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SHORT COMMUNICATION

Low temperature regulates Arabidopsis *Lhcb* gene expression in a light-independent manner

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Summary

Low temperature treatment of dark-grown seedlings of *Arabidopsis thaliana* results in a rapid increase in the amount of mRNAs encoding for the major polypeptides of the light-harvesting complex of photosystem II *(Lhcb1* genes). This increase is transient and seems to be due mainly to the accumulation of *Lhcb1*3* transcripts, indicating that low temperature differentially regulates the expression of the Arabidopsis *Lhcb1* gene family in the dark. A 1.34 kb fragment of the *Lhcb1*3* promoter is sufficient to confer low temperature regulation to a reporter gene in transgenic Arabidopsis etiolated seedlings, suggesting that the regulation is occurring at the transcriptional level. The cold-induced accumulation of *Lhcb1*3* mRNA is not part of a general response to stressful conditions since no accumulation is detected in response to water stress, anaerobiosis or salt stress. The amount of *Lhcb1*3* mRNA decrease in response to exogenous abscisic acid (ABA) suggesting that this phytohormone acts as a negative regulator. Moreover, the accumulation of *Lhcb1*3* mRNAs in cold-treated ABA deficient etiolated seedlings is higher than that of wild-type and ABA insensitive etiolated seedlings, indicating that low temperature regulation of *Lhcb1*3* is not mediated by ABA.

Introduction

Genes encoding the light-harvesting chlorophyll a/b binding proteins of photosystem II *(Lhcb* genes, formerly known as *cab* genes; Jansson *et al.*, 1992) constitute a family of nuclear genes the expression of which has been extensively studied in higher plants. *Lhcb* genes have been shown to be mainly regulated by phytochrome, under the control of red/far red light (Silvertorne and Tobin, 1984; Thompson and White, 1991), blue light (Fluhr and Chua, 1986) and circadian rhythms (Nagy *et al.*, 1988a). However, published results indicate that factors other than light also regulate the expression of these genes. Thus, *Lhcb* genes are subjected to endogenous control during different stages of development. In *Sinapis alba, Lhcb* mRNAs can be detected in the dark during the first days of seedling growth when *Lhcb* genes become competent to respond to phytochrome (Schmidt *et al.*, 1987). In a similar way, *Lhcb* transcripts increase in cotyledons of soybean before they emerge from the soil and decrease when cotyledons senesce (Chang and Walling, 1992). In Arabidopsis, it has also been shown that *Lhcb* gene expression is regulated in a light-independent manner during the first days of seedling development (Brusslan and Tobin, 1992). Later in development, after bolting, *Lhcb* gene expression is reduced in the rosette leaves (Chory *et al.*, 1991). Similar developmental control of *Lhcb* mRNA levels has been seen in maize (Sullivan *et al.*, 1989), barley (Rapp and Mullet, 1991) and pine (Yamamoto *et al.*, 1993).

Plant growth regulators, the levels of which change during plant development, also modulate the expression of *Lhcb* genes. Abscisic acid (ABA) and methyl jasmonate cause a decrease in the levels of *Lhcb* transcripts (Bartholomew *et al.*, 1991; Chang and Walling, 1991; Reinbothe *et al.*, 1993; Weatherwax *et al.*, 1996), whereas plants treated with cytokinins maintained increased levels of transcripts in the dark (Flores and Tobin, 1986). Environmental factors have also been described that control *Lhcb* gene expression. Water-deficit conditions repress the expression of *Lhcb* genes in leaves of tomato plants in an ABA-dependent manner (Bartholomew *et al.*, 1991). On the other hand, increases in cytosolic calcium levels positively affect the expression of *Lhcb1* genes, since microinjection of calcium into individual hypocotil cells from *aurea* tomato seedings activates a β-*glucuronidase* (*GUS*) reporter gene fused to the promoter region from the wheat *Lhcb1*1* gene (Neuhaus *et al.*, 1993).

Cytosolic calcium has been implicated in the transduction of a variety of external stimuli such as red light, mechanical stress, low temperature, fungal elicitors and gravity (Hepler and Wayne, 1985; Knight *et al.*, 1991; Reddy and Poovaiah,

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1987; Shacklock *et al.*, 1992). Regarding low temperature, it has been shown to induce an increase in cytosolic calcium levels (Knight *et al.*, 1991, 1996), which could play an essential role in the cold acclimation process (Knight *et al.*, 1996; Minorsky, 1985; Monroy *et al.*, 1993; Monroy and Dhindsa, 1995), a process by which many chilling-tolerant plants increase their freezing tolerance in response to low non-freezing temperatures (Steponkus, 1984). Furthermore, experiments with alfalfa protoplasts and Arabidopsis seedlings have revealed that the cold-induced cytosolic calcium increase is sufficient to induce the expression of different cold-inducible genes (Knight *et al.*, 1996; Monroy and Dhindsa, 1995).

