA20ox1* and *AtGA20ox2* in their response to daylength and light quality, a petiole sample was taken just before the start of the main 8-h-SD light period (predawn) when *AtGA20ox2* expression should be elevated. A second sample was taken at the end of the main 8-h-light period (predusk) when *AtGA20ox1* expression should be elevated. The results in Figure 4 confirm these expectations and, most importantly,

show that only an FR-rich incandescent LD exposure activated *AtGA20ox2* expression; a comparable low irradiance LD exposure ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) from R-rich fluorescent lamps gave no LD increase. Furthermore, in the same experiment, the R-rich fluorescent light gave no enhancement of petiole elongation (Fig. 4). Thus, not only are these LD photoresponses linked closely to FR wavelengths and not to the R of fluorescent lamps, but these differences indicate that the blue wavebands of the fluorescent lamps may also be unimportant. Later, we return to the identity of photoreceptors. A further implication of these findings is that blocking *AtGA20ox2* expression should impact on the LD enhancement of petiole elongation, while mutation of *AtGA20ox1* should only impact on petiole growth in SD and we consider these predictions below.

Does Decreasing the Expression of GA Biosynthetic Genes Affect Petiole Elongation?

A Salk line (Alonso et al., 2003) with a T-DNA insert in the intron of the *AtGA20ox1* gene was selected in the Columbia background and this mutant is subsequently designated *ga5-3*. Based on reverse transcription-PCR assays with petioles of *ga5-3*, we could detect no expression of *AtGA20ox1* (Fig. 5A), as might be predicted. Conversely, expression of *AtGA20ox2* did not decrease but increased by about 50% in the *ga5-3* mutant (Fig. 5B), which may indicate a gene-specific reduction in feedback control of 20-oxidase gene expression by a lowering of bioactive GA product.

We were unable to obtain a T-DNA insertional mutant of *AtGA20ox2*, although we examined two candidate lines available in the Salk Collection. Therefore, we used an RNA silencing approach and obtained 13 stably transformed second generation transgenic hairpin lines. As shown in Figure 5B, relative to wild type, *AtGA20ox2* expression was reduced by 90% in line 3, to about 50% in line 2 and by about 10% in line 12 that was included as a transformed control. There was no effect of reduction in expression of *AtGA20ox2* on expression of *AtGA20ox1* (Fig. 5B).

For these lines with their reduced 20-oxidase expression, petiole growth in SD or following an EOD FR exposure is shown in Figure 5, C and D. For *ga5-3*, its elongation in SD was reduced by approximately 25% compared to the wild type (Fig. 5C). However, *ga5-3* showed a normal increase in response to an EOD FR exposure (Fig. 5D) and to an FR-rich LD (data not shown), this claim being based on first removing the SD effect that we have done here by calculating the LD increment over that of the same line in SD. With the *AtGA20ox2* transgenic hairpin lines, the converse response was evident. They responded as well as the control in SD (Fig. 5C) but showed reduced FR (Fig. 5D) or LD response (data not shown). Importantly, the extent of gene silencing for the three *AtGA20ox2* lines (Fig. 5B) matches the reduction in their petiole elongation on exposure to EOD FR (Fig. 5D). This finding further supports our claim that *AtGA20ox2* expression

