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Abscisic acid deficiency prevents development of freezing tolerance in *Arabidopsis thaliana* (L.) Heynh.

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Summary. Abscisic acid (ABA) has been implicated as a regulatory factor in plant cold acclimation. In the present work, the cold-acclimation properties of an ABA-deficient mutant (*aba*) of *Arabidopsis thaliana* (L.) Heynh. were analyzed. The mutant had apparently lost its capability to cold acclimate: the freezing tolerance of the mutant was not increased by low temperature treatment but stayed at the level of the nonacclimated wild type. The mutational defect could be complemented by the addition of exogenous ABA to the growth medium, restoring freezing tolerance close to the wild-type level. This suggests that ABA might have a central regulatory function in the development of freezing tolerance in plants. Cold acclimation has been previously correlated to the induction of a specific set of proteins that have been suggested to have a role in freezing tolerance. However, these proteins were also induced in the *aba* mutant by low temperature treatment.

Key words: Freezing tolerance – Cold acclimation – Cold-induced proteins – Abscisic acid – *aba* mutant

Introduction

Plants growing in temperate regions are frequently exposed to freezing temperatures. Two basic mechanisms to survive subzero temperatures have developed in plants: freezing avoidance and freezing tolerance (Levitt 1980). Freezing avoidance seems not to be a major surviving mechanism in herbaceous plants, although some protection can be obtained by supercooling (Levitt 1980; Li 1984). Instead, the main mechanism for low temperature resistance seems to be the tolerance to extracellular freezing and subsequent freeze-induced dehydration (Levitt 1980).

Many plants have the capability to cold acclimate: exposure to low but nonfreezing temperatures can increase the freezing tolerance of the plant substantially (Levitt 1980; Sakai and Larcher 1987). Freezing tolerance is also induced by exogenously added abscisic acid (ABA) (Chen and Gusta 1983; Chen et al. 1983; Reaney and Gusta 1987; Orr et al. 1986; Lång et al. 1989). Furthermore, analysis of the intracellular levels of ABA during cold-acclimation treatment shows a peak in ABA early in the acclimation process (Chen et al. 1983). This, together with the induction of freezing tolerance by exogenously added ABA, has been taken to suggest that ABA may function as a trigger to initiate the acclimation process (Chen et al. 1983).

Cold acclimation has been correlated to the induction of a specific set of proteins, low-temperature-induced proteins, that have been suggested to play a role in the increased freezing tolerance (Cloutier 1984; Guy et al. 1985; Meza-Basso et al. 1986; Mohapatra et al. 1987; Kurkela et al. 1988; Gilmour et al. 1988; Perras and Sarhan 1989; Lång et al. 1989). The induction of some of these cold-acclimation-related proteins has been shown to take place at the gene expression level (Guy et al. 1985; Meza-Basso et al. 1986; Tseng and Li 1987; Mohapatra et al. 1987; Kurkela et al. 1988; Gilmour et al. 1988).

We have recently shown that the small Cruciferae *Arabidopsis thaliana* (L.) Heynh. exhibits a synthesis of about 10–15 cold-acclimation-related proteins (Kurkela et al. 1988; Lång et al. 1989). A subset of these proteins is also induced by treatment of *A. thaliana* plantlets with exogenously added ABA (Lång et al. 1989). The addition of exogenous ABA simultaneously leads to increased freezing tolerance of *A. thaliana* (Lång et al. 1989). Although this and similar studies with other plants do not directly demonstrate that ABA regulates cold acclimation they, however, suggest that ABA has a regulatory

function in this process (Robertson and Gusta 1986; Robertson et al. 1987, 1988; Johnson-Flanagan and Singh 1987; Tseng and Li 1987).

To more directly assess the function of ABA in cold acclimation, we employed an ABA-deficient mutant of *A. thaliana* (Koornneef et al. 1982). We report here that this *aba* mutant has lost the capability to increase its freezing tolerance, and that this can be fully restored by addition of exogenous ABA, suggesting that ABA plays a central role in plant cold acclimation. Furthermore, the use of this acclimation-deficient *aba* mutant has allowed us to assess the role of the cold-induced proteins in freezing tolerance.

