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The Interaction of Light and Abscisic Acid in the Regulation of Plant Gene Expression[1]

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Extended dark treatments of light-grown plants of both *Lemna gibba* and *Arabidopsis thaliana* resulted in substantial increases in abscisic acid (ABA) concentrations. The concentration of ABA could be negatively regulated by phytochrome action in *Lemna*. As has been noted in other species, ABA treatment reduced *Lemna rbcS* and *Lhcb* RNA levels, which are positively regulated by phytochrome in many species. In view of these observations, the possibility that phytochrome effects on gene expression may be mediated primarily by changes in ABA was tested using a transient assay in intact plants. The phytochrome responsiveness of the *Lemna Lhcb2*1* promoter was still apparent in the presence of exogenous ABA. Additionally, when 2-bp mutations were introduced into this promoter so that phytochrome responsiveness was lost, a response to exogenous ABA was still present. We conclude that phytochrome- and ABA-response elements are separable in the *Lhcb2*1* promoter. We tested whether the effects of ABA on RNA abundance could be inhibited by treatment with gibberellin and found no evidence for such an inhibition. We have also found that the ABA-responsive *Em* promoter of wheat can be negatively regulated by phytochrome action. It is likely that this regulation is mediated at least in part by phytochrome-induced changes in ABA levels. Our results demonstrate that it is essential to take into account that dark treatments and the phytochrome system can affect ABA levels when interpreting studies of light-regulated genes.

Light regulation of gene expression in plants has been an important and active area of investigation for many years (reviewed by Tobin and Silverthorne, 1985; Thompson and White, 1991; Terzaghi and Cashmore, 1995). One class of the photoreceptors involved in such regulation, the phytochrome family, has been studied particularly intensively (reviewed by Quail et al., 1995). Phytochrome action has been shown to regulate the transcription of a number of genes, including genes encoding two major kinds of chloroplast proteins, the light-harvesting chlorophyll *a/b* proteins, and the Rubisco small subunits (*Lhcb* and *rbcS* genes, respectively). Phytochrome action can also negatively regulate the transcription of other genes, including, in some species, genes for phytochrome proteins. Many of the genes that can be regulated by phytochrome also can respond to other environmental and developmental signals; however, not all studies of light regulation have identified the photoreceptor involved (reviewed by Tobin and Kehoe, 1994).

While studying several genes from *Lemna gibba* that were negatively regulated by phytochrome action (*NPR* genes), we found that the NPR proteins were related to a family of proteins from late-embryogenesis genes that are positively regulated by ABA in other plant species (Okubara et al., 1993). Additional work demonstrated that ABA could also positively regulate the *NPR* genes (Williams et al., 1994). Furthermore, when light-grown plants were placed into D for 24 h, which results in an increase in the transcription of the *NPR* genes (Okubara and Tobin, 1991), the level of endogenous ABA was found to increase substantially (Williams et al., 1994). These results raised the strong possibility that the dark-induced increase in *NPR* transcription was a result of the increased ABA level.

ABA-responsive genes involved in stress responses as varied as cold, desiccation, and wounding have been studied by many groups (reviewed by DeLisle and Ferl, 1990; Skriver and Mundy, 1990; Chandler and Robertson, 1994; Dolferus et al., 1994). Short promoter motifs that are involved in responsiveness to ABA have been identified (Skriver et al., 1991; Marcotte et al., 1992; Iwasaki et al., 1995), and at least some of these include a core motif of ACGT. One such ABA-responsive gene is the wheat *Em* gene, whose RNA is abundant in developing embryos (Williamson et al., 1985). A transcription factor (EmBP1) of the basic-region Leu-zipper class designated bZIP has been shown to bind to a region including the ACGT motif in the *Em* promoter (Guiltinan et al., 1990; Niu and Guiltinan, 1994). Such a motif also exists in the *NPR1* and *NPR2* promoters (Okubara et al., 1993).

There have been a number of reports of ABA repression of the expression of genes that are known to be positively regulated by phytochrome. Quatrano et al. (1983) reported

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Abbreviations: D, darkness; FR, far red light; LUC, luciferase; R, red light.

that ABA inhibited the appearance of *rbcS* mRNA in germinating wheat seedlings, and ABA was also reported to inhibit the appearance of the rbcS protein in embryonic cotyledons of bean (Medford and Sussex, 1989). More recently, two groups reported that ABA could repress transcription of both *rbcS* and *Lhcb* genes in tomato and of *Lhcb* genes in soybean (Bartholomew et al., 1991; Chang and Walling, 1991). Because the expression of these genes is known to decline in dark-treated plants, it seemed essential to investigate the possible interrelationships between regulation of genes by light and by ABA in a single species.