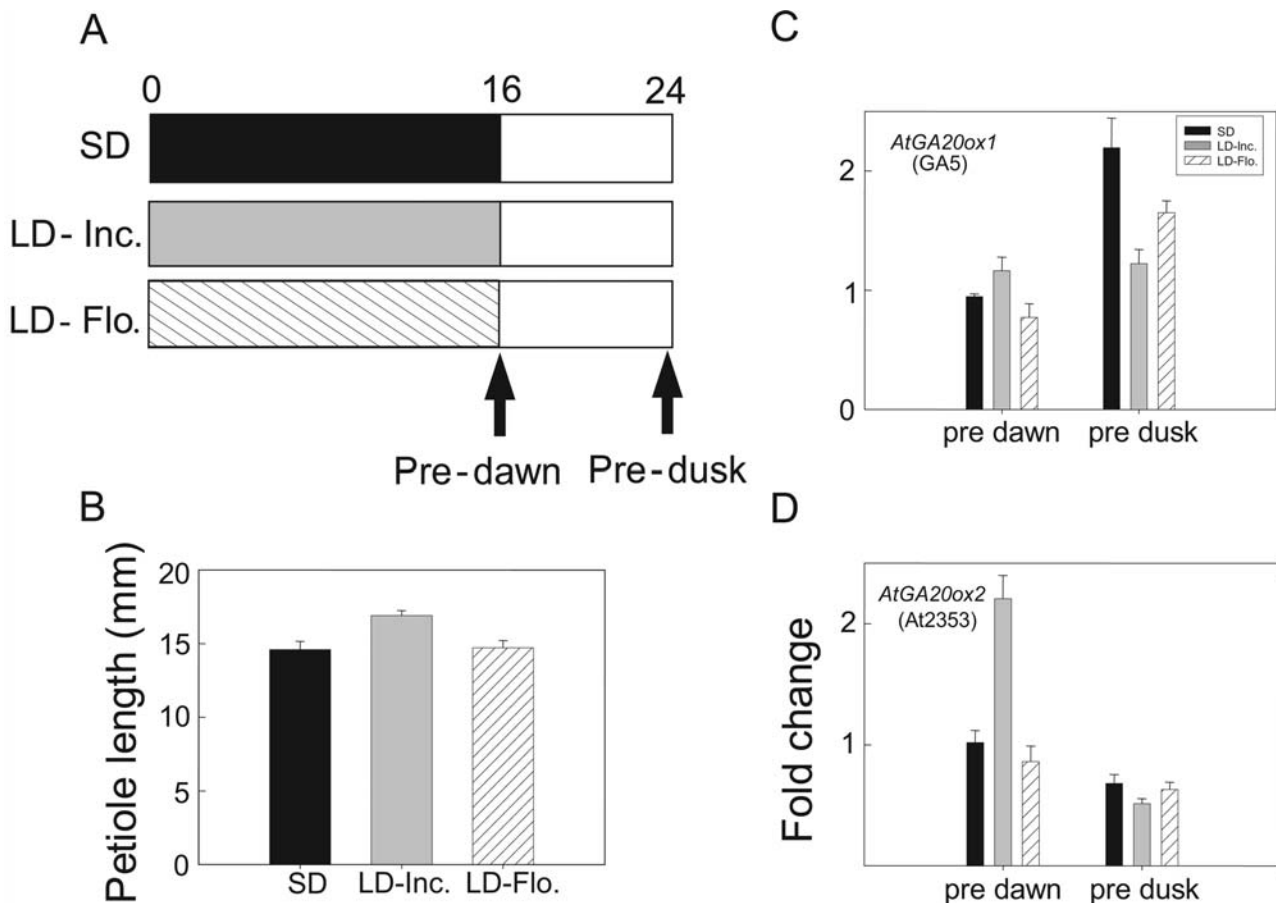


Figure 4. Effect of LD light quality on petiole elongation and on expression in the petiole of two GA 20-oxidase genes in ecotype Columbia. The LD was at a low irradiance of $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ from red-rich fluorescent (Flo) or FR-rich incandescent (Inc) lamps as shown in A. The arrows in A indicate harvest times of the harvests shown in C and D for analysis of gene expression. The predawn, morning harvests were taken after a single overnight LD or at the same time in SD conditions. The predusk samples were taken 8 h later at the end of the daily 8-h fluorescent light period. Petiole length (B) was determined 4 d after the start of the LD exposure. Other conditions as in Figure 2.

induced by FR-light is important for the synthesis of GAs required for the LD stimulation of petiole elongation. No other "growth" phenotypes were obvious in these gene silencing lines.

In a further, preliminary study with *GA20ox* antisense lines provided to us by A. Phillips and P. Hedden (Biotechnology and Biological Science Research Council, Rothamsted, UK), we have found a similar daylength differential in the regulation of petiole elongation. The *AtGA20ox1* antisense line showed a 22% reduction in its petiole elongation in SD and no reduction for the *AtGA20ox2* antisense line (data not shown). Conversely, LD response was reduced somewhat in the *AtGA20ox2* antisense line but there was no reduction in LD response for the *AtGA20ox1* antisense line (data not shown). That petiole elongation is reduced in these antisense lines was noted by Coles et al. (1999) based on photographic records with young seedlings, but no effects of daylength were established in that study.

Overall, circadian changes regulate *AtGA20ox1* so that it expresses most in the "daytime" of SD. By

contrast, *AtGA20ox2* lacks this circadian pattern of response and shows a FR-rich light, LD response over the "nighttime" hours of each daily cycle. Thus, there could be significant differences between these genes in their circadian regulation and in their response to different light treatments. We examine the photoregulation of these genes in more detail in the following section.

Photoreceptors, Expression of GA Biosynthetic Genes, and Regulation of Petiole Elongation

Of the various photoreceptors, we have examined only PHY action since petiole elongation and 20-oxidase expression increased in FR-rich LD or EOD FR conditions and not in R-rich conditions (Figs. 1 and 4). In an initial experiment, after exposure to a single 10-min EOD FR, *AtGA20ox2* expression increased rapidly (within 3 h) and dramatically (up to 40-fold), and this increase was duplicated when the FR exposure was repeated the next day (Fig. 6). Importantly, the results in Table I show that the EOD FR promotion of *AtGA20ox2* expression and of petiole elongation