Since *Lhcb1* gene expression is positively regulated by cytosolic calcium, and low temperature exposure provokes an influx of extracelular calcium responsible for the expression of different cold-inducible genes, we decided to investigate whether *Lhcb1* gene expression could also be induced by low temperature in a light-independent manner. The results presented here demonstrate that low temperature exposure causes an accumulation of *Lhcb1* mRNAs in dark-grown seedlings of Arabidopsis. This accumulation is transient and restricted to etioplast containing cells. Furthermore, it is not part of a general response to stress situations since it is not detected when etiolated seedlings are exposed to water stress, anaerobiosis or salt stress. In addition, we show that individual members of the *Lhcb1* gene family are differentially regulated by low temperature, that this regulation occurs at transcriptional level, and that is not mediated by ABA. All these findings provide an interesting perspective to understand how common signalling intermediates can control different responses.

Results

Low temperature induces the accumulation of Lhcb1 *transcripts in etiolated seedlings of Arabidopsis*

In order to determine whether *Lhcb1* gene expression was regulated by low temperature in a light-independent manner, Arabidopsis seeds were sown on Petri dishes and allowed to germinate in total darkness at room temperature. After 4 days of growth, half of the seedlings were transferred to 4°C, with the rest being maintained at room temperature. In all cases, seedling development was allowed to proceed for 3 additional days. At different times over the 7-day developmental period, poly $(A)^+$ RNA was prepared from the etiolated seedlings and the levels of *Lhcb1* transcripts determined by RNA blot hybridization. The probe used was a fragment from the cDNA corresponding to the *Lhcb1*3* gene that recognizes transcripts from all members of the *Lhcb1* gene family (Sun and Tobin, 1990). The results obtained are shown in Figure 1(a).

One-day-old etiolated seedlings showed low, but detectable, levels of *Lhcb1* transcripts. After 2 days of dark growth at room temperature, there was more than a 10-fold increase in the *Lhcb1* mRNA levels compared with the first day of development. This increase was transient with the level of transcripts diminishing gradually during the development until the seventh day, when *Lhcb1* mRNAs were almost undetectable. This pattern of *Lhcb1* transcript accumulation in etiolated seedlings of Arabidopsis during the first days of development is similar to that described by Brusslan and Tobin (1992).

When 4-day-old etiolated seedlings were transferred to 4°C in the dark, *Lhcb1* transcript levels continued almost undetectable during the first 12 h. After 24 h of exposure