MATERIALS AND METHODS

Growth and Treatment of Plants

Lemna gibba L. G-3 was grown aseptically on liquid E medium (Tobin, 1981) in continuous white light at 27°C; etiolated plants were supplemented with 3 μM kinetin and grown in intermittent (2 min/8 h) R. Plants were treated for 10 min with FR to convert Pfr to Pr before being placed in D. *Arabidopsis thaliana* (var Columbia) was grown in soil under continuous white light at 23°C. Plants were watered 1 d before and 2 d after being placed in D to ensure that they were not water stressed.

Determination of ABA Content

Samples were harvested under a dim green safelight and immediately frozen in liquid N_2. The determination of ABA content was carried out by radioimmunoassay as previously described (Bray and Beachy, 1985).

RNA Blot Analysis

Total RNA was isolated as previously described (Williams et al., 1994). After fractionation of 5 or 10 μg of each sample of RNA on a 1% agarose gel containing 6% formaldehyde, samples were transferred onto ZetaProbe nylon membrane (Bio-Rad) or nitrocellulose (Schleicher & Schuell) and hybridized according to manufacturer's protocols. *NPR1*, *Lhcb*, *rbcS*, and rRNA (Stiekema et al., 1983; Okubara and Tobin, 1991; Williams et al., 1994) probes were labeled by random priming with [α-^{32}P]dCTP (Feinberg and Vogelstein, 1983) to a specific activity of 1×10^6 to 5×10^6 cpm/μg and hybridized to immobilized RNA in 50% formamide, 0.25 M $NaHPO_4$, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 7% SDS (nylon membrane), or in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 0.1 mg/mL herring sperm DNA (nitrocellulose) at 42°C overnight. Nylon membranes were washed in 0.5× SSC, 0.1% SDS once at room temperature, then at 65°C. Nitrocellulose membranes were washed in 2× SSC, 0.1% SDS twice at 42°C, then in 0.1× SSPE, 0.1% SDS at 65°C. Membranes were then exposed to preflashed Kodak XAR5 film at −70°C using a Cronex (DuPont) intensifying screen.

Transient Assay

Microprojectile bombardments were performed as previously described (Okubara et al., 1993; Williams et al., 1994), with some modifications. A sample of fronds of 9- to 11-week-old cultures grown with intermittent R was plated onto agar medium and given 10 min FR before being placed in D. The D treatment was for 1 d for bombardment with the wheat *Em* promoter construct and for 2 d before bombardment with the *Lhcb* promoter construct. To the designated samples, 1 mL of 10 μM ABA was added 4 to 6 h before bombardment. If the ABA fully diffused into the agar, the final concentration would be 0.5 μM. All bombardments were performed with 1.0 μm (Analytical Scientific Instruments, Alameda, CA) or 1.6-μm gold particles (Bio-Rad). Plants were bombarded with 3.9 μg of pBM113kp, a construct containing a 650-bp fragment (−560 to +90) of the wheat *Em* promoter fused to the *uidA* reporter gene encoding GUS (Marcotte et al., 1988) and 0.12 μg of an internal standard construct, pAHC18, a minimal ubiquitin promoter fused to the *luc* gene (Bruce and Quail, 1990). Alternatively, plants were bombarded with 3 μg of an *Lhcb*::*luc* gene fusion construct containing either a fragment (−592 to +88) of the *Lemna Lhcb2*1* promoter (wild type) or a construct containing the same fragment but with a 2-bp substitution at positions −130 and −132 from the start of transcription (nonphytochrome-responsive mutant) (Kehoe et al., 1994); 50 ng of a minimal rice actin promoter fused to the *uidA* reporter (McElroy et al., 1990) was included as an internal control. Following bombardment, plants remained in D and some samples received either 2 min of R or 2 min of R followed by 10 min of FR; plants were then returned to D for 16 to 18 h before being assayed for LUC and GUS activities (Okubara et al., 1993). Background activities from plants bombarded with gold particles only were subtracted. The reporter activity for each treatment (at least five independent transformations) was determined by analysis of covariance using the internal standard activity as the dependent variable. Differences between the average reporter/internal standard activity were tested for significance by the *t* test. Data from multiple experiments are shown.