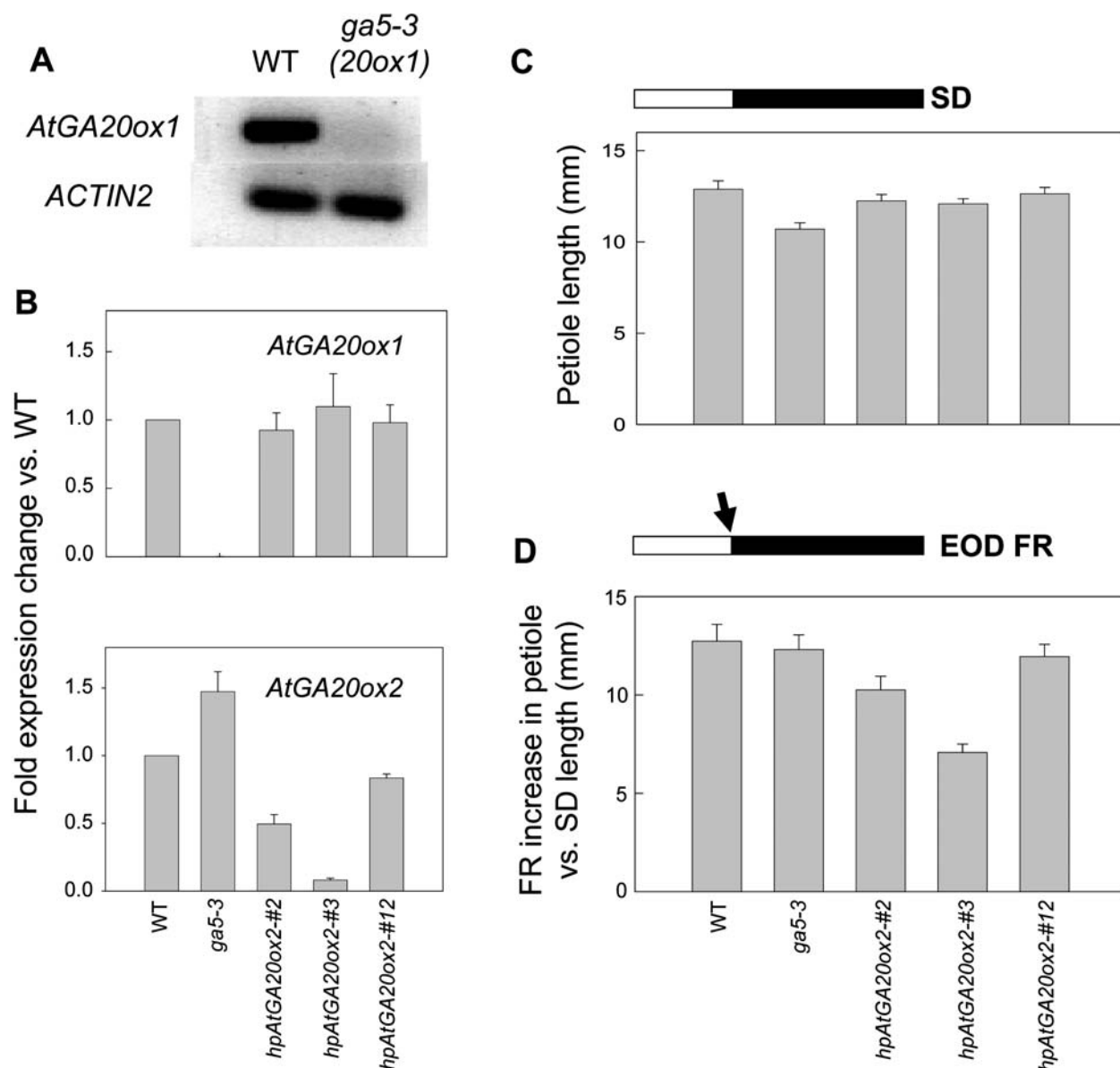


Figure 5. Expression in petioles of GA 20-oxidases (A and B) and petiole elongation in SD (C) or with EOD FR (D). For *AtGA20ox1* gene expression, petioles were harvested predusk in SD and, for *AtGA20ox2*, after 3 h in the dark following an EOD FR exposure. Petiole elongation is shown in D as the FR increment over that in SD (C). The lines examined were: WT, ecotype Columbia; *ga5-3*, a TDNA-insertional mutant of *AtGA20ox1* in Columbia; and three Columbia lines, *hpAtGA20ox2-2*, *-3*, and *-12*, transformed with a hairpin construct designed to specifically block *AtGA20ox2* expression. The extent of inhibition of *AtGA20ox2* expression (virtually no effect in no. 12 to a 90% reduction in no. 3) is matched by a reduced FR increment in petiole growth.

was fully reversed by a subsequent exposure to 10 min of R light. Both *AtGA20ox* genes showed increased expression after FR in the experiment shown in (Fig. 6), but *AtGA20ox1* showed a weaker response and sometimes no change after FR whereas *AtGA20ox2* always responded strongly (Fig. 6; Table I; a third experiment not shown here). In a further study, but with *AtGA3ox1* that regulates a different GA biosynthetic step, there was no effect of R/FR on its expression in the petiole (Fig. 7), which confirms the evidence in Figure 2 for plants exposed to a FR-rich LD.

The R/FR photoreversible control of gene expression as shown in Table I is diagnostic for PHY action (Borthwick et al., 1952) and, specifically, for the light stable PHY of the PHYB class (PHYB, C, D, and E; see Quail, 2002). To reinforce this claim, the wild type and a *phyA* mutant showed identical EOD FR effects on 20-oxidase expression and petiole elongation (Figs. 7 and 8). Thus, PHYA is not part of the response to either EOD FR or to FR-rich LD exposures. In a *phyB* null mutant, we expected the converse response, greater petiole elongation, and higher levels of gene expres-