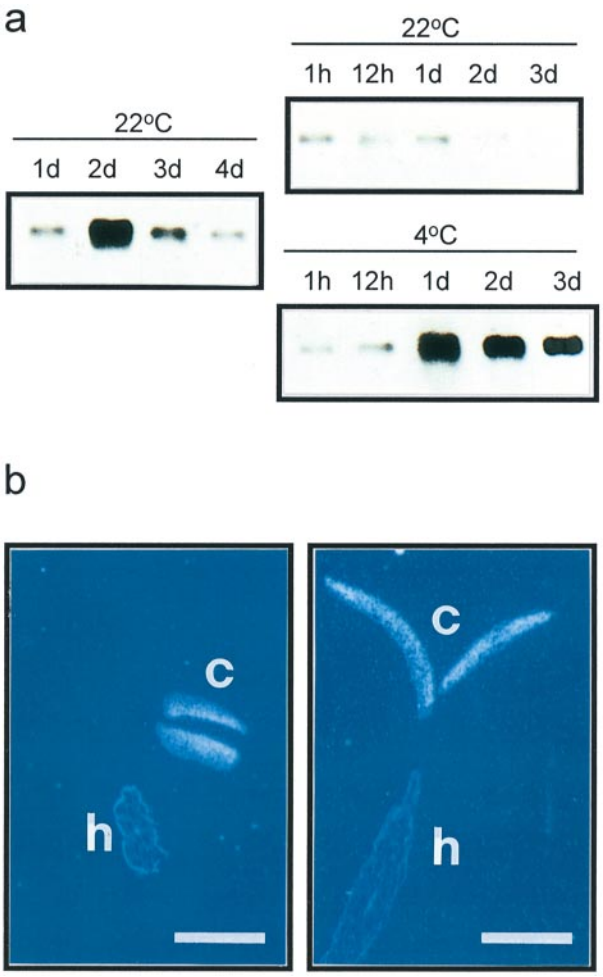

Figure 1. Low temperature induces *Lhcb1* mRNA accumulation in etiolated seedlings of Arabidopsis.
(a) RNA blot hybridizations of 1 μg poly $(A)^+$ RNA extracted from etiolated seedlings grown at 22°C from 1 to 4 days and then exposed to 4°C or maintained at 22°C at the times indicated (h, hours; d, days). The probe used was a 3′ fragment from the cDNA corresponding to the *Lhcb1*3* gene.
(b) *In situ* hybridizations of longitudinal sections of Arabidopsis seedlings with a ^{35}S-labeled antisense *Lhcb1*3* probe. Sections were stained with Calcofluor and examined under UV epifluorescence. Photomicrographs show the expression of *Lhcb1*3* in 4-day-old etiolated seedlings exposed one additional day in the dark to 4°C (left) or in the light at room temperature (right) (c, cotyledons; h, hypocotyls). Bar equals 1 mm.

to 4°C, the level of *Lhcb1* mRNAs increased to levels similar to those detected in 2-day-old etiolated seedlings (see above). After 48 h of cold treatment, the *Lhcb1* transcript levels started to decrease. These results indicate that low temperature induces a light-independent transient accumulation of *Lhcb1* transcripts in seedlings of Arabidopsis. In a similar way, low temperature also induces the accumulation of *Lhcb1* transcripts in adult plants of Arabidopsis (data not shown) suggesting that this response is independent of the developmental stage.

Lhcb1 gene expression in response to light is subjected to cell-type control, taking place only in chloroplast-contaning cells (Chory and Susek, 1994). This raised the question of whether low-temperature induction of *Lhcb1* expression is also restricted in a similar way. Cell-type specific accumulation of *Lhcb1* transcripts by low temperature was analysed by *in situ* hybridization in longitudinal sections of 4-day-old etiolated seedlings of Arabidopsis exposed for 24 h to 4°C in the dark with a *Lhcb1**3 antisense probe (Figure 1b, left). As a control, accumulation of *Lhcb1* transcripts in response to light was analysed in 4-day-old etiolated seedlings of Arabidopsis exposed for 24 h in the light at room temperature (Figure 1b, right). The expression of *Lhcb1* in cold-treated etiolated seedlings was restricted to the mesophyll cells of the cotyledons, no signal being detected in epidermal cells, hypocotyls or roots. This expression pattern was the same as that observed in light-exposed seedlings, indicating that *Lhcb1* gene expression in response to low temperature is also subjected to cell-type control, being restricted to etioplast containing cells. No hybridization was obtained when using a sense strand *Lhcb1*3* probe (not shown).

Low temperature regulates the accumulation of Lhcb1*3 *transcripts in etiolated seedlings of Arabidopsis at the transcriptional level*

In Arabidopsis, proteins forming the type 1 antenna complex of photosystem II are encoded by at least five genes *(Lhcb1*1–Lhcb1*5)* that show high levels of homology (McGrath *et al.*, 1992; Sun and Tobin, 1990). Expression analyses have just been performed with *Lhcb1*1, Lhcb1*2* and *Lhcb1*3*, formerly named *cab2* or *AB165, cab3* or *AB180* and *cab1* or *AB140*, respectively (Karlin-Neumann *et al.*, 1988; Leutwiler *et al.*, 1986). RNase protection assays suitable for differentiating the transcripts deriving from the individual *Lhcb1* gene members were used to determine the accumulation of corresponding transcripts in response to low temperature. Two antisense RNA probes were generated *in vitro* to protect fragments corresponding to *Lhcb1*3*, and *Lhcb1*1* and *Lhcb1*2* RNAs, respectively (Brusslan and Tobin, 1992; Karlin-Neumann *et al.*, 1991). A ubiquitin probe protecting the *ubq3* RNA served as a constitutive control (Brusslan and Tobin, 1992).