RESULTS

Dark Treatment of Plants Affects Endogenous ABA Levels

We examined how dark treatments could affect ABA levels in two species, a monocot (*L. gibba*) and a dicot (*A. thaliana*), extending our initial observation for a single time point in *L. gibba* (Williams et al., 1994). Figure 1A shows the rapid increase in ABA levels in a representative experiment after light-grown plants were transferred to D. The level of ABA had nearly doubled by 8 h and continued to increase for the duration of the experiment. We also tested whether an increase in ABA in response to an extended dark treatment occurred in 3-week-old seedlings of Arabidopsis. Figure 1B shows the increase in ABA levels in these seedlings grown in white light and placed into D for 4 d. When these seedlings were returned to light, the ABA levels declined to the original level within 2 d. Thus, in the absence of any stress, a dark treatment of light-grown plants resulted in a substantial increase in ABA concentration.

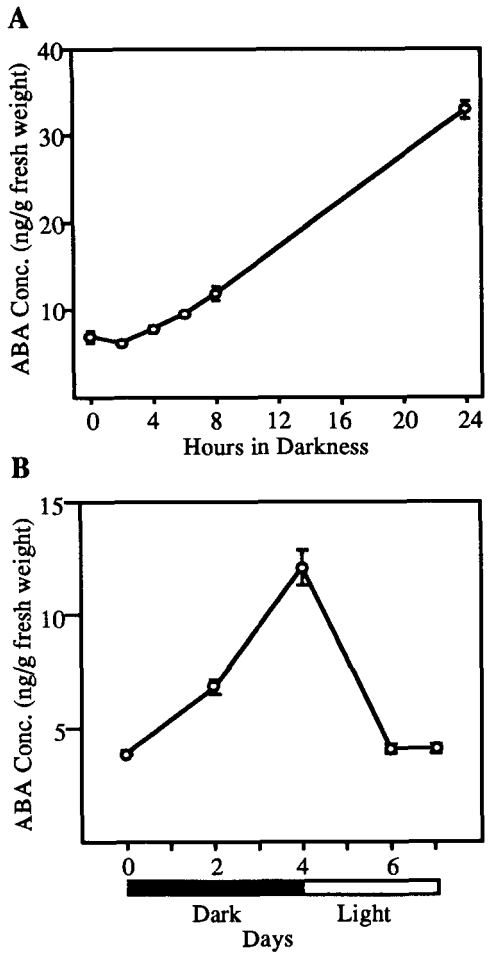

Figure 1. ABA levels increase in response to dark treatments in both *Lemna* and Arabidopsis. A, Three-week-old cultures of green *L. gibba* were given 10 min of FR to convert Pfr to Pr and then placed in D for the time indicated. B, Three-week-old Arabidopsis plants were placed in D (black bar) and subsequently returned to white light (white bar) for the times indicated. SE values are shown.

Phytochrome Action Can Reduce Endogenous ABA Levels in *Lemna*

We next investigated whether phytochrome was involved in mediating changes in ABA levels. We tested the effect of a brief R treatment on the endogenous ABA concentration in dark-treated *L. gibba*. A representative experiment is shown in Figure 2. A significant decrease in ABA was observed 4 h after a brief R illumination, and this effect of R was reversible by FR. The initial concentration of ABA differed among experiments, as did the amount of the decline. The decrease in five experiments ranged from 28 to 65%, with an average value of 44%. In three of these experiments we also gave a FR treatment immediately after R, and the effect of R was partially reversed in each instance. The extent of this reversal ranged from 33 to 88%. Thus, we conclude that phytochrome action can result in a substantial decrease of endogenous ABA levels in *Lemna*.

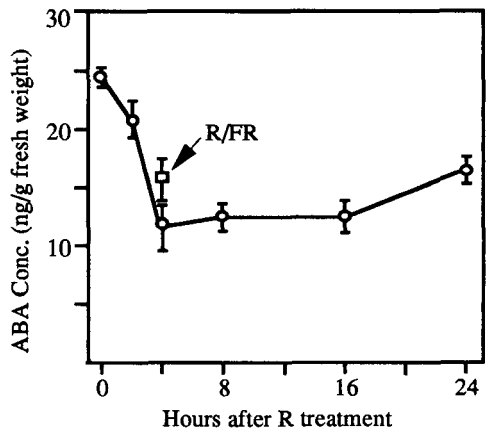

Figure 2. ABA levels can be negatively regulated by phytochrome action. Eight-week-old cultures of intermittent-R-grown *L. gibba* were given 10 min of FR and placed into D for 24 h. Plants were then treated with 2 min of R (○) or 2 min of R followed immediately by 10 min of FR (□) and returned to D for the time indicated. SE values are shown.