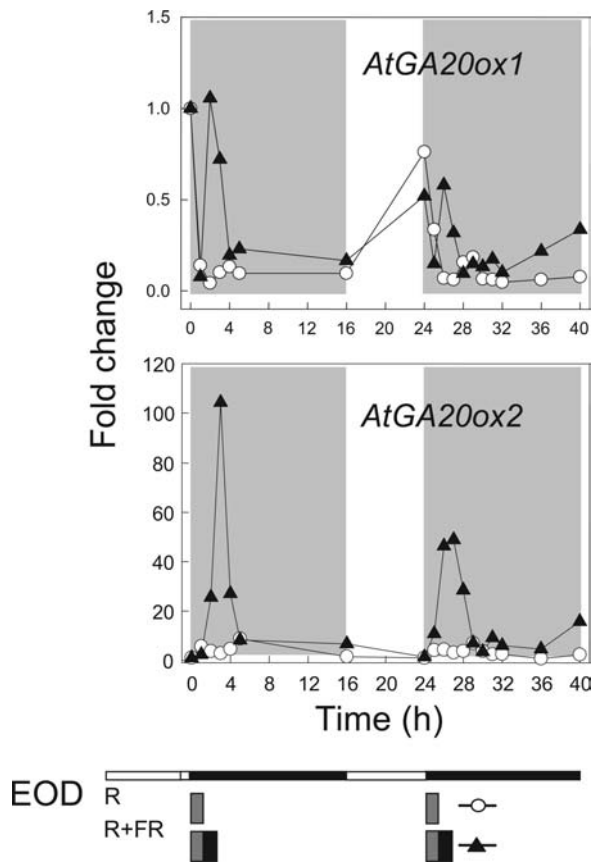


Figure 6. Enhancement by FR light of GA 20-oxidase gene expression in elongating petioles exposed twice over 2 d to 10 min EOD R or EOD R/FR light terminating the first and second of two 8-h SD (ecotype Columbia). Other conditions as in Figure 2.

sion in R light, particularly since hypocotyl elongation in young seedlings of this mutant is almost as great as for etiolated (dark) controls (Reed et al., 1993). The fact that light grown plants of the same mutant showed little or no increases in elongation or of gene expression when PHYB input was reduced in the mutant or when, in addition, the mutant was exposed to FR (Figs. 7 and 8), indicates that in adult plants, secondary, pleiotropic effects of the mutant make it unsuitable for testing for PHYB input. Nevertheless, our physiological demonstration of photoreversibility in the wild

type stands as unequivocal evidence for the action of PHYB or of related PHYs.

The complex pattern of diurnal changes in expression of *AtGA20ox1*, particularly at the end of the SD (Figs. 2 and 6), could make it difficult to identify responses to FR exposure. Therefore, in further studies, the time of giving FR was either delayed by 3 h after the start of darkness or was given early when the main 8-h-SD light period was terminated after 4 h. The times to peak expression after such changes in the time of FR exposure were the same as before in Figures 6 and 7 (2 h in dark after FR for *AtGA20ox1* and after 3 h for *AtGA20ox2*) and, again, the more substantial increases were for *AtGA20ox2* (data not shown). Thus, the diurnal and circadian patterns of gene expression do not appear to influence the response to EOD FR.

DISCUSSION

Here, based both on gene expression analysis and on responses in a mutant or in gene silencing lines, we show that GA 20-oxidase biosynthetic genes are involved in the petiole elongation in Arabidopsis associated with exposure to EOD FR light or to LD from FR-rich incandescent lamps. The light input involves PHYB and related PHYs and not PHYA since responses were the same in the wild type and a *phyA* mutant. Furthermore, it is unlikely that there was an additional blue light action because petiole elongation and 20-oxidase expression could be accounted for as an R/FR response, an LD exposure from fluorescent tubes having the same effect as an R EOD treatment.

The rapid increase in elongation on exposure to FR fits with the scenario that increased expression of GA 20-oxidases caused increased GA synthesis and, in turn, greater elongation; gene expression increased within 3 h of a 10-min EOD FR exposure (Figs. 6 and 7) or within 4 h of the start of the first of two FR-rich LD exposures (Fig. 2). The first sign of increased petiole elongation is at 2 d (Fig. 1) by which time the GA₁ and GA₄ contents of the young petioles have increased up to 3-fold (Gocal et al., 2001). It is clear that GA is essential for elongation as the response is reduced in a mutant and in gene silencing lines (Fig. 5). Furthermore, in our earlier studies (Gocal et al., 2001), we showed that

Table I. R/FR photoreversible regulation of elongation of young petioles and of the expression of two GA 20-oxidase genes

At the end of each 8-h SD cycle from fluorescent lamps, plants were exposed sequentially to 10 min of R and FR light. Petiole elongation was determined after 16 h in darkness following the second daily exposure to R/FR. Values are means \pm SE. Harvests for assays of gene expression were 3 h after the start of the dark period and these values have been normalized to the time zero value 3 h earlier. The differences shown for DAY I were also evident on DAY II (data not shown) and were replicated in other more detailed time courses shown in Figures 6 and 7.

	R	R + FR	R + FR + R
Petiole length at 2 d (mm)	10.0 \pm 0.4	14.6 \pm 0.7	10.6 \pm 0.4
<i>AtGA20ox1</i> expression versus time zero	0.4	1.4	0.5
<i>AtGA20ox2</i> expression versus time zero	3.0	35.3	3.0