Materials and methods

Plant material and growth conditions

Two ecotypes or *Arabidopsis thaliana* (L.) Heynh. (2n = 10) were used. The ecotype Columbia (C) came originally from Chris Sommerville (Michigan State University, East Lansing/MI). The ecotype Landsberg erecta (LE) and mutants in ABA metabolism generated in this background – *abi-1, abi-3* (Koornneef et al. 1984) and *aba* (Koornneef et al. 1982) – were kindly provided by Maarten Koornneef (Agricultural University, Wageningen, The Netherlands). Surface sterilization of seeds, axenic growth of plants in 24-well tissue culture plates, cold acclimation, and ABA treatment (15 mg/l final concentration) were as described previously (Lång et al. 1989).

Greenhouse-grown plants were kept in pots in a mixture of soil and perlite and watered daily. The temperature was 20 °C day and 18 °C night, with additional light given during the winter months.

Assessment of freezing tolerance

Freezing tolerance was determined by freezing detached leaves (from greenhouse-grown plants) or whole plants without roots (from the plants cultivated in vitro) wrapped in Miracloth (Calbiochem) in a controlled temperature bath, as described previously (Lång et al. 1989). Extracellular freezing was initiated at −1.5 °C by touching the samples with a frosted wire. After 1-h equilibration period the temperature was lowered at a rate of 2 °C/h. Samples were withdrawn at 1 °C intervals and thawed on ice overnight. Freezing injury was determined by measuring the electrolyte leakage essentially as described earlier (Sukumaran and Weiser 1972; Lång et al. 1989). Tissue showing 50% (LT_{50}) or more electrolyte leakage was considered killed. Visual determination of freezing injury was as previously (Lång et al. 1989).

Radiolabelling and extraction of soluble proteins

To obtain radioactively labelled proteins, [^{35}S]-methionine was added to the growth medium (50–150 μCi/ml) of in vitro-grown plantlets and the plants were further incubated for 5–8 h. Extraction of proteins was as described previously (Lång et al. 1989).

Isolation of RNA and in vitro translation

RNA was extracted by grinding plant tissue in liquid nitrogen and then homogenizing in a buffer containing 8 *M* guanidinium hydrochloride. After extraction with phenol/chloroform/isoamylalcohol, RNA was precipitated and washed with ethanol. This was followed by resuspending the precipitate in 3 *M* KAc and then RNA was washed again with ethanol, dried, and dissolved in water (Logemann et al. 1987).

Total RNA was in vitro-translated using rabbit reticulocyte lysate (Amersham), following essentially the protocol provided by the manufacturer.

Electrophoretic analysis of proteins

Electrophoretic separation of plant proteins was done on 10%–18% linear acrylamide gradient gels using the buffer system described by Laemmli (1970). Two-dimensional electrophoresis was as described previously (Lång et al. 1989). After electrophoresis, the proteins were visualized by staining with Coomassie blue. Dried gels were subjected to autoradiography using Konica X-ray film or fluorographed after amplifier (NEN) treatment.

Results

Freezing tolerance is lost in an ABA-deficient mutant

Two-week-old in vitro-grown plantlets of both ecotypes of *A. thaliana* and of the ABA-deficient mutant (in Landsberg erecta background) were exposed to cold-acclimation conditions for the time periods indicated (Fig. 1), and their freezing tolerance was assessed by ion leakage measurements. The results of this analysis (Fig. 1 A) show that the ABA-deficient mutant (*aba*) was not able to cold acclimate. No increase in freezing tolerance was induced by the acclimation treatment, in contrast to the wild-type parent strain where frost tolerance was rapidly increased to about −7 °C. The Landsberg erecta ecotype was not different in this respect from the previously analyzed Columbia ecotype (Fig. 1; Kurkela et al. 1988). The freezing tolerance of the *aba* mutant stayed at about the nonacclimated wild-type level throughout the acclimation period; the mutant appeared even slightly more sensitive. Essentially similar results were obtained with greenhouse-grown plants, the only difference being that the freezing tolerance was shifted to a somewhat lower temperature regime (Fig. 1 B).