An ABA-Responsive Em Promoter Also Responds to Phytochrome Action

To test whether the observed changes in ABA in response to R could be physiologically relevant, we used the Em promoter from wheat, a well-characterized, ABA-responsive promoter. Figure 3 shows that the expression of the *Em* promoter in the *Lemna* transient assay was significantly reduced by brief R treatment. Although we did not see significant reversal of the effect of R by a subsequent FR treatment, the low level of expression in the absence of added ABA and the size of the SE limits our ability to detect

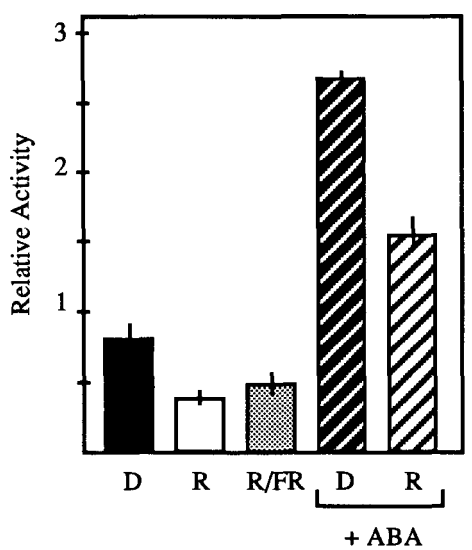

Figure 3. A wheat *Em* promoter responds to both ABA and phytochrome action. Following bombardment with the *Em::uidA* construct, intermittent-R-grown *L. gibba* cultures were treated with D (D), 2 min of R (R), or 2 min of R + 10 min of FR (R/FR) and returned to D for 16 to 18 h before assaying for reporter gene activity. Ten micromolar ABA was applied to the indicated samples. SE values are shown.

small changes. The results also confirm that the *Em* promoter can respond to exogenous ABA in this transient assay. The extent of the response to ABA seen here was not as large as the 15- to 30-fold induction seen in the rice embryo protoplast system (Marcotte et al., 1988), most likely because expression was taking place in vegetative rather than embryonic tissue (cf. Finkelstein, 1993; McCarty, 1995). A significant difference in expression of the reporter gene activity was also observed between samples given dark and R treatments in the presence of added ABA, suggesting that this promoter also contains a phytochrome-responsive element. These results, taken together with the observed R-induced decrease in ABA (Fig. 2), suggests that the expression of *Em* may be regulated by light in part through changes in ABA levels and that the observed R-induced decrease in ABA is sufficient to have physiological consequences. A mutant version of the *Em* promoter, in which both the Em1a and Em2 boxes were altered, was also tested in the transient assay; however, it retained substantial ABA induction as well as the phytochrome response (data not shown). We have not determined whether these responses are mediated at least in part by the remaining functional Em1b box (e.g. Vasil et al., 1995), nor can we rule out the presence of an additional phytochrome-responsive element that is separable from the ABA-responsive element in this promoter.

ABA Affects Specific mRNA Levels in *L. gibba*

Because ABA has been reported to suppress *rbcS* and *Lhcb* gene expression in other species, we tested whether it had a similar effect in *L. gibba*. The decrease of both these mRNAs during a period of dark treatment in the presence or absence of exogenous ABA is shown in Figure 4. The presence of 10 μM ABA clearly accelerated the decline of both mRNAs while stimulating the accumulation of the *NPR1* mRNA.