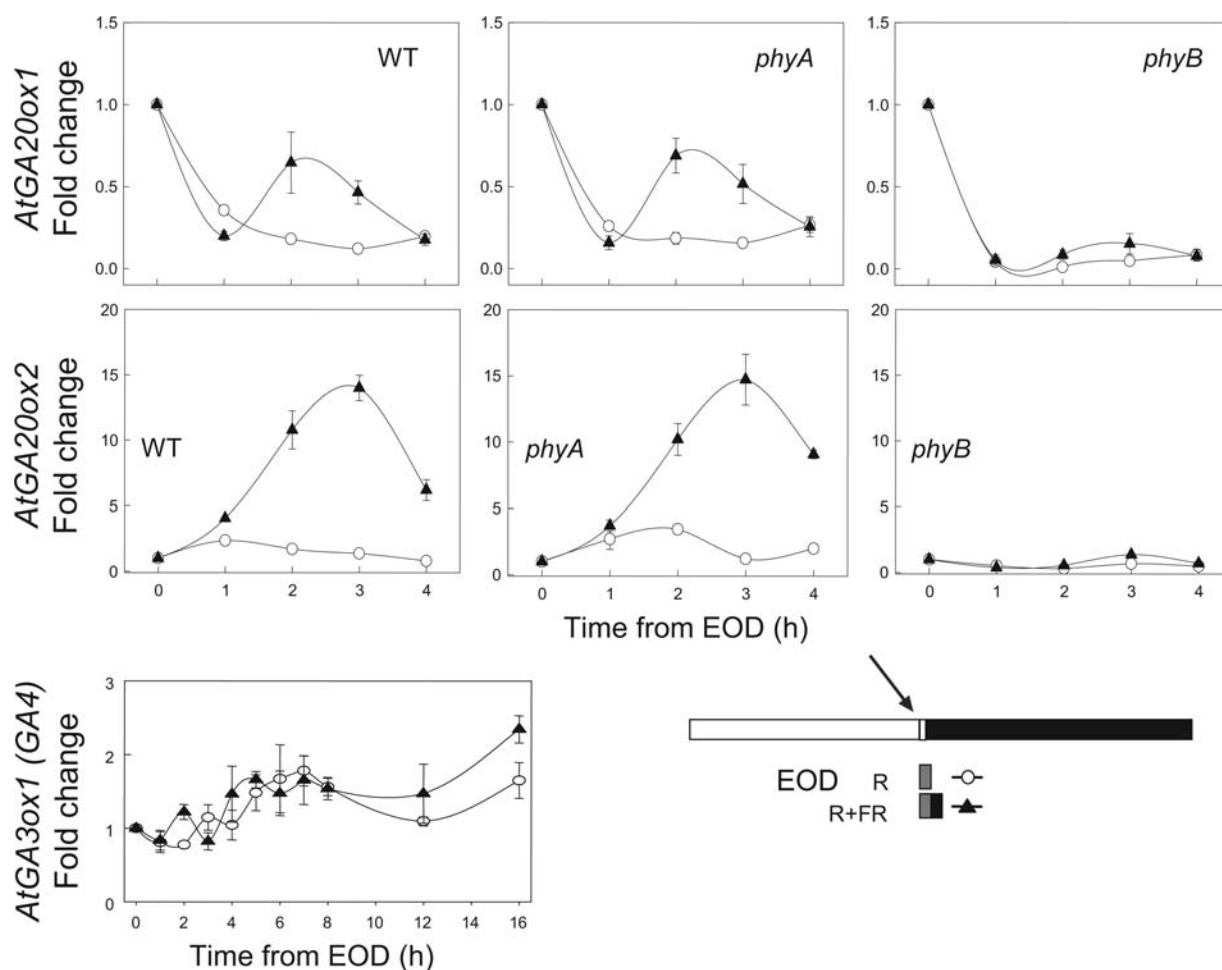


Figure 7. Effect of EOD FR light on GA 20-oxidase gene expression in elongating petioles of the Columbia wild type and of the *phyA-211* and *phyB-9* mutants in the Columbia background. Harvests were made over the first 4 h of the dark period following a single EOD R or an R/FR treatment. A further experiment is included that shows that EOD FR did not enhance expression of the 3-oxidase gene, *AtGA3ox1*, in elongating petioles of Columbia. Other conditions as in Figure 2.

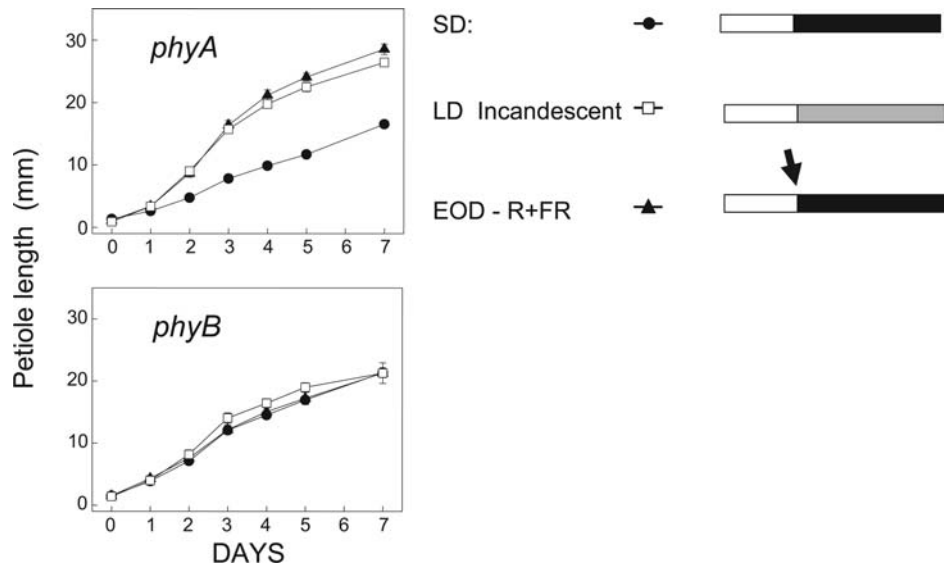
application of a GA biosynthesis inhibitor blocked the increase in petiole elongation on exposure to a FR-rich LD, the effect of the inhibitor being reversed by applied GA_4 , while GA_4 alone applied to plants in SD increased petiole elongation (Gocal et al., 2001). Thus, we have provided substantial evidence that an increase in GA synthesis is central to the enhanced petiole elongation caused by a FR-rich LD or by exposure to EOD FR. It remains to be clarified whether the light acts directly on the petiole to increase GA synthesis there. In the most relevant study, García-Martínez and coworkers (1987) showed that light input and GA increase were directed to the growth-responsive epicotyl tissue of cowpea but they could not exclude an additional action of light via the leaf.