Similar analysis of the ABA-insensitive mutants (*abi-1* and *abi-3*) did not show any major difference from the wild-type behavior; both mutants appeared fully capable of cold acclimation (data not shown).

Exogenous ABA restores freezing tolerance

As we have shown earlier (Lång et al. 1989), exogenously added ABA is apparently taken up from the growth medium and can result in increased freezing tolerance of wild-type *A. thaliana* plants. Addition of exogenous ABA to growth medium of *aba* mutant plants resulted in increase of freezing tolerance (Table 1). This increase was similar, regardless of whether the mutant plants were grown at +20 °C or +4 °C. Mutants treated with ABA

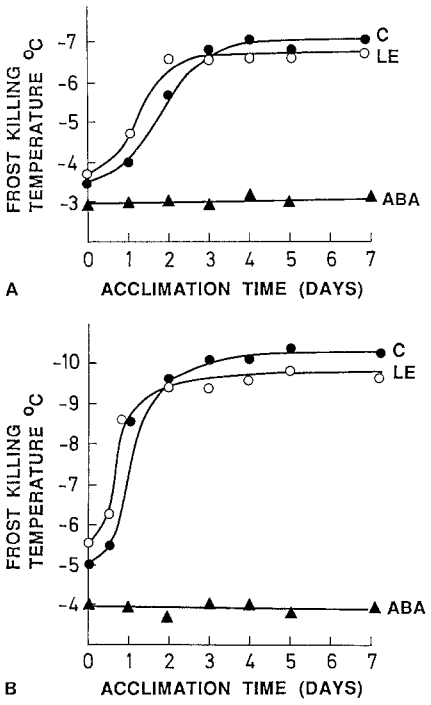

Fig. 1 A and B. Freezing tolerance of *A. thaliana* wild-type (C and LE, ecotype Columbia and Landsberg erecta, respectively) and *aba* mutant (ABA). **A** Two-week-old axenically grown plantlets were exposed to cold-acclimation conditions (+4 °C day/+2 °C night) for the time periods indicated and their freezing tolerance was assayed. **B** Results from similar experiments but with greenhouse grown plants. The killing temperatures indicated are average values of at least five independent experiments.

Table 1. Effect of exogenous ABA on freezing tolerance of wild-type (C and LE, ecotype Columbia and Landsberg erecta, respectively) and *aba* mutant plants of *Arabidopsis thaliana*. Each value is an average of at least five independent experiments

Plant	Treatment		Frost killing temperature °C
	ABA	Low temperature	
C	−	−	−3.5
C	−	+	−7.0
C	+	−	−7.2
LE	−	−	−3.7
LE	−	+	−6.5
LE	+	−	−6.3
aba	−	−	−3.0
aba	−	+	−3.0
aba	+	−	−6.0

were acclimated to close to the wild-type level (Table 1). Therefore, it appears that the mutant defect can be complemented by exogenously added ABA. This suggests that the impaired cold acclimation of *aba* mutants is indeed caused by the ABA deficiency of these plants.

Cold-induced proteins are still produced in the ABA-deficient mutant

Cold acclimation has been previously correlated with the induction of new proteins in several plant species (Guy and Haskell 1987; Meza-Basso et al. 1986; Mohapatra et al. 1987; Tseng and Li 1987) including *A. thaliana* (Gilmour et al. 1988; Kurkela et al. 1988; Lång et al. 1989). Furthermore, exogenous ABA can both induce freezing tolerance and result in the induction of a subset of these cold-acclimation-related proteins (Tseng and Li 1987; Robertson et al. 1987; Lång et al. 1989). Consequently, it has been proposed that induction of these proteins leads to increased freezing tolerance. To study the protein synthesis in the *aba* mutant during low temperature treatment, the proteins from both the wild-type and *aba* mutant plants were radio-labelled in vivo and analyzed by two-dimensional gel electrophoresis. A set of low-temperature-induced proteins was evident from wild-type plants subjected to acclimation conditions (Fig. 2). Surprisingly, a similar set of proteins was induced even in the *aba* mutant exposed to low temperature. We were unable to detect any differences between the wild-type and the mutant by two-dimensional gel electrophoresis (Fig. 2). We also analyzed the in vitro translation products from mRNA isolated from plants grown at +20 °C or those exposed to acclimation temperatures. The typical low-temperature-induced polypeptides were produced (Kurkela et al. 1988), but again we could not detect any differences between wild-type and the *aba* mutant (data not shown).