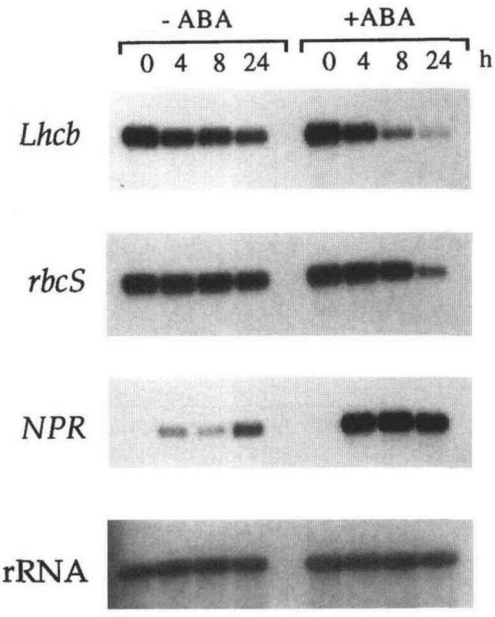

Figure 4. ABA treatment accelerates the dark-induced decline in *Lhcb* and *rbcS* mRNAs in intermittent-R-grown *L. gibba*. RNA blot analysis of samples from plants placed into D for the indicated times in the absence (−ABA) or presence (+ABA) of 10 μM ABA. Identical blots were hybridized with the *rbcS*, *Lhcb*, and *NPR1* probes, as well as a rRNA probe as a loading control.

A *Lemna Lhcb* Promoter Demonstrates Separable Phytochrome and ABA Responses

We next tested whether the effect of phytochrome action on *Lhcb* gene transcription might be accounted for by its effect on endogenous ABA levels. That is, does a decline in ABA concentration in response to R (and thus, presumably, a decrease in ABA-mediated repression of transcription of both *rbcS* and *Lhcb* genes) account for the effect of phytochrome in increasing transcription of the *Lhcb* gene? For this experiment we made use of two *Lhcb::luc* constructs. The first was a wild-type promoter construct containing 592 bp of the native *Lemna Lhcb2*1* promoter, which can confer phytochrome responsiveness to a reporter gene (Kehoe et al., 1994). This regulation is mediated by two separate regulatory elements, the REα (AACCAA) and the REβ (CGGATA) sequences, which are located −134 to −129 bp and −114 to −109 bp from the transcription start site, respectively (Degenhardt and Tobin, 1996). The second construct contains the same 592-bp promoter region but with a 2-bp alteration in the REα element, changing it to AAGCGA, and it has lost phytochrome responsiveness (Degenhardt and Tobin, 1996). Figure 5 shows that expression of the wild-type promoter construct was repressed in the transient assay by ABA treatment. Because the amount of ABA given has been shown to greatly increase internal ABA levels (to 4.2 μg/g fresh weight; Williams et al., 1994), and because the magnitude of the decline seen after a R treatment would not significantly affect that level, this result demonstrates that a phytochrome-responsive element(s) must exist separately from an element that allows the response to the increased ABA level. Furthermore, when the same treatments were given to the mutant construct, which did not respond to R, ABA treatment still caused a decline in transcription. Experiments using a construct in which the REβ element was changed to CGTTTA gave similar results (data not shown). We conclude that the *Lhcb2*1* promoter can respond to both ABA and phytochrome action and that the DNA sequence elements for these two responses are separable.

GAs Cannot Reverse the Effect of ABA

In view of the opposing effects of GA and ABA on seed germination (reviewed by Chandler and Robertson, 1994), we tested whether GA could abrogate the ABA responses we observed in *L. gibba*. We repeated our transient assay experiment with the *L. gibba Lhcb2*1::luc* wild-type construct in the presence and absence of ABA and GA; we observed neither any effect of GA alone nor GA antagonism of ABA repression of *Lhcb2*1* activity (data not shown). Because there has been a recent report of a barley dehydrin gene in which the effects of GA were exhibited only at the posttranscriptional level (Robertson et al., 1995),

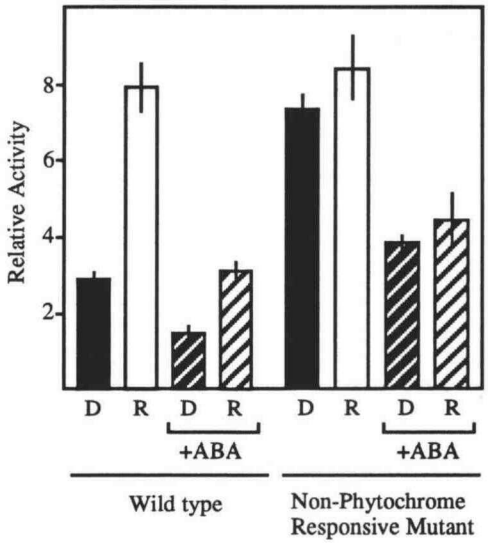

Figure 5. Phytochrome- and ABA-responsive elements are separable in an *L. gibba Lhcb* promoter. Following bombardment with the wild-type or nonphytochrome-responsive mutant *Lhcb::luc* construct, intermittent-R-grown plants remained in D (D) or were treated with 2 min of R (R) and returned to D. Ten micromolar ABA was applied to the indicated samples. SE values are shown.