Expression profiling of elongation-related genes associated with exposure of *Arabidopsis* to FR-rich light (Devlin et al., 2003) indicated that a broad spectrum of genes regulate or are regulated by FR but, without detailed mutant and phenotype analysis, few conclusions can be drawn from such findings. On the other hand, the FR phenotype is consistently linked with

increases in GA 20-oxidases and in GA content (see ref. in García-Martínez and Gil, 2002). Our data confirm this linkage as, based on analysis of 10 *Arabidopsis* GA biosynthetic genes, the 20-oxidases responded most to FR-rich LD (Fig. 2). Similarly, from an analysis of the expression of a 2-oxidase, a 3-oxidase, and a 20-oxidase in spinach petioles, Lee and Zeevaart (2002) concluded that a 20-oxidase was most significant. More importantly, however, our findings with a mutant and with hairpin gene silencing lines (Fig. 5) confirm that one 20-oxidase, *AtGA20ox2*, is central to FR-regulation of petiole elongation. Thus, a narrowed focus on the 20-oxidase step of GA biosynthesis is readily justified.

A unique aspect of our study is the contrast apparent between two of the *Arabidopsis* 20-oxidase genes. *AtGA20ox1* responds weakly to FR and is regulated by a circadian clock such that its maximum expression and physiological effectiveness is seen in SD. By contrast, *AtGA20ox2*, which shows no circadian control, is mostly expressed when exposed to FR-rich EOD or LD conditions (Figs. 2 and 3). These distinctions explain the daylength-specific effects of the mutant and si-

Figure 8. Effect of two PHY mutants, *phyA-211* or *phyB-9*, on elongation of young petioles of Columbia exposed to an LD from incandescent lamps or to a 10-min EOD FR light treatment. For the same experiment, petiole elongation of wild type, Columbia, was shown in Figure 1, and it matches almost exactly that of the *phyA* mutant shown here.



lencing lines in that rhythmic gating of gene expression and a weak FR response confer the SD phenotype of *ga5-3* (Fig. 5). Conversely, there is an LD phenotype in the *AtGA20ox2* hairpin silencing lines (Fig. 5) because of the greater FR response of *AtGA20ox2* coupled with its lack of rhythmic gating. The daylength-specific effects of these two 20-oxidase genes also have implications for studies of flowering of Arabidopsis. For example, in an FR-rich LD, *AtGA20ox2* should regulate a GA-dependent pathway and we will present such an analysis of flowering responses in a future publication.

A model summarizing the interrelationships between LD or EOD FR light, GA metabolism, and elongation is presented in Figure 9. In relation to light regulation of GA biosynthesis, it is known that, in R light, PHY Pfr activates seed germination in association with increased expression of the 3-oxidase GA metabolism genes of Arabidopsis (Yamaguchi et al., 1998) and lettuce (Toyomasu et al., 1993, 1998). However, our findings show that expression of 3-oxidase in mature plants was unchanged by R/FR exposures, whereas photoconversion of PHYB to Pr in LD or with

FR EOD increased 20-oxidase expression. Clearly, the lack of Pfr in FR activates a signal cascade that enhances the 20-oxidase step of GA biosynthesis and, so, elongation increases. Conversely, the presence of Pfr in R light activates GA biosynthesis but via increase in 3-oxidases, potentially only during germination and, apparently via a different signal transduction path.

The 20-oxidase, *AtGA20ox2*, is central to the LD/FR regulated PHY response associated with elongation, a claim also supported by our evidence of little or no change for the nine other GA metabolism genes studied here and including two 2-oxidases that regulate GA catabolism. As discussed above, the interaction between LD and circadian regulation of 20-oxidases can be significant but has received too little attention previously. The more significant LD-regulated changes occur rapidly, overnight (Fig. 2), and apparently independently of circadian regulation (Fig. 3). However, in earlier studies of Lee and Zeevaart (2002), 20-oxidase expression in LD was examined only at dawn and dusk, while the analysis of 20-oxidase rhythms by Carrera et al. (1999), although

Figure 9. A model for the interactions between PHY-regulated light input, GA 20-oxidase expression, and EOD/LD regulation of petiole elongation in Arabidopsis. The shading emphasizes those changes shown to be essential for elongation in response to FR-rich conditions. Negative regulation is shown by the bar replacing an arrowhead.