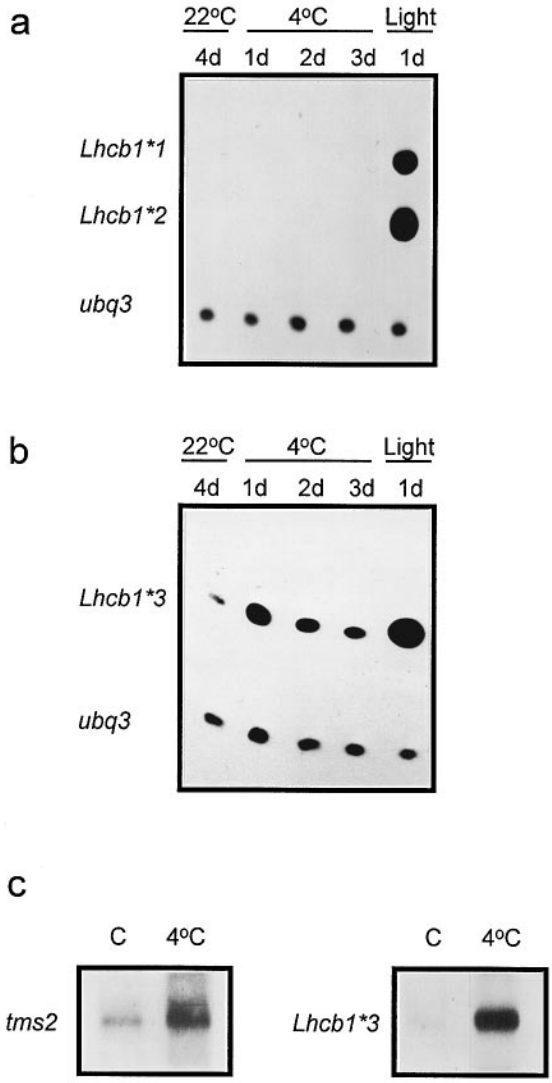

Figure 2. Low temperature regulates the accumulation of *Lhcb1*3* transcripts in etiolated seedlings of Arabidopsis at the transcriptional level.
(a and b) RNase protection analysis with Poly $(A)^+$ RNA (0.5 μg) extracted from 4-day-old etiolated seedlings grown at 22°C, from 4-day-old etiolated seedlings exposed for 1–3 days to 4°C in the dark, and from 4-day-old etiolated seedlings exposed to the light for 1 day at 22°C. The probes used to protect *Lhcb1*1*, *Lhcb1*2*, *Lhcb1*3* and *ubq3* transcripts have been described previously (Brusslan and Tobin, 1992; Karlin-Neumann *et al.*, 1991).
(c) RNA blot hybridizations of 10 μg of total RNA extracted from 4-day-old *Lhcb1*3*::*tms2* transgenic etiolated seedlings exposed for 24 h to 4°C, or maintained for an additional day in the dark at 22°C (C). The probe used was a fragment of the coding region from the *tms2* gene (left). After exposure and dehybridization, the blot was rehybridized with the *Lhcb1*3* probe as described in Figure 1 (right).

Figure 2 shows the results obtained when using these probes with poly $(A)^+$ RNA from 4-day-old etiolated seedlings grown at 22°C or exposed to 4°C in the dark for 1, 2, or 3 additional days. As a positive control, poly $(A)^+$ RNA from 4-day-old etiolated seedlings grown one additional day in the light was also used. The *ubq3* mRNA levels

only just showed differences among the samples analyzed indicating that the RNA amounts used in the protection assays were very similar. Protected fragments corresponding to all the probes employed were detected in the positive controls denoting they worked properly. Whereas the levels of *Lhcb1*1* and *Lhcb1*2* mRNAs were undetectable in 4-day-old etiolated seedlings and did not increase by low temperature treatment (Figure 2a), *Lhcb1*3* mRNA levels increased after 24 h of cold exposure (Figure 2b). Longer exposure of etiolated seedlings to 4°C resulted in a decrease of *Lhcb1*3* transcript levels. These results suggest that only one member of the *Lhcb1* gene family, the *Lhcb1*3* gene, could be regulated by low temperature in dark-grown seedlings of Arabidopsis.

To test whether the *Lhcb1*3* promoter could confer low temperature regulation upon a reporter gene, we used an Arabidopsis transgenic line containing the *Lhcb1*3*::*tms2* fusion (Karlin-Neumann *et al.*, 1991). This fusion consists in 1.34 kb of the *Lhcb1*3* promoter, including the initial 14 bp of the *Lhcb1*3* transcript, fused to the *tms2* gene which encodes an enzyme, the indole-3-acetamide hydrolase, involved in the bacterial pathway of auxin biosynthesis from tryptophan (Schröder *et al.*, 1984). Transgenic seeds were sown on Petri dishes and allowed to germinate in total darkness at room temperature. After 4 days, etiolated seedlings were transferred to 4°C for 1 additional day (time of maximum *Lhcb1*3* mRNA accumulation, see above). The *Lhcb1*3*::*tms2* transcripts were identified by RNA blot hybridizations with a probe corresponding to the *tms2* gene (see Materials and methods). Figure 2 (c, left) shows that *Lhcb1*3*::*tms2* mRNAs increased at high levels 24 h after low temperature exposure. This expression pattern paralleled that of the endogenous *Lhcb1*3* gene (Figure 2c, right), indicating that 1.34 kb of the *Lhcb1*3* upstream region is sufficient to confer responsiveness to low temperature treatment.