we also tested the effect of GA in concert with ABA on accumulation of *Lhcb* and *NPR* mRNAs in *L. gibba*. We administered 50 μM GA in the presence or absence of 10 μM ABA to intermittent-R-grown *L. gibba* placed in D for 4 or 24 h and measured specific mRNA levels (Fig. 6). We saw evidence of neither a direct effect of GA on specific mRNA accumulation nor antagonism of the ABA-induced accelerated decline of *Lhcb* and increase of *NPR* messages. Thus, it seems unlikely that any effects of phytochrome on the ABA pathway are mediated via GA.

DISCUSSION

We have shown that the endogenous concentration of the hormone ABA in mature plants can be affected simply by a transfer of plants to D, resulting in increased ABA levels in both *L. gibba* and *A. thaliana*. Furthermore, we have shown that the phytochrome system can affect the ABA concentration in *L. gibba*, with a brief R treatment leading to a decrease in ABA. Whether the effect of a prolonged dark treatment involves additional processes, such as initiating a new developmental pathway (e.g. the beginning of senescence), remains to be determined. However, the effect of phytochrome on ABA levels that we have observed may be sufficient to result in changes in expression of the well-characterized, ABA-responsive wheat *Em* promoter.

Although we observed changes in ABA levels in vegetative tissue of mature plants, the observed decrease seen after a R treatment is consistent with the R-induced decrease seen in endogenous ABA levels in Scots pine seeds (Tillberg, 1992) and germinating lettuce seeds (Toyomasu et al., 1994a), as well as the reported increased ABA levels in a phytochrome-deficient mutant of *Nicotiana plumbaginifolia* (Kraepiel et al., 1994). However, the opposite effect of R on ABA levels has been reported in corn roots (Leopold and LaFavre, 1989). It remains to be seen whether phytochrome plays a role in affecting Arabidopsis ABA levels.

We have also confirmed down-regulation of *rbcS* and *Lhcb* mRNAs by application of ABA to intact plants of *L. gibba*. Both of these gene families can be positively regulated by phytochrome (Tobin and Silverthorne, 1985; Quail, 1991; Thompson and White, 1991; Tobin and Kehoe, 1994). Thus, we were able to address the important question raised by these observations of whether phytochrome induction of the expression of these genes was mediated primarily by changes in ABA concentration. We found that in a *Lhcb* promoter, the DNA elements needed for regulation by phytochrome and ABA are separable. This promoter can still respond to phytochrome in the presence of a concentration of ABA so great that the change observed in response to R treatment would not significantly change the endogenous ABA concentration (see Williams et al., 1994). Therefore, in this case, phytochrome must act in a manner independent of its effect on ABA concentration. Furthermore, 2-bp mutations in either of two recently identified phytochrome-regulatory regions of this promoter (Kehoe et al., 1994; Degenhardt and Tobin, 1996) that render it unable to respond to phytochrome do not abolish its response to ABA. These results clearly demonstrate that the DNA elements important for phytochrome and ABA regulation are separable in this *Lhcb* promoter.

The antagonistic effects of GA and ABA have been clearly documented for seed germination, with GA playing a generally stimulatory role and ABA playing an inhibitory role (reviewed by Chandler and Robertson, 1994; McCarty, 1995). The GA-inducible α-amylase genes that mobilize storage carbohydrates in barley aleurone during seed germination have been extensively characterized, and multiple *cis*-acting elements that mediate the opposing transcriptional effects of GA and ABA have been defined (Gubler and Jacobsen, 1992; Rogers and Rogers, 1992). The α-amylase GA/ABA response has been shown to act through a