A distinguishing feature between the wild-type and the *aba* mutant, however, was that some of the proteins (e.g., 160 kD and 45 kD) that were induced by low temperature treatment in the wild type were already produced at elevated levels at +20 °C in the *aba* mutant (Fig. 2 C).

Discussion

It has been implied that abscisic acid, ABA, is a key growth regulator in a variety of cellular processes as diverse as senescence, cellular water balance, seed storage protein synthesis, and cold acclimation (Walton 1980; Addicott 1982; Li 1984). There is quite good, albeit indirect, evidence for the role of ABA in cold acclimation. Firstly, the endogenous ABA level appears to peak early during acclimation treatment and this peak is not evident in plants that cannot acclimate (Chen et al. 1983). Secondly, exogenously added ABA has been shown to induce freezing tolerance both in plant cell cultures (Chen and Gusta 1983; Orr et al. 1986; Reaney and Gusta 1987) as well as in whole plants (Chen et al. 1983; Tseng and Li 1987; Lång et al. 1989). Consequently, it has been sug-

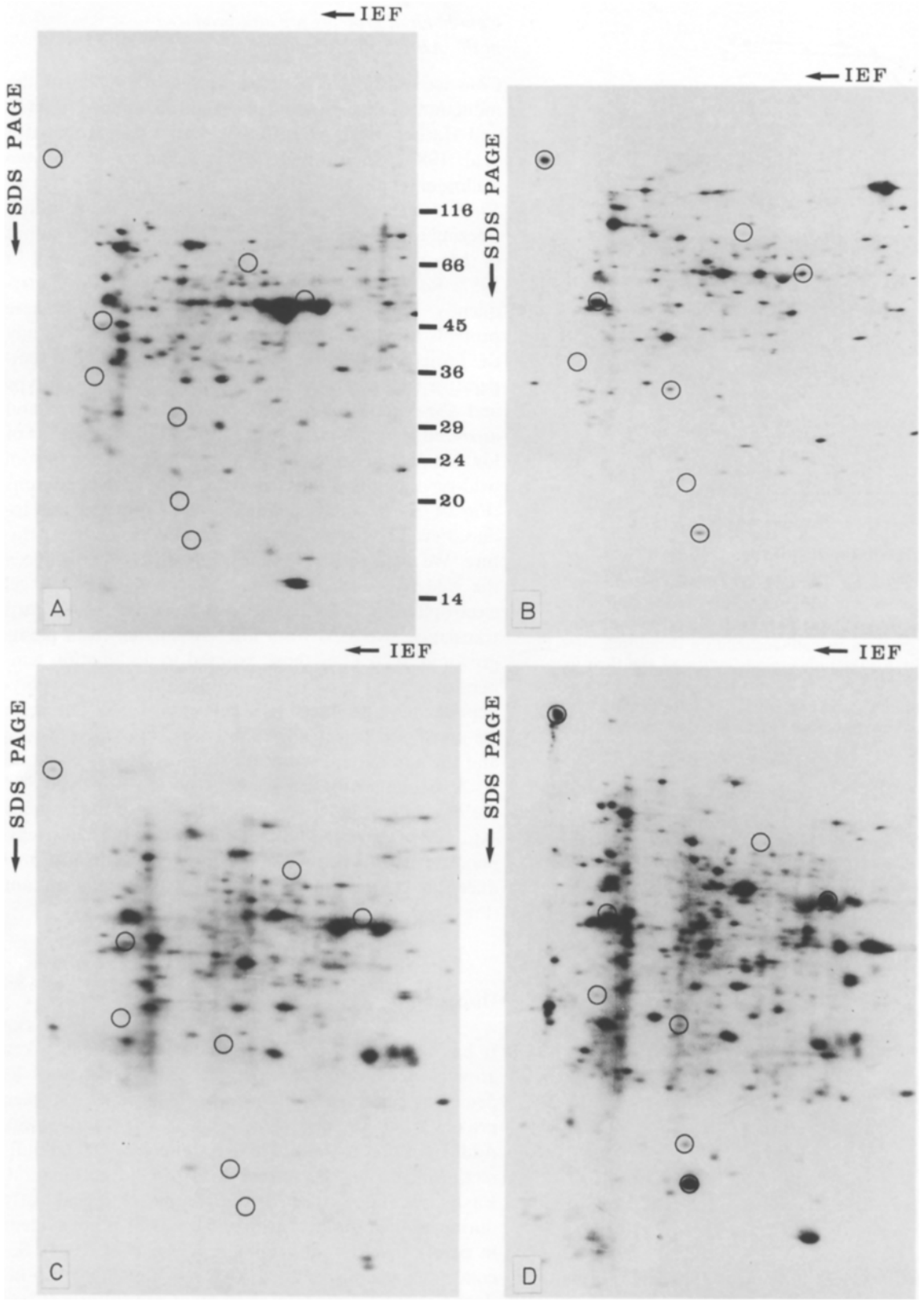

Fig. 2 A–D. Analysis of cold-induced proteins by two-dimensional gel electrophoresis and fluorography. The wild-type and *aba* mutant plants were subjected to low temperature treatment for 5 days, the proteins were radio-labelled, extracted, and subjected to electrophoretic analysis. Low-temperature-induced proteins are indicated by circles. **A** – wild-type grown at normal temperature, **B** – low temperature treated wild-type, **C** – *aba* mutant grown at normal temperature, **D** – low-temperature-treated *aba* mutant

gested that the increase in endogenous ABA concentration triggers the cold acclimation, probably by altering gene expression (Chen et al. 1983).

In this work we have tried to clarify the role of ABA in cold acclimation by employing an ABA-deficient mutant of *A. thaliana*. This mutant was impaired in cold acclimation; no increase in freezing tolerance was observed following low temperature treatment. This provides the first direct evidence that ABA is indeed involved in the cold-acclimation process. Further evidence is provided by the results that the cold-acclimation defect could be complemented by adding exogenous ABA to the mutant plants. Consequently, ABA appears to have a central role in cold acclimation and enhancement of freezing tolerance in plants. It is not, however, clear whether the increased ABA level itself functions as a trigger of gene expression necessary for the increased freezing tolerance, or whether it merely induces a second messenger in the cell.