Low temperature induced accumulation of Lhcb1*3 *transcripts in etiolated seedlings of Arabidopsis is not the consequence of a general stress response*

To determine whether the accumulation of *Lhcb1*3* mRNA in etiolated seedlings of Arabidopsis was specifically regulated by low temperature or only part of a general response to stressful conditions, we analyzed the effect of water stress, salt stress and anaerobiosis on the *Lhcb1*3* mRNA levels. RNA blot experiments were performed (Figure 3) using poly $(A)^+$ RNAs isolated from 4-day-old etiolated seedlings grown at 22°C (C1) and from 4-day-old etiolated seedlings that had been dehydrated until they lost 50% of their initial fresh weight (D), treated with a 250 mm NaCl solution during 24 h (Na), or exposed to anaerobiosis for the same time (AN). Poly $(A)^+$ RNAs from 4-day-old etiolated seedlings grown 1 additional day either in the dark (C2) or in the light (L) were used as controls. The probe used was a cDNA fragment corresponding to the *Lhcb1*3* gene. Figure 3 shows that the levels of *Lhcb1*3* mRNA did not accumulate in response to any of the stress treatments. As expected, *Lhcb1* gene expression was detected in etiolated seedlings that had been exposed for 24 h to light but was not detected in dark-grown seedlings. Hybridizations with the *RD29* probe, which recognizes two homologous Arabidopsis genes inducible by water and salt stresses (Yamaguchi-Shinozaki and Shinozaki, 1993), and the ADH probe, which recognizes an Arabidopsis gene responsive to anaerobiosis (Chang and Meyerowitz, 1986), were realized as controls for these experiments and exhibited the expected induction profile. These results indicate that the accumulation of *Lhcb1*3* transcripts in etiolated seedlings of Arabidopsis is specifically induced by low temperature and is not part of a general response to stress conditions.

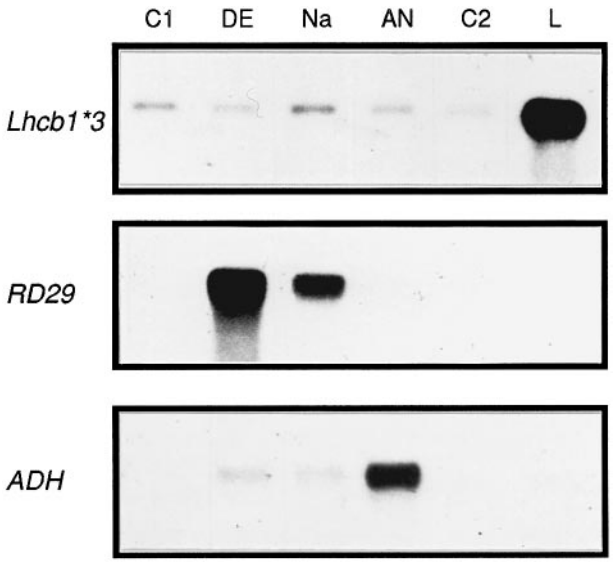

Figure 3. Accumulation of *Lhcb1*3* transcripts in response to low temperature is not a general response to stressful conditions.
RNA blot hybridizations of 1 μg poly $(A)^+$ RNA isolated from 4-day-old etiolated seedlings grown at 22°C (C1) or from seedlings that had been dehydrated until losing 50% of their initial fresh weight (DE), treated with a 250 mm NaCl solution during 24 h (Na), or exposed to anaerobiosis for the same time (AN). Poly $(A)^+$ RNAs from 4-day-old etiolated seedlings grown for 1 additional day either in the dark (C2) or in the light (L) were used as controls. The *Lhcb1*3* probe was the one described in Figure 1. The *RD29* and ADH mRNAs were detected with probes corresponding to the *RD29* A coding region (Yamaguchi-Shinozaki and Shinozaki, 1993), and to a fragment of the ADH gene (Chang and Meyerowitz, 1986), respectively.