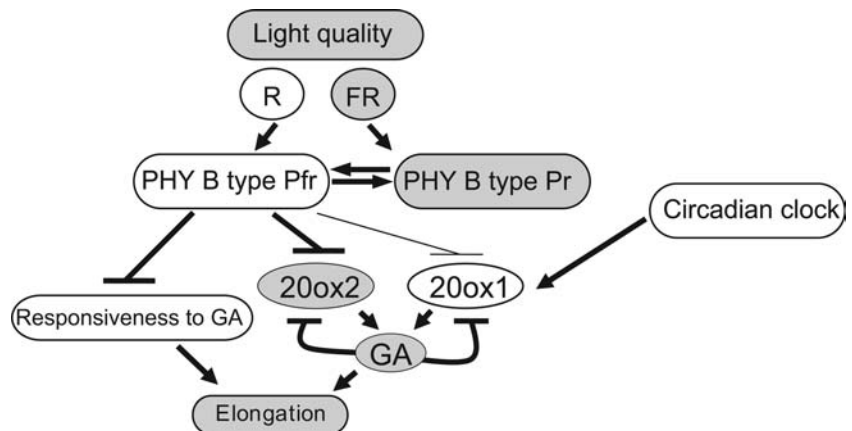


Table II. Genes assayed and primers used for Q-PCR assays

Gene	AGI Gene Code	Forward Primer	Reverse Primer
<i>AtCPS</i>	(GA1) At4g02780	CTAAGCTTACAAGGATACC	GACATTGGGAAGCTCTCC
<i>AtKS</i>	(GA2) At1g79460	GGATCTTAAATGTGATAGTG	AAGGAACTGCAGCCTCCG
<i>AtKO</i>	(GA3) At5g25900	TGCCAAAGAGGCCATGG	TTCGTTTCTGTGCATTAGC
<i>AtKAO1</i>	(CYP88A3) At1g05160	AGAGCACTCAAGGCTAGG	CTTGTTCCAGCCTTTGCTC
<i>AtKAO2</i>	(CYP88A4) At2g32440	AGAGCTTTGAAGGCAAGG	TCTCTTGTCTTCTCTTAGC
<i>AtGA20ox1</i>	(GA5) At4g25420	CTCATGAATACACGAGCC	TGATACACCTTCCCAAATG
<i>AtGA20ox2</i>	(At2353) At5g51810	ATGCTCACCGTTTGATGG	CCTTCCCAAAGTGTCTCG
<i>AtGA20ox3</i>	(YAP169) At5g07200	CCTATCTGCATATGGACTC	AAACCTTCCCGAAATCTTC
<i>AtGA3ox1</i>	(GA4) At1g15550	CCACGGCGTGCCTTTGG	GATATCGCAGTAGTTGAGG
<i>AtGA3ox2</i>	(GA4H) At1g80340	CCTCGCGACTTCTCGAC	AATAATTTACAGTATTTGAGG
<i>AtGA2ox1</i>	At1g78440	CGGGAACCTTCAGAAACGC	ACATTCTTACCACCATGG
<i>AtGA2ox2</i>	At1g30040	TTCCGTGAGTCGGTGG	CTCCGCCTTCTCTCCG
<i>AtGA2ox3</i>	At2g34500	GCAATTTTCAGAGAGGCAG	CTTCTCTTGACCGGAG

involving frequent sampling, did not focus on changes occurring during the switch between daylengths.

The complex ways PHY regulates different GA biosynthetic genes, different signal transduction pathways, and different physiological responses is further heightened by studies of deetiolation. In pea, R light acting via Pfr inhibits growth of etiolated plants and blocks GA synthesis (Reid et al., 2002), the expected converse of the effect of removal of Pfr to allow growth of Arabidopsis petioles. However, unlike the evidence that PHYB and not PHYA regulate petiole elongation (Figs. 7 and 8) and seed germination of Arabidopsis (Yamaguchi et al., 1998), for pea, PHYA is active and it regulates a decrease in a 3-oxidase. That R/FR photoreversible PHYB regulates both EOD FR and LD responses (Figs. 1, 6, 7, and 8; Table I) also raises questions about claims that flowering of Arabidopsis is mediated by PHYA; this claim must be treated with some skepticism.

An explanation for the clear evidence of R/FR photoreversible control of petiole elongation but the complete loss of response in the *phyB* mutant (Figs. 7 and 8) is not obvious. Were GA content to increase, as it appears to do in a sorghum *phyB* mutant (Lee et al., 1998), then 20-oxidase expression might be sufficiently down-regulated to become unresponsive to the removal of PHYB Pfr. Nevertheless, for an Arabidopsis *phyB* mutant, GA content of elongating hypocotyls is the same as the wild type (Reed et al., 1996). Perhaps PHYB also regulates some other component of GA signaling that alters responsiveness to GA but too little is known of this pathway for any conclusions to be drawn. Also, we cannot exclude the possibility of secondary changes given the well-documented pleiotropic effects of the *phyB* mutation (Reed et al., 1993).

In conclusion, for Arabidopsis, we have shown that FR-rich LD or EOD FR conditions stimulate petiole elongation by regulating expression of GA20-oxidase biosynthetic genes, as was also implied in earlier studies of elongation of petioles or stems (for review, see García-Martínez and Gil, 2002). Here, by using PHY mutants and R/FR photoreversibility assays, we have established that both EOD FR treatment and FR-

rich LD act through PHYB-type PHYs and not PHYA. In addition, our examination of expression of two 20-oxidase genes and of responses in their mutants has shown that their role in petiole elongation depends on light conditions: the one, *AtGA20ox1*, being more relevant in SD, the other, *AtGA20ox2*, responding to FR-rich LD daylengths/light qualities. There are important implications of these findings for studies of the role of GAs in LD-regulated flowering and this issue will be considered in a later publication.