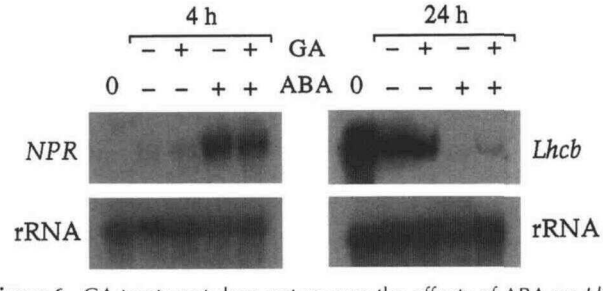

Figure 6. GA treatment does not reverse the effects of ABA on *Lhcb* and *NPR* mRNAs. Intermittent-R-grown *L. gibba* cultures were placed into D in the absence (−) or presence (+) of 10 μM ABA and 50 μM GA. Shown is RNA blot analysis of the initial untreated plants (lane 0) or from samples isolated 4 or 24 h after hormone treatment and hybridized to *NPR1* or *Lhcb* probes, respectively. Hybridization to a rRNA probe as a loading control is shown below.

single TAACAAA promoter element; this is in contrast with our demonstration of separable ABA- and phytochrome-responsive elements in the *L. gibba Lhcb* promoter. In fact, we find no evidence for a homologous GA-/ABA-response element in the *L. gibba Lhcb* promoter. Other investigators have reported GA inhibition of ABA-induced specific mRNA accumulation (Bartels et al., 1991; Robertson et al., 1995); however, this may likely be occurring through posttranscriptional regulation. The lack of effect of GA (either alone or in concert with ABA) on *Lhcb* transcription or mRNA accumulation may suggest that different pathways are involved in phytochrome and ABA signaling than in GA and ABA action.

The response of the wheat *Em* promoter to phytochrome action is presumably occurring at least in part through an ABA-responsive element that has been defined in previous studies (Marcotte et al., 1992). We note that motifs similar to this element (containing an ACGT core) are also found in some *rbcS* and *Lhcb* genes and have been called G boxes (Schindler et al., 1992). In the case of the Arabidopsis *rbcS* gene, the sequence was found to have a strong enhancing function (Donald and Cashmore, 1990). A family of plant bZIP proteins has been found that binds to regions containing this core element in promoters of genes that respond to diverse signals such as UV, visible light, and anaerobic stress (reviewed by Foster et al., 1994). The *L. gibba Lhcb2*1* promoter does not contain this core motif, although it can respond to ABA. In addition, recent reports have identified novel ABA-responsive elements that do not contain ACGT core sequences (Cejudo et al., 1992; Iwasaki et al., 1995; Shen and Ho, 1995). It remains to be determined whether these sequences might function in some way to mediate a response to ABA in the *Lhcb* and *NPR* genes.

The regulation of plant gene expression by light involves multiple regulatory sequence elements and protein factors. In this work we have clearly shown that light/dark treatments can also affect the hormone ABA, with important consequences for dissecting out individual signaling pathways. It is not yet possible to establish a clear causal or sequential relationship between the two stimuli. Our observations suggest that light and ABA effects are antagonistic, and we have shown that DNA sequence elements for the two signals are separable in the *Lhcb* gene. However, this antagonism was not seen in the regulation of a desiccation-related "early-light-inducible" gene whose ABA-mediated gene activation was reported to occur only in the presence of light (Bartels et al., 1992).

The molecular mechanisms by which either phytochrome or ABA mediates its various physiological responses are not known. Calcium ions have been implicated as a second messenger for both phytochrome (reviewed by Millar et al., 1994; Tobin and Kehoe, 1994; Quail et al., 1995; Terzaghi and Cashmore, 1995) and ABA (Wang et al., 1991; Bartels et al., 1992; Gilroy and Jones, 1992; Assman, 1994), but the precise modes of action have not been fully elucidated. Other plant hormones, including GAs (e.g. Nick and Furuya, 1993; Derkx et al., 1994; Toyomasu et al., 1994b) and cytokinins (e.g. Binns, 1994; Sano and Youssefian, 1994) have also been reported to interact with or be affected by light conditions. Thus, light-induced changes in the endogenous concentrations of ABA and possibly other hormones must be considered in interpreting experiments involving light-induced signal transduction, and a reevaluation of earlier experiments with transgenic plants (e.g. Herrera-Estrella et al., 1984; Ha and An, 1988; Elliott et al., 1989; Gidoni et al., 1989; Lam and Chua, 1990) could prove useful. Ultimately, more detailed experiments will be required to separate direct and indirect effects of light on the expression of individual highly regulated genes.

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