The accumulation of Lhcb1*3 *transcripts by low temperature treatment in etiolated seedlings of Arabidopsis is not mediated by ABA*

Since most of the cold-inducible genes are also induced by ABA (Thomashow, 1994), we performed two different

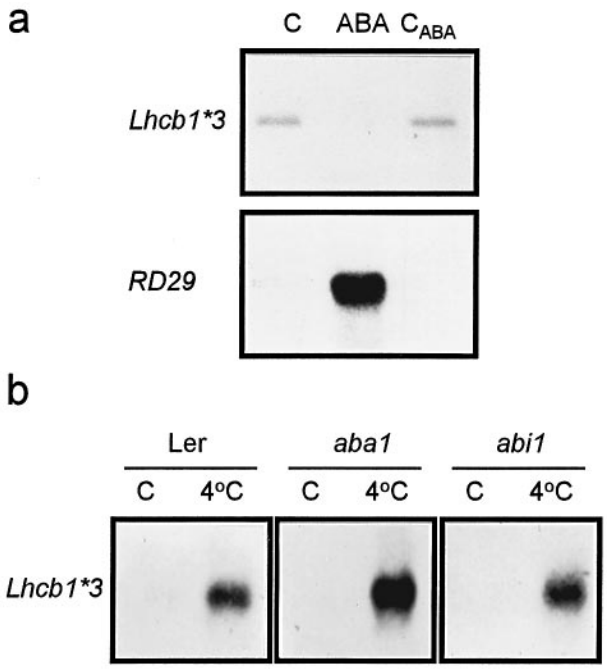

Figure 4. Effect of ABA on the accumulation of *Lhcb1*3* mRNA in etiolated seedlings of Arabidopsis.
(a) RNA blot hybridizations of 1 μg poly $(A)^+$ RNA isolated from 4-day-old etiolated seedlings (C), or from seedlings that had been sprayed either with a 100 μm solution of ABA (ABA) or with water containing the same concentration of the ABA solvent, 0.1% DMSO, 0.025% Triton (C_{ABA}).
(b) RNA blot hybridizations of 1 μg poly $(A)^+$ RNA isolated from 5-day-old L*er*, *abi1* and *aba1* etiolated seedlings (C), and from 4-day-old etiolated seedlings that had been exposed to 4°C in the dark for 1 additional day (4°C). The probes used for *Lhcb1*3* and *RD29* were those described in Figures 1 and 3, respectively.

experiments to determine whether the accumulation of *Lhcb1*3* mRNA by low temperature exposure in etiolated seedlings of Arabidopsis was mediated by this phytohormone. In a first experiment (Figure 4a), we analyzed the effect of exogenous ABA treatment on the levels of *Lhcb1*3* transcripts. Poly $(A)^+$ RNAs were isolated from 4-day-old etiolated seedlings (C), and from 4-day-old etiolated seedlings that had been sprayed either with a 100 μm solution of ABA (ABA) or with the ABA solvent (C_{ABA}). In the second experiment (Figure 4b), we analyzed the accumulation of *Lhcb1*3* mRNA in etiolated seedlings of ABA insensitive (*abi1*) or deficient (*aba1*) Arabidopsis mutants after low temperature treatment. Poly $(A)^+$ RNAs were isolated from 5-day-old L*er*, *abi1* and *aba1* etiolated seedlings grown at 22°C (C), and from 4-day-old seedlings that had been exposed to 4°C in the dark for 1 additional day (4°C). Northern-blot hybridizations were performed with the probe for the *Lhcb1*3* gene.

Figure 4(a) shows that exogenous application of ABA provoked a decrease in the levels of *Lhcb1*3* transcript. As a control, hybridization with a probe for the *RD29* genes, which are also ABA inducible (Yamaguchi-Shinozaki and Shinozaki, 1993), confirmed that the ABA treatment worked properly. Figure 4(b) shows that the accumulation of *Lhcb1*3* mRNA in *abi1* seedlings when exposed to low temperatures was similar to that observed in the wild-type. In the case of *aba1* seedlings, the levels of *Lhcb1*3* mRNA after low temperature treatment were higher than those from the wild-type or the *abi1* mutant. Thus, the accumulation of *Lhcb1*3* transcripts by low temperature exposure in etiolated seedlings of Arabidopsis is not mediated by ABA. Moreover, these results indicate that ABA could act as a negative regulator of this accumulation.