The increase in freezing tolerance by low temperature treatment or by exogenously added ABA at a normal temperature regime has been previously correlated to induction of new mRNAs and synthesis of new proteins, a subset of which is common to both treatments (Johnson-Flanagan and Singh 1987; Robertson and Gusta 1986; Robertson et al. 1987; Tseng and Li 1987; Lång et al. 1989). It has been proposed that these proteins may be involved in the increased freezing tolerance obtained by these treatments. Alternatively, the induction could merely reflect the adjustment of the cellular metabolism to a lower growth temperature, or the proteins may be induced in response to signals generated by disturbances in the cellular water balance. Our results, indicating that these proteins are induced by low temperature treatment even in the *aba* mutant, do not support the hypothesis that these proteins would be directly responsible for the increased freezing tolerance. Therefore, some of these proteins could indeed be involved in the adjustment of tissue metabolism to lower growth temperature. On the other hand, the fact that several of the proteins were induced in the *aba* mutant already at the normal growth temperature suggests that these proteins could be involved in the maintenance of the cellular water balance. The *aba* mutant has been reported to exhibit symptoms of water stress even in normal growth conditions (Koornneef et al. 1982).

We have previously shown that a subset of these low-temperature-induced proteins is also induced by exogenously added ABA in *A. thaliana* at normal growth temperatures (Lång et al. 1989). In this study we show that the same proteins are also induced in the *aba* mutant by low temperature. These results suggest that there are two induction systems for these proteins, one which is ABA-mediated and the other which is ABA-independent and triggered by low temperature.

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