MATERIALS AND METHODS

Plant Material, Growing Conditions, and Light Treatments

Plants of Arabidopsis (*Arabidopsis thaliana*) L. Heynh. ecotype Columbia and various mutant and transgenic lines of Columbia were grown for 5 to 7 weeks in 8-h SD at an irradiance of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 22°C. There was no flowering for plants held for up to 10 weeks in these SD conditions in light from red/blue-rich fluorescent tubes; however, flowering occurred within 3 weeks on transfer of 5-week-old plants to a 24-h LD from incandescent lamps (data not shown; Bagnall and King, 2001).

For studies of petiole growth, plants were either kept in the standard 8-h SD from fluorescent lamps or exposed to a LD given as an additional 16 h of light either from incandescent bulbs (FR-enriched light) or from fluorescent lamps (R-rich light) and both at an irradiance of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For 10-min R or FR light EOD exposures terminating the 8-h SD, FR light was obtained using a plastic cutoff filter and R was from red fluorescent lamps, as we described previously (Bagnall and King, 2001). In one experiment, an LL exposure was given and this was from fluorescent lamps at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Although we found no evidence of a touch effect, to minimize any touching we used calipers for daily measurements of petiole elongation. Single young petioles from 16 or more plants were measured in studies of elongation and, for studies of gene expression, young elongating petioles were harvested from 16 or more plants. The studies of gene expression required the use of a green safe light when young petioles were harvested during a dark period. All our findings were confirmed in duplicate experiments some of which are presented here.

Q-PCR Analysis of RNA Expression

Total RNA was extracted from young elongating petioles using an RNeasy Plant Mini kit (Qiagen, Clifton Hills, Australia) and treated with RNase-free DNase (Qiagen) according to the manufacturer's instruction. One or two micrograms of total RNA were reverse-transcribed using Super Script II (Invitrogen, Mt. Waverley, Australia) according to the manufacturer's instruction. The cDNA was diluted 5- or 25-fold and 4 μL used in a 20- μL Q-PCR with QuantiTect SYBR Green PCR Master Mix (Qiagen) or in a 10- μL Q-PCR

with SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich, Castle Hill, Australia). Q-PCR was performed on a Rotor-Gene 2000 Real-Time Cycler (Corbett Research, Sydney). Cycling and reaction conditions were as described in Klok et al. (2002). The sequences of the forward and reverse primers are shown in Table II along with gene information. Product sizes matched those expected from the known gene sequence. Amplicon sequence was confirmed for each primer set. At least one of each primer pair bridged an intron so there was no amplification of genomic DNA and all assays included a no-template sample to ensure Q-PCR results were not influenced by primer dimer formation. The Q-PCR assays were repeated three times and for any claimed treatment effects the result was confirmed in at least one further independent experiment. All samples were normalized using the Comparative Quantification analysis method (Rotogene-5 software, Corbett Research, Sydney). This method uses information about the start of the exponential phase of amplification (take-off point) and the average reaction efficiency. Concentrations were compared directly after normalization against a loading standard and to the value at lights-on of the first SD. The loading standard was *ACTIN-2* (At3g18780 forward primer, TCAGATGCCAGAAAGTCTGTTC and reverse, CCGTACAGATCCTTCCTGATATCC). Its expression was stable within and across experiments; as an example, in one experiment the normalized value was 0.9 ± 0.06 over 69 assays.

Mutant Selection and Production of Gene Silencing Lines

The *phyA-211* and *phyB-9* mutants in Columbia were described previously (Reed et al., 1993; Bagnall and King, 2001). A T-DNA-insertional mutant for *AtGA20ox1* was isolated in the Columbia background using Salk lines (accession no. 016701) obtained from ABRC (see Alonso et al., 2003). Subsequently we refer to this line as *ga5-3*. Transgenic hairpin lines for silencing *AtGA20ox2* were produced in the Columbia background using routine transvection techniques. Transformants were selected for kanamycin resistance and confirmation of the insert obtained by PCR for both the T-DNA and the hairpin sequence. Hairpin constructs were made using pHellsgate12 following the methods outlined by Helliwell and Waterhouse (2003). Primers for the hairpin construct (forward, GCACAACAACATCTCCGGCAG and reverse, AACTCTGTCTAATGTATCCG) were designed to span a unique coding region of *AtGA20ox2* with no blocks of sequence identity of over 18 bases between the construct and nontarget gene sequences. Expression analysis was performed as above.

ACKNOWLEDGMENTS

We thank Cheryl Blundell for her help with maintaining plants and Drs. Tony Miller, Masumi Robertson, and Peter Waterhouse for guidance with molecular techniques. Drs. Peter Hedden and Andy Phillips (BBSRC, UK) are thanked for supplying the Columbia 20-oxidase antisense lines that were used previously in the study of Coles et al. (1999). For helpful comments on the manuscript, we thank Drs. Lloyd Evans, Neil Olszewski, and Masumi Robertson. During his stay in Canberra, T.H. was supported by a NARO Research Abroad Grant for Young Scientists.

Received January 12, 2005; revised February 10, 2005; accepted February 15, 2005; published May 27, 2005.

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