Discussion

The experiments presented here demonstrate that *Lhcb1* mRNAs accumulate in etiolated seedlings of Arabidopsis in response to low temperature. This accumulation is transient with *Lhcb1* transcripts peaking after 24 h of exposure to 4°C and decreasing thereafter. Furthermore, low temperature differentially regulates the expression of the individual members of the *Lhcb1* gene family. Comparison of the RNase protection data with those of the total gene family indicates that the accumulation of *Lhcb1*3* transcript accounts for at least a part of the *Lhcb1* gene family response to low temperature. These experiments indicate that *Lhcb1*1* and *Lhcb1*2* genes are not responsive to low temperatures under our conditions. The two remaining cloned members of the Arabidopsis *Lhcb1* gene family, *Lhcb1*4* and *Lhcb1*5* (formerly know as *Cab4* or Lhb1B1 and *Cab5* or *Lhb1B2*, respectively) (McGrath *et al.*, 1992) were not examined in this study. Interestingly, *Lhcb1*3* is also the sole gene of the family that is regulated by blue light (Gao and Kaufman, 1994), and the most sensitive and responsive to white and red light (Karlin-Neumann *et al.*, 1988). Accumulation of *Lhcb1*3* mRNAs in the dark has been observed in Arabidopsis mutants affected in the phytochrome transduction pathway, the genes involved in these mutations being proposed to negatively regulate such accumulation (see Chory and Susek, 1994 for review). The possibility that the cold-induced accumulation of *Lhcb1*3* we have observed could be a consequence of the inactivation of these regulators by low temperature can be ruled out since etiolated seedlings grown at low temperature do not show all the other characteristics displayed by those mutants.

We have determined that a 1.34 kb fragment of the *Lhcb1*3* promoter confers low temperature responsiveness in etiolated seedlings. This fragment does not contain the elements TACCGACAT or TGGCCGAC that have been proposed to be involved in low temperature-regulated gene expression (Baker *et al.*, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994), indicating that a different element(s) mediates the cold response shown by *Lhcb1*3*. The fact that a *Lhcb1*3* promoter fragment confers low temperature regulation to the *tms2* gene suggests that a transcriptional

control is involved in this regulation. The possibility of a post-transcriptional control is unlikely since the *Lhcb1*3*::*tms2* transcript only contains 14 bp of upstream untranslated region from the *Lhcb1*3* promoter, which is not enough to stabilize the transcript (Karlin-Neumann *et al.*, 1988).

RNA blot hybridizations indicate that the accumulation of *Lhcb1*3* mRNAs in etiolated seedlings of Arabidopsis is specifically induced by low temperature and not part of a general response to stress conditions. *Lhcb1*3* mRNA levels decrease in response to ABA suggesting that this phytohormone could act as a negative regulator. In a similar way, the expression of *Lhcb* genes from tomato, soybean, *Lemna gibba* and Arabidopsis has also been shown to be repressed by ABA treatments (Bartholomew *et al.*, 1991; Chang and Walling, 1991; Weatherwax *et al.*, 1996). Furthermore, the accumulation of *Lhcb1*3* mRNAs in cold treated ABA-deficient (*aba1*) etiolated seedlings is higher than that observed in L*er* wild-type and ABA-insensitive (*abi1*) etiolated seedlings, indicating that ABA is neither sufficient nor necessary for this accumulation. Specific low temperature regulation has also been described for Arabidopsis *RCI1* and *bbcI* genes (Jarillo *et al.*, 1994; Saez-Vasquez *et al.*, 1993), wheat *Wcs120* and *Wcs19* genes (Houde *et al.*, 1992), barley *blt101* gene (Goddard *et al.*, 1993), and alfalfa *cas18* gene (Wolfraim *et al.*, 1993). However, to our knowledge, *Lhcb1*3* is the first gene thus far where expression is regulated positively by low temperature but negatively by ABA.

The dual regulation of *Lhcb1*3* by low temperature and light raises the question of whether the signal transduction pathways triggered by these stimuli interact through common intermediates. Recent results on calcium and protein phosphorilation signalling suggest that this could be the case (Bowler *et al.*, 1994; Knight *et al.*, 1996; Monroy *et al.*, 1993; Monroy and Dhindsa, 1995; Neuhaus *et al.*, 1993). In this way, our data from the *in situ* hybridization experiments reveal that the distribution of *Lhcb1*3* transcripts in etiolated seedlings exposed to low temperature is identical to the distribution observed in control light grown seedlings, being restricted to etioplast-containing cells. The coupling of low temperature with the control of *Lhcb1*3* gene expression which we have revealed can be interpreted as being advantageous for a seedling experiencing low temperature to accumulate *Lhcb1*3* transcripts which might allow the rapid synthesis of the photosynthetic apparatus antenna in more appropriated developing conditions. This, in turn, would prevent photo-oxidative damage (Thompson and White, 199 l), and would help maximize light energy utilization (Grossman *et al.*, 1995).

Since *Lhcb1* are, in all likelihood, the genes whose expression has been most extensively studied, the findings reported here can be very useful in discerning signal intermediates in low temperature response. In addition, they provide a simple experimental system for evaluating the significance of cross-talk between pathways, and to study the specificity of plant responses to individual stimuli and how common signalling intermediates can control different responses.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col) and Landsberg *erecta* (L*er*), ABA deficient (*aba1*) and insensitive (*abi1*) Arabidopsis mutants, and a Col derivative transgenic line containing a *Lhcb1*3*::*tms2* fusion (Karlin Neuman *et al.*, 199 l) were used in this study. Seeds were sown, under sterile conditions, onto filter papers which were placed in Petri dishes containing mineral solution (Haugunn and Somerville, 1986) solidified with 0.8% agar, and germinated for 4 days in the dark at room temperature. Cold treatment of etiolated seedlings was carried out at 4°C, in the dark, for different time periods. Anaerobiosis treatment was given by exposing 4-day-old etiolated seedlings in a nitrogen atmosphere for 24 h. To induce water stress, 4-day-old etiolated seedlings were taken out of the Petri dishes and allowed to dehydrate until the loss of 50% of their initial fresh weight. Salt stress was induced by treating 4-day-old etiolated seedlings with a 250 mM NaCl solution for 24 h. Finally, ABA treatments were performed by spraying 4-day-old etiolated seedlings with a 100 μM solution of ABA in 0.025% Triton X-100. The ABA stock solution was prepared as a 100 mM solution in DMSO, and control treatments were given with water containing the same concentrations of DMSO and Triton X-100. Seedlings were collected 3 h after the start of treatment. To ensure total darkness, all manipulations were achieved under dim-green safe light.

RNA extraction and blot hybridization

Frozen material was ground in liquid N_2 by using a small mortar and pestle. Total RNA was isolated as described by Nagy *et al.* (1988b) and Poly $(A)^+$ RNA selected by using the Quick Prep mRNA purification kit (Pharmacia, Sweden). For Northern hybridizations, 1 μg of Poly $(A)^+$ RNA was denatured in formamide and formaldehyde, and separated by electrophoresis on l% formaldehyde gels. Transfer of RNA to nitrocellulose filters (Schleicher & Schuell, USA) was performed as recommended by the manufacturer. The probes used were a 0.3 kb 3′ fragment from the cDNA corresponding to the *Lhcb1*3* gene (Karlin-Neumann *et al.*, 1988), the *tms2* coding region (Schröder *et al.*, 1984), a 2.5 kb fragment from the Arabidopsis ADH gene (Chang and Meyerowitz, 1986) and the *RD29*A coding region (Yamaguchi-Shinozaki and Shinozaki, 1993).

Probes were labelled by random primer extension with (α-^{32}P)dCTP (Feinberg and Vogelstein, 1983). Hybridizations were carried out following standard protocols (Sambrook *et al.*, 1989). To check for mRNA integrity and equal loading in each lane of the RNA blots, filters were hybridized with a (α-^{32}P)-labelled 20-mer oligo-dT prepared using terminal transferase (Ausubel *et al.*, 1987). The hybridization signals were quantified by using a Howteck Scanmaster 3 + scanner and a Bioimage 3.3 software from Millipore. Poly $(A)^+$ RNA samples from each experiment were analysed in at least two independent blots, and each experiment was repeated at least twice.

Probes for RNase protection and RNase protection analysis

The three probes used in this study, cab1, cab3,2 and ubq3, have previously been described (Brusslan and Tobin, 1992; Karlin-Neumann *et al*., 1991). RNA probes were synthesized by using the 'Maxiscript system' (Ambion, USA) with (α-^{32}P)UTP as described by the manufacturer. RNase protections were done by employing the 'Guardian kit' (Clontech, USA) according to the protocol provided. Protected fragments were electrophoresed on 8% sequencing gels, detected in a radiographic film after exposure at – 80°C, and quantified as described above for RNA blot hybridization signals. Two independent repeats of the RNase protection analyses showed that the results obtained were very consistent.

In situ *hybridization*

In situ RNA hybridization of Arabidopsis seedlings was carried out following the procedure described previously by Huijser *et al.* (1992). Tissue was fixed in the dark in ethanol:acetic acid:DMSO (70%: 20%: 10%) for 1 h at room temperature. Sections of paraffin embedded tissue were hybridized with a mix containing 1×10^3 cpm μl^{-1} of ^{35}S- labelled sense or antisense RNA probes. The probes were synthetized from in vitro transcription of pCABI40 plasmid (Karlin-Neumann *et al*., 1991), using the 'Maxiscript system' (Ambion, USA) as described by the manufacturer. For autoradiography, hybridized slides were dipped in Kodak NTB2 emulsion and stored for 2 weeks at 4°C before developing. The distribution of silver grains was observed using dark field illumination and the underlying tissue, stained with Calcofluor, was examined using UV epifluorescence. In situ hybridizations experiments were repeated twice.

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